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Abstracts:
Poster Presentations

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Advances in Cell Biology - General Topics

B001/P1416
Expanded Double Negative T Cells in NOD Mice Reveal Novel Immunopathology in Type 1 Diabetes
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Non-Obese Diabetic (NOD) mice are a translational model for Type 1 Diabetes due to many shared hallmarks of disease progression, including immune cell infiltration of the pancreatic islets and hyperglycemia. Pathogenesis occurs as a result of autoreactive T cell infiltration into the pancreas and subsequent ß-cell death. Through single cell RNA sequencing, we have previously identified a transcriptionally distinct subset of CD3+CD4-CD8- Double Negative T Cells (DNTs) that are significantly expanded in NOD mice and concomitant with disease progression. In the periphery, DNTs are inversely correlated only with CD4+ T Cells, while CD8 development remains unaffected. Splenic and thymic DNTs significantly downregulate TCRß while showing aberrant expression of the B Cell marker B220. In-vitro analyses of NOD splenocytes showed that while significantly more activated at rest compared to CD4/CD8 T Cells, DNTs have a delayed activation response when treated with chemical activators such as αCD3/CD28, Concanavalin A, and PMA/Ionomycin. Further, peripheral analyses demonstrated that DNTs upregulate inhibitory receptors LAG-3 and CTLA-4, which suggests that DNTs have an immunosuppressive or regulatory role. Our findings suggest that DNTs represent novel immunopathology in NOD mice, which may resemble conserved mechanistic pathways in human Type 1 Diabetes. By determining the development and function of DNTs, our work provides insights into translational disease pathogenesis. DNTs have been implicated in human disease initiation and represent potential therapeutic interventions.

B002/P1027
Finding Clarity in Alzheimer's Brains Whole-Brain Spatialized Tau Staining in PS19 Mice
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Objective: Tau aggregation into neurofibrillary tangles (NFTs) is a pathological hallmark of Alzheimer’s disease (AD). Standard histological methods remain largely two-dimensional, which inadequately represent network-level spatial relationships of tau pathology. We aimed to optimize three-dimensional whole-brain imaging of tau pathology in the PS19 mouse model using iDISCO clearing and lightsheet microscopy to faithfully portray intact architectures of AD mouse brains. This approach minimizes sampling bias during pathology analysis and enables more accurate, system-level assessments of therapeutic effects. Methods: Eight-month-old PS19 mouse hemispheres were immunolabeled with AT8 antibody (anti-phospho-tau, 1:200) and Alexa Fluor 647 F(ab’)2 secondary antibody (1:500), counterstained with propidium iodide (1:1500), cleared via iDISCO, and imaged using a LaVision Ultramicroscope. In parallel, Thioflavin-S (0.05%) was tested as a small-molecule dye alternative for whole-brain tau visualization. Results: Volumetric imaging revealed dense phospho-tau enrichment in cortical layers II/III and hippocampal pyramidal neurons, displaying distinct somatic and neuritic morphologies that would be obscured in thin-section histology. Three-dimensional reconstructions exposed spatial gradients and regional connectivity patterns of tau accumulation across the cortex and hippocampus—features not discernible in traditional two-dimensional analyses. While Thioflavin-S colocalized with AT8 in sectioned tissue, whole-brain applications were limited by autofluorescence and solvent-induced leaching. Nevertheless, small-molecule staining accelerated labeling from 12 to 4 days and reduced reagent cost by ~20,000-fold relative to antibody-based methods, suggesting strong potential for high-throughput screening.

Conclusion: iDISCO-enabled 3D imaging provides a transformative advance over conventional IHC by preserving intact neural networks and enabling spatially resolved quantification of tau pathology across the entire brain. This platform captures organizational complexity lost in 2D sections and establishes a scalable framework for evaluating AD pathogenesis and therapeutic interventions with unprecedented spatial fidelity.

B003/P1521
In Vitro Modeling of Beta-Propeller Protein-Associated Neurodegeneration to Explore Natural Chelator-Based Therapies
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Beta-Propeller Protein-Associated Neurodegeneration (BPAN) is a rare and devastating neurodegenerative disorder that typically manifests in early childhood or adolescence. It is caused by mutations in the WDR45 gene, which encodes a protein critical for regulating autophagy and iron homeostasis. Clinically, BPAN is characterized by progressive cognitive and motor decline, movement disorders, and psychiatric symptoms, often leading to severe disability and reduced lifespan. A hallmark of BPAN pathogenesis is abnormal iron accumulation, which drives neurodegeneration. Iron chelation therapy, using agents that bind and remove excess iron, has shown therapeutic promise in other neurodegeneration with brain iron accumulation (NBIA) disorders by reducing iron-induced toxicity and improving cellular function. In this study, we directly reprogrammed BPAN patient fibroblasts into neurons using a chemical conversion approach. These patient-derived neurons exhibited distinct disease-associated phenotypes compared with healthy controls. To date, no in vitro studies have evaluated the relative efficacy of different iron chelators in BPAN. We therefore tested several naturally occurring iron chelators, including caffeic acid, ferulic acid, and baicalin. Treatment resulted in modest but measurable improvements in neuronal disease phenotype. Our findings provide the first in vitro evidence that natural iron chelators can partially ameliorate disease-associated phenotypes in BPAN neurons. This work highlights the therapeutic potential of iron chelation as a strategy to mitigate BPAN-related neurodegeneration and underscores the need for further preclinical investigation.

Advances in Cell Biology Tools and Technologies - Advanced Imaging and Super-Resolution Microscopy

B464/P1030
3D morphological characterization of extracellular vesicles using deep-learning-based localization
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Extracellular vesicles (EVs) released by cells are thought to play an important role in intercellular communication, with their functional contents and shape indicative of specific pathologies. Therefore, it is critical to develop quantitative methods for classifying EVs as diagnostic biomarkers. Previous work in this area has primarily focused on two-dimensional measurements, revealing approximately circular EVs. However, EVs are complexly-shaped three-dimensional particles (~30–200 nm in diameter) that are composed of a lipid bilayer membrane, genetic information, and surface receptors. In this poster, we present quantitative methods for characterizing EVs imaged in 3D using single-molecule localization microscopy (SMLM) powered by Double Helix Optics' engineered point spread function technology. We image labeled tetraspanin proteins on the

surfaces of EVs and then localize emitters using newly developed convolutional-neural-network-based localization models. With these methods, we localize 3x faster than conventional software algorithms, and achieve 3D isotropic spatial resolution with a 42% increase in localized emitter yield. Then, we extend 2D metrics of diameter, circularity, and eccentricity to 3D, and compare the results of 2D and 3D imaging for the same EV samples. We find that 2D imaging of EVs is not sufficient to predict their 3D morphologies.

B468/P1034
Characterizing the bioenergetics of epithelial remodeling via multiphoton microscopy and fluorescence lifetime imaging in Drosophila early embryogenesis
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Mitochondria exist inside the cell as dynamic networks that can change topology in response to different stimuli or cellular demands. Fragmented or fused mitochondrial networks are often correlated with distinct bioenergetic states (e.g., glycolysis vs. oxidative phosphorylation) and stress conditions (high vs. low ROS), but the role of mitochondrial dynamics (fission and fusion) during normal embryonic development is not well understood. Fluorescence lifetime imaging (FLIM) offers the ability to both visualize mitochondria and quantify cellular bioenergetics by taking advantage of the endogenous autofluorescence of mitochondrial cofactors (e.g., NADH). Combined with the flexibility of multiphoton microscopy, FLIM can be used to study mitochondrial network morphology and bioenergetics in a label-free, non-invasive manner at subcellular resolution. We used FLIM to measure NADH fluorescence lifetime in live Drosophila embryos during convergent extension (CE) of the head-to-tail axis—a conserved process of tissue remodeling in which thousands of epithelial cells undergo coordinated cell intercalation to drive tissue elongation. We show that NADH has a relatively short lifetime before, during, and after CE in wild-type embryos. Short NADH lifetimes are correlated with glycolysis and fragmented mitochondrial networks, suggesting that fused mitochondria and highly efficient ATP generation are not required for optimal CE. To test this, we inhibited mitochondrial fission using RNAi to knock down Drp1, and we observed significant increases in NADH lifetime and dramatic changes in mitochondrial morphology. Interestingly, we also observed significant defects in the cell behaviors that drive CE, showing that mitochondrial fission is required for efficient tissue remodeling. By contrast, inhibiting mitochondrial fusion (Opa1 knockdown), did not have strong effects on NADH lifetime and caused relatively mild developmental defects in the early embryo. Together, our data indicate that mitochondrial fission is dominant over fusion during Drosophila CE, perhaps because fused networks are incompatible with rapid changes in cell morphology and/or glycolytic environments are more conducive to cytoskeletal rearrangements. These experiments demonstrate novel FLIM-based applications for characterizing normal embryonic development, which could yield a better understanding of the mitochondrial underpinnings of pathologies involving epithelial remodeling, such as spina bifida, impaired wound healing, and cancer progression.

B470/P1036
De novo designed bright, hyperstable rhodamine binders for fluorescence microscopy
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Fluorescence microscopy is an essential technique for studying biomolecules in their native environment, offering high spatio-temporal resolution, but relying on fluorescent labels. Current labeling approaches compatible with live imaging primarily repurpose natural systems like fluorescent proteins or proteins that bind fluorescent ligands. Progress has been made to develop labels with minimal size, high brightness, and high photostability by engineering such natural proteins. However, selecting a labeling approach typically involves compromises among properties due to limitations in re-engineering features of natural proteins. Here, we introduce Rhodamine binder (Rhobin) tags – a series of de novo designed proteins capable of binding rhodamine-derived fluorophores. Rhobin tags were developed by considering desirable features directly during the protein design process, resulting in compact binders with nanomolar substrate affinity. They further exhibit excellent thermostability, rapid labeling kinetics and are orthogonal to existing labeling systems such as HaloTag or SNAP-tag. Rhobin tags readily enable live and fixed cell imaging of a wide range of subcellular targets in mammalian cells. The transient binding of fluorophores by Rhobin tags further enables live super-resolution STED microscopy with low photobleaching, as well as PAINT-type single-molecule localization microscopy. To demonstrate the stability of Rhobin tags under extreme environmental conditions, we showcase protein tagging and timelapse imaging in the extremophile Sulfolobus acidocaldarius living at 75°C, an application previously inaccessible with existing tags. We anticipate that Rhobin tags will become a central component in the toolbox of fluorescent labels and will pave the way for a new generation of protein tags and biosensors with tailor-made properties.

B458/P1024

Enhanced 8-Color Live Cell Imaging: Overcoming Spinning Disk Confocal Spectral Limitations Through New Generation Fluorochrome Selection

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Background: Spinning disk confocal microscopy provides superior temporal resolution and reduced phototoxicity compared to point scanner systems, making it optimal for live cell imaging. However, conventional spinning disk systems are constrained to 4-color multiplexing due to fixed laser lines and emission filters, limiting comprehensive analysis of complex cellular interactions. Objective: We hypothesized that next-generation fluorochromes, including tandem dyes and large Stokes shift compounds, originally developed for flow cytometry, could overcome these spectral limitations, while maintaining dual-platform compatibility for integrated imaging and cytometry workflows. Methods: We systematically screened fluorochromes based on photostability, narrow excitation/emission profiles, and Stokes shift properties. Selection prioritized minimal spectral crosstalk to maximize multiplexing potential. Validation was performed on standard spinning disk confocal systems using both fixed and live human peripheral blood mononuclear cells to identify CD4+ and CD8+ T cells, NK cells, B cells and monocytes. Results: New generation tandem and large stock shift fluorochromes have displayed significantly better photostability compared to legacy counterparts. Strategic fluorochrome selection enabled development of an 8-color imaging panel, doubling conventional spinning disk multiplexing capacity. The optimized panel demonstrated good spectral separation with minimal crosstalk and maintained photostability during overnight live imaging sessions. All selected fluorochromes retained full flow cytometry compatibility, enabling parallel analysis of identical samples across both platforms. Conclusions: This approach successfully combines spinning disk confocal temporal advantages with enhanced multiplexing capabilities, enabling comprehensive analysis of complex multicellular interactions. Dual platform compatibility

unlocks strong experimental flexibility, such as live imaging of flow-sorted cell populations without additional labelling for studying dynamic cellular processes across diverse immune and cancer populations. This methodology opens new avenues for understanding intricate cell-cell communication networks in real-time while maintaining quantitative flow cytometry validation of the same experimental samples.

B473/P1039
FAST: Filamentous Actin Segmentation Tool for quantifying cytoskeletal organization
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Studying how actin filaments are assembled into different subcellular structures can provide insights into both physiological processes and the mechanisms of disease. However, quantifying the size, abundance, and organization of different classes of actin structure from optical microscopy data remains a challenge. To address this, we developed a deep learning based Filamentous Actin Segmentation Tool (FAST) to accurately and efficiently segment and quantify different classes of actin structure from phalloidin stained confocal microscopy images. We evaluated the performance of this tool to segment and quantify the abundance of different classes of actin structure in different cell lines and with dynamic changes in actin organization using lifeact-GFP during drug treatments. FAST enables quantification of different classes of actin structure from actin images alone, without the need for specific antibodies against proteins in different actin structures and hence can be a useful tool for researchers studying for studying actin related pathways involved in cell motility, cancer metastasis, and drug development.

B474/P1040
FLEXTAG: A Small and Self-renewable Protein Labeling System for Anti-fading Multi-color Super-resolution Imaging
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Super-resolution fluorescence imaging enables visualization of subcellular structures and molecular interactions at the nanoscale, but its broader application has been hindered by long-standing limitations in current protein tagging systems, including rapid photobleaching, tag-induced artifacts, poor post-fixation labeling efficiency, and restricted multiplexing capability. Here, we present FLEXTAG (Fluorescent Labeling for Exchangeable, X-resilient Tagging in Advanced Generic Nanoscopy), a comprehensive protein labeling system comprising three orthogonal, ultrasmall (12-18 kDa), and self-renewable protein tags that collectively overcome these major limitations of existing tagging systems, enabling optimized multi-color super-resolution imaging. Through continuous exchange of organic fluorophores, FLEXTAG supports unprecedented durations of high-resolution imaging in both live and fixed cells with minimal photobleaching. It is compatible with major super-resolution modalities, such as SIM, STED, STORM, and PAINT, and is applicable to a wide range of subcellar targets. To further address fixation-induced labeling inefficiency and background fluorescence, we developed a novel protection-based fixation method and chemical blocking strategies that significantly preserve tag accessibility and enhance signal-to-noise ratio —improvements that are broadly applicable to other protein tagging systems. Altogether, FLEXTAG enables long-term tracking of dynamic behaviors and interactions of subcellular targets, as well as mapping of nanoscale protein organizations and cellular architecture, advancing both basic research and translational applications in cell biology.

B465/P1031

Gentle and rapid 3D live-cell imaging using extended depth of field engineered point spread functions (ePSFs)

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With its combination of high resolution, axial sectioning, and intrinsic rejection of out-of-focus blur, 3D confocal imaging by sequential capture of 2D slices through a volume remains the gold standard in live-cell fluorescence imaging despite the cost of induced phototoxicity and photobleaching. The photodamage caused by confocal imaging reduces the brightness of fluorescent markers over time and often leaves cells either in a phototoxic repair state or dying, rather than in their natural growth state. Here, we demonstrate 3D imaging of mitochondrial networks in live cells using engineered point spread functions (ePSFs) and advanced computational optics to reduce the number of required axial slices. While ePSFs have previously shown significant improvements in the 3D position encoding of fluorescence microscopes and the ability to extend depth of field, we find that they can also be used to reduce the number of axial slices required for 3D volume imaging by a factor of 4 or more, and as a result, to proportionally decrease the acquisition time and total light dose. Furthermore, the reduction in acquisition time effectively increases the temporal resolution available for the capture of fast dynamics of cellular processes. We present correlated results from ePSF and spinning disk confocal methods to illustrate these advantages for imaging 3D volumes.

B462/P1028

Integrating Light, X-ray, and Electron Microscopy to Resolve Legionella Infection Sites in Zebrafish Larvae

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Objective: Legionella pneumophila, the bacterium responsible for Legionnaires' disease, is an intracellular pathogen able to exploit unexpected host environments. In zebrafish (Danio rerio) larvae, we recently found that L. pneumophila colonizes the yolk syncytium, a nutrient-rich compartment not previously associated with infection. This study aimed to characterize the three-dimensional organization of these infection sites using a correlative multimodal imaging workflow combining fluorescence microscopy, laboratory-based X-ray tomography, and electron microscopy. Methods: Zebrafish larvae were infected at 24 hours post-fertilization and fixed 48 hours after infection. Infection loci were identified by fluorescence microscopy before sample preparation for electron microscopy. Resin-embedded larvae were imaged by laboratory-based X-ray tomography to map the entire zebrafish and regions of interest within the yolk and guide subsequent focused ion beam–scanning electron microscopy (FIB-SEM) analysis. Results: X-ray tomography provided volumetric datasets that revealed the general morphology of the yolk and the whole zebrafish larvae. Correlation of fluorescence and X-ray data allowed targeted selection of regions for ultrastructural analysis. FIB-SEM imaging confirmed the presence of bacterial clusters enclosed within membranous compartments in the yolk syncytial layer, revealing how L. pneumophila can persist within this metabolically active tissue. Conclusion: This correlative multimodal workflow demonstrates the power of X-ray tomography to bridge light and electron microscopy for studying infection processes in whole organisms. It establishes the zebrafish larva as a versatile vertebrate model for visualizing bacterial colonization in vivo and provides a framework for investigating host–pathogen interactions across multiple spatial scales.

B459/P1025
Mapping Microscopy Performance for Next-Gen Biomedical Imaging
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We systematically benchmarked the performance of core optical microscopy modalities to identify their strengths, limitations, and potential complementarity with emerging quantitative phase imaging methods. Using calibrated microspheres ranging from 0.5 to 15 µm, we evaluated brightfield, darkfield, phase-contrast, and fluorescence microscopy systems. Each modality was assessed in terms of spatial resolution, contrast generation, quantitative accuracy, and operational constraints, and the results were compared to those obtained with a custom-built Quantitative Phase Microscopy (QPM) system developed in our laboratory at UNC Charlotte. The QPM system operates under low-intensity illumination with single-shot capture, allowing high-resolution mapping of optical path length shifts correlated to refractive index and cell mass. The benchmark revealed that brightfield microscopy achieved a lateral resolution of 0.35 µm (60×/0.95 NA) but exhibited minimal visibility (Michelson contrast −0.20 to −0.01, CNR −20 to −0.3). Phase contrast improved image visibility (Michelson up to 0.72, CNR ≈ 37) yet retained halo artifacts, and the contrast in this technique is not reliable for objects exceeding π/3 rad. Fluorescence imaging reached wavelength-dependent lateral resolutions from 0.54 µm (DAPI) to 0.76 µm (Cy5), with CNR values from 7 to 25 and SNRs up to 82, confirming superior detectability at the cost of labeling complexity. Darkfield offered moderate edge enhancement (Michelson ≈ 0.8, CNR ≈ 12) but remained sensitive to background noise. The QPM system achieved label-free quantitative phase and intensity recovery with a measured lateral resolution of ~0.5 µm, axial sensitivity of ~3 nm (for n = 1.52), and size accuracy within ±3 %, demonstrating quantitative precision without labeling or phototoxic effects. Through this benchmark comparison, we demonstrate how our approach simultaneously captures phase and intensity information, enabling multimodal workflows. The significance of this study lies in guiding both end users and developers toward integrated imaging strategies: by systematically quantifying performance across core modalities, we establish a foundation for multimodal approaches in which QPM and conventional methods together provide richer, quantitative insights for interdisciplinary biomedical research.

B471/P1037
Multimodal imaging enables identification of a unique spatial organization of extracellular matrix surrounding megakaryocytes
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Megakaryocytes, the progenitor cells of blood platelets, play a crucial role in hemostasis and ensuring continuous platelet production in the bone marrow. Unlike other hematopoietic cells, megakaryocytes do not enter the blood circulation intact. They remain anchored within the bone marrow while extending cytoplasmic protrusions called proplatelets through the sinusoidal endothelial barrier. These proplatelets subsequently fragment into functional platelets in the blood stream. This unique process of intravasation facilitates efficient platelet production while maintaining the megakaryocyte cell body within the bone marrow niche, thus preventing potential thrombotic complications. How the extracellular matrix (ECM) influences the delicate balance between megakaryocyte retention and proplatelet extension remains largely unknown. Here, we investigate the spatial organization and functional role of ECM components in the megakaryocyte vascular niche. To approach this aspect in a native environment, we combined high-resolution methods on bone

marrow samples: immunofluorescence on 250 nm thin cryosections, large whole-mount 3D confocal reconstruction, and transmission electron microscopy. In vivo intravital two-photon microscopy was used to describe MK intravasation in real time. Ex vivo primary MK from bone marrow were notably screened with scanning electron microscopy and photonic microscopy to assess attachment, spreading and remodeling on ECM. This multimodal strategy revealed that laminin and collagen IV form 3 Dimensional ECM cages encompassing megakaryocytes and anchor them to the sinusoidal basement membrane. Notably, megakaryocytes actively contribute to the ECM cage assembly through integrins. We used β1/β3 integrin knockout mice demonstrating a more fragile 3D cage resulting in increased intravasation and entire megakaryocyte entry into circulation. Thus, the ECM cage represents a novel concept of an active and dynamic 3D microenvironment that is essential for maintaining megakaryocyte perivascular positioning. In conclusion, integrating complementary imaging modalities allowed to characterize a specific microarchitecture which guides megakaryocyte maturation and intravasation, underscoring the critical role of ECM microarchitecture and dynamics in megakaryocyte function.

B477/P1043

Objective Single-Molecule Nuclear Architecture Profiler (O-SNAP): A comprehensive pipeline for spatial profiling of chromatin architecture

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The eukaryotic genome is hierarchically organized across multiple spatial and genomic scales. Understanding how this organization relates to cell state is central to elucidating the molecular basis of development, cellular reprogramming, and disease. SMLM has been instrumental in revealing previously inaccessible aspects of chromatin organization and remodeling during physiological and pathological transitions. While data generated by SMLM are complex and spatially rich, current analysis approaches fail to capitalize on this wealth of information. We present O-SNAP (Objective Single-Molecule Nuclear Architecture Profiler), a comprehensive pipeline for automated extraction and classification of nuclear features from SMLM data. O-SNAP quantifies 144 biologically grounded features describing chromatin or histone mark organization at nanoscale resolution. It incorporates statistical tools inspired by RNA-Seq differential expression analysis, such as pairwise comparisons via volcano plots, feature set enrichment analysis, and pseudotime trajectory inference – enhancing biological interpretability and allowing for mechanistic insight into how specific chromatin features contribute to cell identity. Additionally, O-SNAP classifies cell states with high accuracy by training machine learning models on nuclear SMLM images. We applied O-SNAP to study chromatin remodeling in broad biological contexts including human tenocyte cells from tendinosis patients, reprogramming human fibroblasts, and mammary epithelial cells with elevated levels of reactive oxygen species. In all cases, O-SNAP identified subtle changes to chromatin spatial organization and classified distinct cell states with >80% accuracy. O-SNAP offers a powerful framework to analyze physiological and pathological nuclear architecture transitions, providing mechanistic insights into the underlying chromatin dynamics and potentially enabling rational design of interventions to modulate cell state transitions.

B476/P1042

Single-shot label-free nanoscopy for quantitative organelle visualization on standard commercial microscopes

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Label-free microscopy, which overcomes the limitations of fluorescence imaging, is increasingly required in cell biology. Our objective was to develop a practical, high-resolution, and high-speed label-free imaging method that can be implemented on standard commercial microscopes and is capable of quantitative visualization of intracellular structures. We developed upgraded polarization-resolved differential phase contrast nanoscopy (UpDPC), a single-shot computational phase microscopy technique that enhances both resolution and sensitivity. The method requires only minimal modifications: an inexpensive polarization mask and a polarization-separating camera, and enables simultaneous use with conventional fluorescence microscopy. Using a resolution-optimized setup, UpDPC achieved gap separation below 100 nm with 445 nm illumination, as validated by Siemens star test patterns, and provided quantitative phase measurements that matched theoretical predictions. The system enables real-time phase retrieval in ~100 ms per frame on a standard PC, allowing continuous live-cell imaging. Temporal resolution reached 125 Hz, which enabled us to capture vesicle transport driven by molecular motors. We confirmed that UpDPC transmitted higher spatial frequencies than the Zernike phase contrast microscope and avoided the directional bias of the DIC microscope. In vitro, single microtubules (25 nm diameter; ~3.1 nm optical path difference in water) became visible in label-free images. In cells, we visualized mitochondrial membrane structure, density variations inside nucleoli, endoplasmic reticulum, actin bundles, peroxisomes, and stress granules, and tracked stepwise vesicle motions matching the molecular motor parameters. Low phototoxicity enabled continuous mitosis recordings >1 hour at 30 ms exposure, where chromosomes, centrosomes, and spindle fibers were discernible. In conclusion, UpDPC provides high-speed, high-resolution, and quantitative label-free imaging that extends the capability of standard commercial microscopes. By enabling reproducible visualization of a wide variety of organelles and dynamic cellular processes with minimal phototoxicity, this approach offers a practical route to comprehensive, label-free studies of organelle dynamics and interactions in cell biology.

B469/P1035

Tracking tubule flow reveals compensation, not obstruction, during PKD progression

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Polycystic kidney disease (PKD) is characterized by progressive cyst formation that disrupts normal nephron structure and leads to a decline in kidney function. A widely cited but untested hypothesis suggests cyst initiation occurs due to tubule flow obstruction, leading to fluid entrapment and expansion. The objective of this study was to directly test whether loss of tubule flow is an initiating mechanism of cystogenesis or a consequence of cyst expansion. To address this, we developed a method to longitudinally track tubule flow using low molecular weight fluorescent dextran, which is selectively absorbed by proximal tubule cells via the scavenger receptor megalin. Single and dual dextran injections at distinct time points allowed us to quantify flow-receiving tubules by flow cytometry and confirm spatial uptake by fluorescence imaging. Longitudinal labeling enabled us to determine whether tubules retained or lost flow over time and whether new tubules become active as disease progresses. We complemented these findings with live intravital imaging in controls and cystic mice to visualize flow rates and dynamics in real time. Our results show that proximal tubules

consistently received dextran uptake during cyst initiation and expansion. Dextran-positive cells from an initial injection were also positive for the second dextran injection at later time points, indicating continuous flow. By the cystic timepoint in Pkd2 mutants, there was a significant increase in tubules that were not labeled with the first but positive for the second dextran, suggesting functional compensatory recruitment of additional nephrons from the kidney reservoir. Live imaging confirmed that while flow persisted, the rate of dextran perfusion was reduced in cystic kidneys compared to controls, associated with reduced glomerular filtration rates. Importantly, we found no evidence of tubule obstruction or disconnection preceding cyst initiation, in contrast to the prevailing hypothesis of cyst formation. In conclusion, this study provides the first direct in vivo evidence that proximal tubule flow is preserved during cyst initiation and early expansion in PKD. Rather than being lost at the onset of cystogenesis, tubule flow persists, but at a reduced rate as cysts enlarge. These findings challenge the prevailing early obstruction model and instead support a compensatory recruitment model in which additional nephrons are activated to sustain filtration. This work establishes low molecular weight dextran labeling as a powerful tool for tracking nephron function during both health and disease and suggests that therapeutic strategies should focus on preserving nephron integrity and compensatory capacity, rather than solely on preventing early obstruction.

B466/P1032

Ultra-Bright Fluorescent Probes For Biological Assays Using Plasmon-Enhanced Fluorescence (PEF) Nanoconjugates.

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Here we present a simple, but novel nanoparticle-based fluorescent probe with vastly increased signal compared to the typical fluorophore or quantum dot, while maintaining similar or lower background noise due to nonspecific binding, and adding facile options for functionalization the aid in biological assays. Fluorescence-based assays are some of the most crucial tools of modern biological research, diagnostics, and therapeutic development. Techniques such as fluorescence in situ hybridization (FISH), immunohistochemistry (IHC), and flow cytometry are indispensable, enabling precise detection and quantification of target biomolecules, which provides vital insights into cellular mechanisms in research, and guides treatment decisions in the clinic. However, despite their profound importance these techniques are still significantly hindered by the low innate signal of fluorescent probes. In this research, we report the development and performance of the Nanoparticle-oligo Array (NOA) platform, which consists of nanoparticle core templated with up to 100 fluorophore-carrying oligomers such as DNA and PEG. These probes have a specifically arranged three-dimensional architecture that holds fluorophores at a distance from the nanoparticle core that results in Plasmon Enhanced Fluorescence (PEF), and when measured in solution these conjugates demonstrate fluorescence levels up to 65,000 times brighter than the same number of fluorophores without this spatial arrangement. The architecture also exploits the close proximity of the templated fluorophores to generate incredibly bright, large stokes shift probes through Förster resonance energy transfer (FRET). By combining FRET pairs on the nanoparticle surface, and a FRET donor paired to the plasmon resonance peak of the nanoparticle core, NOA-based FRET probes exhibited a fluorescence response ranging from tens to hundreds of times that of FRET pairs from complementary DNA in solution. The development of these plasmon-enhanced probes offers a transformative advancement in the field of fluorescence assays. With this probe's exceptional physical, chemical, and photonic properties which emerge from its 3D architecture, the integration of NOA probes into common assays such as FISH, IHC could offer a bright, amplification-free methodology for the detection of biomolecules.

B463/P1029

Unlocking Insights in Cellular Research: Evaluating Therapeutic Effects on 3D Cell Models with Live-Cell Confocal Imaging and Quantitative Analysis

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As cellular research continues to evolve towards more complex and translational biological models, the development of companion bioanalytical tools is critical to extract clear insights and enable faster, smarter decisions for improving clinical application and treatment. The Incucyte® CX3's compact spinning disk confocal technology facilitates acquisition and analysis of complex 2D and 3D cell models, such as spheroids and organoids, under physiologically relevant conditions. The integration of optimized protocols and automated analysis tools, requiring minimal user input, accelerate insight into complex biological systems, supporting therapeutic development. This poster illustrates the application of these tools in evaluating the effect of therapeutic compounds on multiparametric readouts of cell health and function. Spheroids generated from cancer cells stably expressing a genetically encoded fluorescent ubiquitination-based cell cycle indicator (FUCCI) were monitored over 96 hours. Confocal microscopy enabled concurrent evaluation of apoptosis utilizing dye-based cell health readouts, alongside cell cycle dynamics following exposure to chemotherapeutic agents, Fluorouracil (5-FU) and Cisplatin. 5-FU induced cell cycle arrest in the G1 phase, as observed through increased orange mean fluorescence measured from max projection images, without significant cell death at the tested concentrations. Conversely, Cisplatin caused cell cycle arrest in the G2/M phase, marked by increased green mean fluorescence at lower concentrations and induced apoptosis at higher concentrations, indicated with elevated NIR Annexin fluorescence. As expected, treatment with Camptothecin, used as a positive control, caused cell cycle arrest and cell death. Similar case study datasets collected in organoid and other complex 3D models will be presented to illustrate the value of live cell confocal imaging to provide fast, simple insights from complex cellular models.

B475/P1041

Using Spatial Transcriptomics to Investigate Microglial Signaling After an Injury

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Microglia, the resident immune cells of the brain, play important roles in traumatic brain injury and neurodegeneration. However, they are difficult to study due to their sensitivity to invasive methods, functional heterogeneity, and sparse distribution within tissues. Although single-cell RNA sequencing captures the transcriptomic complexity of microglia, the method undersamples the transcriptome, lacks 3D resolution, and loses spatial context. In contrast, Single Molecule Fluorescent in Situ Hybridization (smFISH) achieves the subcellular resolution, sensitivity to rare transcripts, and multiplexing needed to study single cells in context. Still, large-scale data collection for statistical power remains a challenge. To address this, we developed a machine learning-based smFISH analysis pipeline optimized for detection and spatial profiling of RNA in microglia. To optimize spot detection, we simulated smFISH data with known ground truth and used part of the open-source QI2lab merfish3d-analysis pipeline to transform our images into spatially mapped RNA "spots." F1 scores were used to establish thresholds for distinguishing true RNA spots from background. For experimental validation, we generated probes for 16 genes that exhibit dynamic expression in microglia

following injury. Tissue from pre- and post-injury mice was collected and prepared for smFISH, then imaged on a widefield confocal microscope equipped with adaptive optics. 16 unique RNA species were visualized across eight imaging rounds using sequential hybridization and binary barcoding. 3D quantitative smFISH data were post-processed using the full merfish3d-analysis pipeline, which includes deconvolution, registration, cell segmentation, and decoding into unique transcripts based on our thresholds. Thresholding minimized imaging artifacts and false positives, with sufficient sampling for well-powered statistical analysis. We split the processed smFISH images into 16 gene-specific images for easier analysis. A poly(dT) probe outlined the cell bodies, enabling cell segmentation and identification of anatomical regions such as the corpus callosum. Comparing transcript expression in microglia pre- and post-injury revealed distinct immune activation profiles. Quantitative smFISH can robustly profile rare cell types like microglia due to its subcellular resolution, spatial fidelity, and statistical rigor.

B460/P1026

Visualizing Inner Ear Ribbon Synapses: Anatomical Complexity and Challenges in High-Resolution Electron Microscopy

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High-resolution electron microscopy (EM) is a powerful analytical technique that enables the visualization of biological and material structures at the nanometer scale. By surpassing the resolution limits of light microscopy, EM provides unprecedented insights into the ultrastructure and organization of complex systems. Its capacity to reveal fine morphological details makes it indispensable across disciplines, from fundamental biology to materials science, where understanding nanoscale architecture is essential for linking structure to function. Ribbon synapses are specialized neuronal junctions prensent in the inner ear, they are essential for a rapid and sustained neurotransmitted release in the auditory processing. Their ultrastructure is characterized by an electron-dense ribbon surrounded by tethered synaptic vesicles and precisely aligned with postsynaptic elements. These synapses are extremely small, typically measuring only 200–500 nm in length, yet their dense packing and highly organized structure make them a major challenge to visualize with traditional imaging methods. Conventional TEM/SEM imaging methods are mainly used to conduct studies on the ribbons synapses. But by using classical EM methods we might undermine the strutural complexity of the ribbon synapses. This is why the use of volume EM imaging methods could be benefical. By combining the high resolution of EM and the information of volume acquisition we can aim at a better understanding of the anatomical complexity of ribbon synapses. Thus providing critical insight and information for studies on auditory functions and dieases.

B472/P1038

XYZ isotropic expansion microscopy enables detailed organelle structural analysis on conventional fluorescence microscopes

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High resolution imaging of intracellular organelles is essential for evaluating cellular function and disease mechanisms. Electron microscopy such as SEM and TEM and super resolution methods such as STED provide the needed resolution but require specialized and expensive instruments, while conventional fluorescence

microscopes have a resolution limit that prevents resolving very small details. In 2015, Boyden et al. reported expansion microscopy, which enlarges specimens by the water uptake of the gel, allowing finer structural details to be observed with standard fluorescence microscopes. Recently, many researchers reported original methods for expansion microscopy, and within these reports expansions beyond 20-fold are described. However, achieving more than 10-fold often requires vacuum degassing and repeated expansion steps, which increase procedural complexity and may reduce reproducibility. In addition, many reported methods expand mainly in the plane. As a result, conventional approaches are not always suitable for detailed analysis of organelle structures. To address this issue, Suzuki et al. at the University of Wisconsin reported a one-step method that enables isotropic expansion in the XYZ axes and improves reproducibility. The method eliminates the need for vacuum equipment by using Parafilm and employs glutaraldehyde as a crosslinker with optimized enzymatic processing to support simple and reproducible expansion. Here, we report a kit based on the Suzuki et al. method with procedural optimizations to reduce complexity and improve reproducibility. The kit enables isotropic expansion in the XYZ axes with simple steps and supports 3-dimensional imaging of organelles on conventional confocal microscopes using expanded samples. We also showed that mitochondrial morphology becomes easy to visualize and that its changes can be evaluated in detail. In conclusion, this study shows that isotropic expansion in the XYZ axes can be implemented through simple kit-based procedures and can provide a useful tool for high resolution organelle imaging on widely available microscopes.

Advances in Cell Biology Tools and Technologies - Artificial Intelligence and Machine Learning Approaches

B498/P1064

Analysis of Therapeutic Response in Hepatocellular Carcinoma with Graph-based Machine Learning Methods on Spatial Transcriptomics Data

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Spatial transcriptomics (ST) enables the investigation of tissue organization by preserving spatial context while profiling gene expression. However, understanding how gene-gene interactions differ between tissue phenotypes remains challenging due to sparsity and noise in gene measurements and patient-specific differences. In this study, we analyzed Visium spatial gene expression data from hepatocellular carcinoma (HCC) tumor microenvironments (TME) to compare spatial gene-gene networks between responder and non-responder patient groups. To do so, we utilized two recently developed methods, spatial Affinity-graph Recovery of Counts (spARC) and Gene Signal Pattern Analysis (GSPA). spARC is a graph-based method that denoises and refines ST data, revealing gene co-expression and simulating communication between cells. GSPA is a method that represents gene measurements as graph signals and uses graph signal processing to create rich gene embeddings given a gene expression matrix. The resulting embeddings represent functional similarity between genes in terms of both transcriptional and spatial concordance. These embeddings were then used to compute localization scores for each gene, which capture the specificity of enrichment patterns. We found that pathways associated with structural remodeling were enriched among genes uniquely localized in responders, whereas pathways associated with extracellular matrix (ECM)-driven resistance and epithelial-mesenchymal transition (EMT) were enriched in non-responders. We additionally created gene-gene similarity graphs utilizing embedding values and analyzed the network structure differences between responder and non-responder samples using the Jaccard Similarity Index, Uniform Manifold Approximation and Projection (UMAP), and random forest classification of gene-centered ego-subgraphs. Furthermore, we introduced a novel metric, the differential response score based on mean Euclidean distance, to further quantify differences between the two groups. Collectively, these analyses indicate that responder and non-responder tissues have

distinct molecular programs, particularly involving ECM interactions, immune regulation, and cell migration. Our findings demonstrate that integrating spatial context into network-based analyses can uncover phenotype-specific molecular organization in the HCC TME, uncovering spatial features that shape therapeutic efficacy.

B497/P1063

Applying AI/LLM Tools in Biological Research: Comparative Approaches and Practical Integration

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Background: Artificial intelligence (AI), particularly large language models (LLMs), are transforming how biological research is conducted. We evaluated different LLMs on core research activities—literature review, experiment management, data analysis, and scientific writing—to assess their current capabilities, limitations, and implications for research use. Our analysis emphasizes how these systems can improve efficiency and accessibility while requiring validation and responsible adoption. Results: In literature review, we compared traditional search engines, LLM-powered search tools, and LLM-integrated computers for their ability to identify relevant information, organize downloads, summarize papers, and inspire related research. While LLMs reduce effort, users must remain cautious of outdated knowledge, hyperfocused searches, and imaginary citations. For experiment design and notebook management, we present workflows where LLMs can aid researchers to generate protocol templates, identify caveats, modify procedures, schedule overlapping experiments, and maintain structured electronic lab notebooks, though one needs to consider data security and intellectual property. In bioinformatics, LLMs can streamline coding and expedite data interpretation, but their performance depends heavily on data quality and prompt design. We analyzed datasets using the LLMs built-in knowledge and found that LLMs tend to align with initial hypotheses and are prone to errors in ambiguous cases. In the emerging area of AI-prompted analysis, connecting LLMs directly to compute with scientific software provides more fine-tuned analysis options and can help bridge the gap between biologists and data analysts. For scientific writing, while LLMs can generate introductions from detailed outlines, the outputs often resemble general literature reviews rather than introductions for novel findings—likely reflecting that LLMs are trained on existing rather than new knowledge. Nonetheless, LLMs serve as valuable peers for editing drafts, aiding reference citations, and reworking existing text for different audiences.Conclusion: We offer practical guidance for laboratories seeking to integrate AI across research practices and emphasize the essential roles of critical evaluation, transparency, and human oversight. Rather than displacing traditional biological expertise, LLMs are emerging as research companions that amplify the capabilities of scientists, transforming once-tedious administrative and analytical tasks into streamlined workflows that free researchers to focus on the creative and interpretive aspects of scientific discovery.

B495/P1061

Benchmarking detection methods toward a label-free AI classifier of pluripotency versus differentiation in iPSCs

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[Background] Rapid, noninvasive assessment of pluripotency/differentiation is pivotal for optimizing passage scheduling in iPSC manufacturing. Implementing a label-free pipeline that does not rely on fluorescent staining

(e.g., Oct4, Hoechst) would substantially improve throughput and operational feasibility. We aim to build an AI framework that forecasts culture quality via undifferentiated cell ratio, by integrating brightfield-only cell detection with classification of differentiation status. [Methods] We benchmarked multiple instance detection approaches on brightfield images and selected Cellpose as the core detector based on superior performance (detected nuclei: 2,762). We then evaluated a hybrid strategy combining Cellpose with a U-Net regression model that predicts Oct4 fluorescence intensity directly from brightfield. The U-Net was trained on 150 paired images and evaluated for predictive accuracy. Cellpose, used without additional fine-tuning, was assessed against manually curated ground truth using mean Intersection over Union (IoU). We further quantified centroid containment, i.e., the fraction of predicted instances that correctly enclosed a single cellular center. [Results] The U-Net's predicted Oct4 intensities exhibited a positive correlation with measured signals (Pearson $r \approx 0.7$), yet the coefficient of determination (R^2) was negative, indicating limited predictive fidelity at this stage. In contrast, Cellpose achieved a mean IoU of 0.48 without retraining, and 76% of detected instances correctly contained a single cell centroid. These outcomes constitute a promising baseline prior to task-specific fine-tuning and support the feasibility of brightfield-only instance segmentation for downstream label-free classification. [Conclusions] Our findings suggest high feasibility of integrating brightfield-based nucleus detection with label-free differentiation assessment to estimate undifferentiated cell ratios. For practical deployment, improving classifier performance and generalization—quantified via IoU and related metrics—will be critical. We plan to refine annotation strategies, expand training across diverse culture conditions including bFGF (+/−), and integrate detector and regressor outputs into a unified pipeline. Label-free AI–driven estimation of undifferentiated ratios is poised to inform prediction and optimization of passage timing, enabling scalable, routine quality control in iPSC culture.

B504/P1070

Building an AI-assisted automatic pipeline for large FOV image analysis (FOVIA)

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In recent years, advances in fluorescence microscopy systems enable high-throughout collection of large field of view (FOV) images of tens of thousands of cells from mammalian tissues under various genetic and/or pharmacological treatments. Quantitative analyses of these images across these different treatments are essential to identifying candidates of interest for further downstream investigations. However, these large FOV images often exhibit highly variable backgrounds and heterogeneous signal contexts, making quantification and comparison challenging by traditional intensity thresholding methods. Here, to analyze hundreds of confocal-level images of the entire mouse lung tissue section, we developed a new quantification algorithm that built off simple regression in QuPath using GEOJSON objects to outlining regions of interest for automatic quantification. In parallel, using CellPose, we trained AI models to segment individual cells. Integrating the two algorithms, we built an automatic pipeline to generate fluorescent intensity heatmap images of lung sections. The application of this pipeline for quantitative comparisons among different genetic perturbations led to the identification of new genetic regulators of lung infections. This method can be easily generalized to analyze large FOV fluorescent images of other tissues and facilitate data analysis from high-throughput genetic and drug screens.

B494/P1060

Cell-context dependent in silico organelle localization in label-free microscopy images

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In silico labeling prediction of organelle fluorescence from label-free microscopy images has the potential to revolutionize our understanding of cells as integrated complex systems. However, out-of-distribution data caused by changes in the intracellular organization across cell types, cellular processes or perturbations, can lead to altered label-free images and impaired in silico labeling. We demonstrated that incorporating biological meaningful cell contexts, via a context-dependent model that we call CELTIC, enhanced in silico labeling prediction and enabled downstream analysis of out-of-distribution data such as cells undergoing mitosis, and cells located at the edge of the colony. These results suggest a link between cell context and intracellular organization. Using CELTIC to generate single cell images transitioning between different contexts enabled us to overcome inter-cell variability toward integrated characterization of organelles' alterations in cellular organization. The explicit inclusion of context has the potential to harmonize multiple datasets, paving the way for generalized in silico labeling foundation models.

B499/P1065

Deep Learning Image Segmentation Across Length Scales: Enhancing Electron Microscopy Data Interpretation

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Recent advancements in deep learning have revolutionized image segmentation, providing powerful tools for interpreting electron microscopy data across various length scales. From cryoEM to cryo and room temperature volume EM, this presentation will explore a range of AI-based deep learning tools and methods, showcasing both proprietary software like Thermo Fisher Scientific's Amira and Dragonfly 3D World, as well as open-source options such as Fiji/ImageJ, MemBrain-Seg V2 and Napari/Empanada. By leveraging these advanced segmentation techniques, researchers can achieve more accurate and efficient data interpretation, gaining detailed insights into the spatial organization and interactions of biological structures. The presentation will showcase the practical applications and benefits of these tools, demonstrating their potential to make electron microscopy data analysis more accessible and impactful for the scientific community.

B501/P1067

Global absolute protein quantification with uncertainty estimates to study cell biology

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Absolute protein concentrations determine the rate and extent of cellular processes and knowing them is essential for a complete description of any cell. Protein concentrations must be carefully controlled to faithfully reconstitute subcellular processes, yet cell biologists often analyze and perturb processes in living cells blind to absolute concentrations. Mass spectrometry is currently the only method that can measure all proteins at once, but is used primarily for relative quantification of proteins between conditions rather than absolute quantification. Current methods to estimate absolute protein abundance via mass spectrometry suffer from 2-fold or more median error, no way to quantify uncertainty, and systematic biases such as underestimating the abundance of scarce proteins. Isotope labeled spike in standards allow accurate absolute measurement but are costly and can only be applied to a few proteins in a sample. In this study, we developed a comprehensive model of how mass spectrometry–based proteomics data is generated and leveraged these insights to build a

Bayesian framework that yields absolute concentration estimates with improved accuracy and precision, while also providing associated confidence intervals. We found that peptide detection in the mass spectrometer depends on both peptide abundance (creating a censoring effect) and the peptide's physicochemical properties (such as its ability to ionize). We further quantified the distribution of ionization efficiencies among peptides from the same protein and developed machine learning predictions for the ionization efficiency of each peptide. Our Bayesian hierarchical model integrates this information to estimate protein concentrations from observed peptide intensities as well as predict error bounds. Applied to exponentially growing S. cerevisiae, the model achieved an R^2 of 74%, outperforming current state-of-the-art methods (R^2 = 66%) and reducing the median fold error from 2.7 to 1.5. The method can also reanalyze existing proteomics datasets effectively. Notably, it eliminates the need for costly spike-in standards and provides confidence intervals for the estimates, facilitating the quantification and propagation of uncertainty in biological studies.

B506/P1821
Harnessing AI/ML for Quantitative Cell Biology
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I will discuss our recent efforts to advance quantitative understanding of cell biology by developing AI-enabled approaches to for analysis and interpretation of biological imaging data. I will discuss approaches we've developed for focal adhesion signaling and cell mechanobiology. I will spend time discussing our current thinking of how scientific AI methods may be developed for building new frameworks for model building and testing of cell dynamics, while also outlining outstanding challenges and unknown. Tackling these represent an exceptional opportunity to leverage recent advances in cell engineering and imaging to advance abilities to predict and engineering cell and tissue dynamics and function at scale.

B502/P1068
Machine Learning-Assisted Evaluation of Drug Toxicity Using 3D Human Intestinal Organoids
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Damage to intestinal cells is one of the most common side effects of chemotherapy, often limiting therapeutic efficacy. To better predict such adverse effects, it is essential to develop in vitro models and analytical tools that enable pre-screening of drugs and drug candidates for toxicity. We developed automated organoid culture protocols and machine learning–assisted analysis methods. Human duodenum organoids were cultured using CellXpress.ai, which enabled automated culture and compound treatment. Organoids were treated with eight anti-cancer drugs known to induce intestinal toxicity. Toxicity evaluation was conducted through morphological and viability assessments using high-content imaging. After 72 hours of compound exposure, organoids were stained for toxicity evaluation using viability, mitochondrial, and nuclear markers. Confocal imaging was performed with HCS.ai system, and image analysis was carried out using IN Carta software. Initially, conventional high-content image analysis was applied. Cells positive for Calcein AM and MitoTracker were classified as intact (live) vs damaged. Positive and negative cells per organoid were counted, also sizes and intensities evaluated. While this approach enables quantification of phenotypic changes and effective concentrations, it is limited by the need to analyze multiple parameters separately to capture biological complexity. Machine learning tools offer a more unbiased and comprehensive approach to analysis by automatically identifying organoids and scoring them as intact or damaged. The analysis extracted 117

quantitative features per organoid. These included morphological descriptors (area, roundness, perimeter), intensities across three fluorescent channels, textural features (entropy, granularity, contrast), and spatial distribution patterns (radial intensity, heterogeneity). Classification was performed using a combination of unsupervised and supervised machine learning. Unsupervised clustering grouped organoids into clusters based on feature similarity, which were then curated into biologically relevant categories by user-defined criteria for morphology and viability. The final classification yielded percentage distributions for each phenotype class allowing evaluation of compound effects in a concentration-dependent manner. Unlike single-parameter readouts, this approach incorporates a broad morphological context, offering deeper insights into phenotypic changes. We demonstrated a fully automated workflow for toxicity evaluation using 3D human duodenum organoids, high-content imaging, and machine learning–based image analysis. The described approach enables AI-driven phenotypic profiling for assessing drug-induced toxicity in organoid models.

B496/P1062

Machine Learning-Based Label-Free Cell Counting Using Transmitted Light Imaging

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Label-free imaging offers significant advantages over traditional fluorescence-based methods, including reduced reagent costs, faster processing, and the ability to monitor live cells without introducing labeling artifacts. In this study, we used customizable AI models within IN Carta Image Analysis Software to develop deep learning-based segmentation models for accurate cell and nuclear detection using transmitted light (TL) images alone. We began by establishing label-free analysis using the U2OS cell line. To develop a TL-based nuclear segmentation model, we first applied a pre-trained AI model to segment nuclei in Hoechst-stained images. The resulting segmentation masks, paired with corresponding TL images, were used to train a new model capable of segmenting nuclei using TL images only. This approach enabled label-free nuclear detection with over 97% accuracy, as validated against Hoechst-based nuclear counts. The model reliably quantified both live and damaged cell phenotypes. Additionally, we developed a whole-cell segmentation model trained on Calcein AM-stained images. This model also demonstrated high performance, achieving 97% accuracy compared to nuclear counts. We further trained the model using images from additional cell lines, including HeLa, HCT116, U2OS, MCF7, and HEK. The model achieved over 90% accuracy across most of these cell types. To evaluate its utility in compound screening, we treated the five cell lines with 10 anti-cancer compounds using a 7-point dilution series. Cell counts obtained via TL-based nuclear segmentation were compared to traditional nuclear staining methods across treatments. The results showed strong correlation and comparable IC_{50} values, confirming the accuracy and reliability of the label-free approach. To further validate the method, we compared IC_{50} values derived from TL-based cell counts with those obtained from ATP-based viability assays. The consistency across methods supports the robustness of TL-based segmentation for compound screening. Overall, this label-free approach offers significant advantages in processing speed and workflow simplicity, enabling real-time analysis of compound effects and making it a powerful tool for high-throughput and phenotypic screening applications.

B500/P1066

Mapping 3D cell surface dynamics and signaling in live cells using physics-based rules

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Cells move and change shape to accomplish diverse biological functions. Immune cells move through tissues to find pathogens, fibroblasts reshape the surrounding matrix during wound healing, and epithelial cells shift their positions during development. In each case, motion and deformation of the cell surface are not merely by-products but active drivers of cell function. Understanding how cells reshape themselves as they migrate, interact, and respond to their environment is therefore a central challenge in modern biology. Yet most quantitative analyses of cell motility have been performed in two dimensions, which misses the complexity of three-dimensional behavior. We present a computational framework for tracking entire cell surfaces from 3D time-lapse fluorescence microscopy movies. Our approach establishes dense point-to-point correspondences across consecutive frames of a cell surface. This enables us to compute per-surface vertex trajectories over time, yielding local surface displacement, as well as track signaling and curvature changes and transient events such as protrusions, blebs, and filopodia. Technically, our method builds on the concept of functional maps, originally developed in computer graphics. Functional maps provide a way to match points on one cell surface to points on another, even when the cell is changing shape. In our implementation, initial functional maps are learned using DiffusionNet, a deep learning model that applies heat-kernel diffusion on cell surface meshes to learn geometry-based features, and then refined with the ZoomOut algorithm to produce high-quality point-to-point mappings. These correspondences are further regularized with biologically or physically interpretable constraints, including conservation of area, curvature, position, and orthogonality, ensuring that the measured motions remain physically meaningful. We demonstrate the utility of our approach on several types of migrating cells, capturing diverse shape dynamics. Overall, our method provides both a technical advance in 3D cell motion tracking and a tool to study how cell shape changes and signaling are coupled via physics-informed modeling and tracking.

B503/P1069

Open-Source AI Algorithms for Geometric Optimization of 3D Scaffolds to Enhance Bone Integration

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New technics for designing scaffolds in regenerative medicine requires creative architectures that combine mechanical stability with the complexity of native bone. Conventional grid-based designs often fail to meet these demands due to geometric limitations and simulation mismatches that generally limit the use in human implant (grafts). This study presents an alternative design strategy based on open-source artificial intelligence (AI) algorithms to optimize 3D-printed scaffold geometries that could be used to improve prothesis production. Our study employs transparent and reproducible machine learning frameworks, novel lattice structures that were generated with features tailored for bone tissue engineering. To assess their biological function and clinical relevance, the AI-optimized scaffolds were tested in cell culture with mesenchymal stem cells (MSCs). Early findings indicate enhanced cell adhesion compared to conventional scaffold patterns, suggesting this could be used for osteogenic differentiation and ensure greater potential for bone integration. In conclusion, the integration of open-source AI with scaffold fabrication and biological validation offers a cost-effective and accessible pathway for advancing regenerative medicine with high clinical value and short-term applicability. This strategy also opens opportunities for applications of AI beyond tissue engineering, including the broader field of mechanical metamaterials.

Advances in Cell Biology Tools and Technologies - CryoEM/CryoET

B451/P1017
A National Network for Cryo-ET Service
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The University of Colorado Boulder Center for cryo-electron tomography (CCET) is a NIGMS NIH-funded service facility for cellular and molecular 3-D imaging and analyses. We are one part of a larger consortium which features a central hub (Univ. of Wisconsin Madison (MCCET), and three spokes. We provide service and training for individual scientists and labs nation-wide. We operate an Aquilos-2 cryo-FIBSEM (Focused ion-Beam Scanning Electron Microscope), a Leica confocal CLEM (correlative light and electron microscopy), plus a long range of relevant peripheral equipment for specimen vitrification and 3-D analysis by cryo-EM. The FIBSEM microscope is our key instrument for vitrified lamellae preparation which will be further imaged in a transmission cryo-EM. The 3-D structure of specimens will, for most parts, be analyzed by tomographic reconstruction. Furthermore, we host numerous trainees for on-site, hands-on training on the FIBSEM, cryo-CLEM, and other relevant technologies.

B456/P1022
Adeno-Associated Virus Capsid Stoichiometry and N-terminal Flexibility: Alternative Structural Configurations through Cryo-Electron Microscopy of AAV2 Assemblies for Gene Therapy Applications
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Adeno-associated viruses (rAAV) are leading vectors for in vivo gene therapy delivery, with broad tissue tropism, low immunogenicity, sustained gene expression, and successful application in FDA-approved treatments and over 225 clinical trials for more than 30 genetic diseases. The AAV2 capsid consists of three structural co-assembled proteins, VP1, VP2, and VP3, expressed from a single cap gene via alternative splicing and start codons, forming a 60-fold symmetric shell. Their identical C-termini form the capsid shell, while VP1 and VP2 possess N-termini extensions that are essential for endosomal escape and nuclear trafficking, yet their conformational states in the capsid remain unresolved due to their low abundance and intrinsic flexibility. Historically, capsids were believed to have a VP1:VP2:VP3 ratio of 1:1:10, though mass spectrometry and biochemical characterization suggest variability and a lack of structural resolution for N-terminal domains in Cryo-EM analyses. Furthermore, precise determination of this ratio within assembled capsids has remained elusive due to unresolved N-terminal density on the inside of AAV2 near 3-fold axis in prior structural studies. Also, previous single-particle reconstruction of AAV2-AAVR (PKD1-5) revealed a βA leading to a hairpin at residue 237, but in AAV2–AAVR (PKD1-2), this βA configuration is absent, suggesting that the unmodeled density is a partially ordered configuration of upstream residues, likely an alternate to the βA strand that connects into βB at residue 237. In this study, we utilized a baculovirus expression system with optimized expression times, producing assemblies with ratios of approximately 2:3:5 (Day 3) and 1:2:7 (Day 5). Our Cryo-EM analyses revealed previously unmodeled internal density projected from VP1/2 N-termini near the 2-fold axis in Day 3 preparations, and a fully resolved βA strand in Day 5 assemblies, lacking the PKD1-2-induced configuration previously observed. Comparison with wild-type stoichiometry (1:1:10) demonstrated that VP ratios influence capsid maturation and the presentation of structural features crucial for infectivity. Our findings clarify the role of VP1/2 stoichiometry in capsid assembly, provide the first structural framework for

flexible N-termini, and offer new strategies for AAV vector engineering. This work addresses a central question in AAV biology and opens new avenues for optimizing gene therapy vectors.

B443/P1009

AI-Driven Enhancements in FIB-SEM for Advanced Cell Biology Imaging

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Focused ion beam scanning electron microscopy (FIB-SEM) enables detailed, three-dimensional (3D) visualization of cell structures with nanometer-scale resolution. This technique allows researchers to explore cellular morphology and interactions with unprecedented detail, offering insights into complex biological processes that are vital for understanding cell behaviour and pathology. The heterogeneity of biological samples and the process of preparing them for analysis present inherent challenges when acquiring 3D data. Furthermore, 3D imaging is very time-consuming, especially when working with larger volumes of interest. To address these issues, we developed two AI-driven strategies to streamline FIB-SEM data acquisition using the Hydra Bio Plasma-FIB system. The first AI approach, dynamic autofunctions, improves autofocus and image quality routines during cryo-volume data acquisition. This ensures that the image focus adapts to the specific morphology of cryo-samples, enabling reliable cryo-volume imaging. The second AI approach is adaptive scanning, which is optimised for acquiring data from resin-embedded samples. This method enables different areas of the sample to be scanned at higher or lower resolutions. Scanning only the relevant areas at a high resolution and the background at a lower resolution significantly reduces scan times, thus decreasing the total acquisition time for obtaining 3D datasets. Consequently, adaptive scanning increases the throughput of volume imaging on resin-embedded samples. Integrating AI-powered automation with the Hydra Bio Plasma FIB increases productivity, reduces the need for manual intervention and improves three-dimensional imaging for cell biology research.

B457/P1023

Atomic models of the conoid, the cell invasion machinery of the human parasite Toxoplasma gondii

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Apicomplexan parasites, responsible for toxoplasmosis, cryptosporidiosis, and malaria, invade host cells through a unique gliding motility mechanism powered by actomyosin motors and a dynamic organelle called the conoid. Using cryo-electron microscopy (cryo-EM) we determined structures of four essential complexes of the Toxoplasma gondii conoid: the preconoidal P2 ring, tubulin-based conoid fibers, and the subpellicular and intraconoidal microtubules. Our analysis identified 40 distinct conoid proteins, several of which are essential for parasite lytic growth, as revealed through genetic disruption studies. Comparative analysis of the tubulin-containing complexes sheds light on their functional specialization by microtubule-associating proteins, while the structure of the preconoidal ring pinpoints the site of actin polymerization and initial translocation, enhancing our mechanistic understanding of gliding motility and therefore parasite invasion.

B444/P1010

Coordination between mitochondrial fission and mitochondrial DNA replication

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Mitochondria contain their own genomes, which are present in multiple copies and distributed throughout the mitochondrial network. Mitochondrial DNA (mtDNA) encodes for proteins that are involved in oxidative phosphorylation (OXPHOS). Perturbations to mtDNA copy number, distribution, or integrity can lead to OXPHOS dysfunction and are known causes of cardiomyopathies and neurodegenerative disorders. Hence, maintenance of mtDNA is essential for cells and entails proper replication and segregation. Replication of mtDNA has been shown to be linked to mitochondrial division (fission), which ensures segregation of newly replicated mtDNA copies. mtDNA does not encode for fission machineries itself. Fission is carried out via recruitment of cytosolic factors such as DRP1 and endoplasmic reticulum. Although these two events are spatially and temporally coordinated, how mtDNA replication occurring 'inside' the mitochondrial matrix is linked to recruitment of mitochondrial fission factors 'outside' on the mitochondrial outer membrane is not known. Using correlative light microscopy and cryo-electron tomography, we show that regions of mitochondria containing replicating mtDNA nucleoids have distinct membrane ultrastructure. Our quantitative membrane analysis reveals that these mitochondria display larger membrane patches with a consistent outer-inner mitochondrial membrane distance. These patches of membrane, also contain tether-like densities which appear to "zipper" between the two membranes. We also find, that these tethers are enriched in mitochondria containing replicating mtDNA, as compared to those containing non-replicating mtDNA. We propose that these tether-like densities could represent the ATPase Family AAA Domain Containing 3A protein, ATAD3A. Using Stimulated Emission Depletion (STED) nanoscopy, we show that ATAD3A is enriched around replicating mtDNA nucleoids. Collectively, our study identifies characteristic changes in the local microenvironment of mtDNA that are associated with replication. We speculate, that changes in membrane ultrastructure and sequestration of ATAD3A at replicating mtDNA helps in recruitment of the mitochondrial fission machinery.

B442/P1008

Cryo-ET reveals the structural cell biology microsporidian parasites

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Microsporidia are a fascinating group of single-celled eukaryotes that infect a wide range of animal species, including humans. These divergent fungal pathogens employ a unique harpoon-like apparatus called the Polar Tube to invade host cells. The Polar Tube—nearly 50 microns long but just 100 nm in diameter—is fired out of the microsporidian spore over the course of just a few hundred milliseconds. Once fired, the Polar Tube is thought to pierce the plasma membrane of a target cell and act as a conduit for the transfer of the parasite cell, from the spore to the inside of the host cell, which initiates infection. The Polar Tube architecture and its association with neighboring organelles within the parasite cell remain poorly understood. In addition, microsporidia are highly diverged from other extant eukaryotes, lack mitochondria, and represent a minimal eukaryotic cell. Here, we will present our latest work using cryoelectron tomography to investigate the structural cell biology of dormant spores from the human-infecting microsporidian species, Encephalitozoon

intestinalis. Segmentation and subtomogram averaging of the Polar Tube reveal at least four layers: two protein-based layers surrounded by a membrane layer and filled with a dense core. Regularly spaced protein filaments form the structural skeleton of the Polar Tube. Combining cryoelectron tomography with cellular modeling, we propose a model for the three-dimensional organization of the polaroplast, a membranous organelle that surrounds the Polar Tube and is continuous with the outermost, membranous layer of the Polar Tube. Our results reveal the ultrastructure of the microsporidian cell and the Polar Tube invasion apparatus in situ, shedding light on the infection mechanism employed by this neglected human pathogen.

B446/P1012

Improved Quality Control of Cryo-EM Grid Prior to Data Collection

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Thorough screening of cryogenic-electron microscopy (cryo-EM) grids is necessary before high-end data collection experiments. However, this screening is not always performed, and protocols vary across cryo-EM facilities. As a result, data collection sessions on a 300-keV Krios microscope may often involve considerable overhead including time consuming last-minute screening and adjusting the data collection strategy based on observed grid quality. These last-minute changes lead to a reduction in data being collected during a Krios session and require more staff time to ensure services are met. Here, we aim to implement a quality control (QC) protocol based in Smart Leginon, a fully automated multi-grid screening software, before each data collection on a high-resolution EM microscope that will standardize grid screening, improve the efficiency of cryo-EM facility's workflow, maximize the time spent collecting data, and increase staff productivity. Here, we describe the QC protocol implemented at Simons Electron Microscopy Center (SEMC) for all data collection sessions. This protocol encompasses a QC session on a Glacios 200-keV microscope using Smart Leginon, a pre-data collection user meeting, and the data collection session itself. We also show how this new protocol has led to improved service to the user community, the challenges that we have encountered, and how the user base has received and reacted to these changes.

B454/P1020

Improving fluorescence guidance during FIB milling for in situ cryo electron tomography

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Cryo focused ion beam (cryo-FIB) milling for cryo lamella preparation is essential to visualize native protein structure and ultra structure inside cells and tissue by cryo electron tomography (cryo-ET). In order to target specific regions and proteins of interest, fluorescence guidance for FIB milling has emerged as a critical step in situ cryo-ET workflows. Yet, achieving high-precision registration between fluorescence microscopy (FM) and cryo-FIB milling remains a significant challenge. Here we present two complementary strategies to improve fluorescence guidance during cryo-FIB milling. First, we systematically characterized registration accuracy in FIB SEM systems where FM and ion/electron beams operate at distinct geometries, requiring stage movement to switch between the FIB and FM imaging modality. By assessing and quantifying the source of the largest

registration errors, we are establishing correction protocols to enhance alignment precision between FM-identified regions of interest and subsequent milling targets. Previous work has shown that higher targeting accuracy can be achieved through simultaneous FM imaging during FIB milling, eliminating the need for time-consuming and often inaccurate registration steps. However, no commercially available system currently has this capability. The geometry of the Thermo Fisher Scientific Arctis FIB-SEM provides coincidence between the LM and FIB, but simultaneous imaging is not natively supported. We modified operation protocols thereby allowing for real-time feedback from FM imaging and iterative refinement of target selection during FIB milling using open source software tools. Using this approach, we demonstrate a significant improvement targeting primary cilia, a rare and small organelle, for subsequent cryo-ET studies. Together, our approaches provide a more robust framework for improving site-specific lamella preparation across different cryo-FIB SEM platforms, advancing the efficiency of cryo-ET workflows for studying rare and spatially constrained biological structures.

B440/P1006
In situ cryo-ET redefines HPV disassembly and transport paradigms
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Human papillomavirus (HPV) causes 4.5% of all human cancers, although the cellular basis of infection remains unclear. Particularly enigmatic are the virus disassembly and transport mechanisms. Here, we use in situ cryo-electron tomography (cryo-ET) to provide direct visualization of HPV16 during infection. Contrary to models predicting progressive disassembly of the L1 capsid during retrograde trafficking, HPV instead remains intact when it reaches the Golgi en route to the nucleus for infection. In the Golgi lumen, the virus undergoes COPI coat-dependent budding to generate vesicles for transport. In the Golgi and vesicles, HPV is connected to the overlaying membrane by proteinaceous structural bridges, possibly formed by the L2 capsid, postulated to be inserted into host membranes to guide virus trafficking. Notably, when HPV reaches the lysosome, the virus remains intact and infectious, contrary to the prevailing view of lysosomal degradation. Together, these findings illuminate and challenge models of HPV entry, revealing the resilience of the virus capsid to disassembly during retrograde trafficking, a coat-dependent budding mechanism for transport, and the lysosome as a reservoir for infectious HPV.

B441/P1007
Investigating Lysosomal Ultrastructural Diversity at High Resolution
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The transient receptor potential mucolipin 1 (TRPML1) ion channel is implicated in diseases with altered lysosomal homeostasis, including Alzheimer's and Parkinson's disease, cancers, Duchenne muscular dystrophy, and lysosomal storage disorders. Lysosomes, although often depicted as spherical, can elongate into tubules or form tubular projections under conditions such as immune activation, nutrient deprivation, and aging. Increasing evidence suggests these tubular lysosomes are heterogeneous and functionally distinct, yet their subpopulations remain poorly defined. The objective of this study is to determine how TRPML1 activity influences the structural diversity of tubular lysosomes. To address this, I am using a combination of live-cell fluorescence imaging, focused ion beam scanning electron microscopy, and electron tomography to characterize lysosomal tubulation. Light microscopy confirmed that TRPML1 activity modulates lysosomal tubulation, while electron tomography is uncovering the subpopulations of tubular lysosomes in situ. Defining

these subpopulations advances our understanding of lysosomal tubulation and clarifies TRPML1's role in lysosomal biology.

B455/P1021
Nanoscale analysis of human G1 and metaphase chromatin in situ
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Chromatin organization plays an important role in cell function. Although many models of chromatin structure have been proposed, it is still not clear how chromatin is organized in situ. In this study, we used cryo-electron tomography (cryo-ET) to examine chromatin structure inside human RPE-1 cells in G1 and metaphase. Subtomogram averaging and classification analysis of our cryo-tomograms allowed us to visualize the chromatin at the nucleosome level. Canonical nucleosomes are concentrated in chromatin domains in G1 and in metaphase chromatids. Our classification analysis detected multiple species of mononucleosomes, and a stacked dinucleosome class average for both G1 and metaphase. We did not detect any other multi-nucleosome class average in our analysis beyond the stacked dinucleosome, suggesting that nucleosome packing in chromatin is largely heterogenous. The accurate detection and assignment of most of the nucleosome densities in the cryo-tomograms is important for a deeper analysis of chromatin packing. However, subtomogram analysis of a crowded environment such as those found in chromatin domains and metaphase chromatids is challenging. Our future analyses will aim to tackle this challenge to facilitate comparative studies of chromatin packing between cells of different states.

B448/P1014
Nanoscale Biopsy of Biomolecular Condensates: SOLIST Unlocks Phase Separation in Cancer and Beyond
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Biomolecular condensates (BMCs) formed by liquid–liquid phase separation (LLPS) regulate key cellular processes and are implicated in diseases from cancer to neurodegeneration. Understanding their molecular organization in a native-like state requires high-resolution techniques such as cryo-electron tomography (cryo-ET). However, classical cryo-ET workflows suffer from low throughput, ice contamination, and poor stability, often restricting its use to simple cell culture. Furthermore, classical approaches would destroy the ultrastructure of in vitro reconstituted systems, where all components can be precisely controlled, which would allow us to finally dissect LLPS both biochemically and structurally. To overcome these limitations in cryo-ET sample preparation, we recently developed Serialized On-Grid Lift-In Sectioning for Tomography (SOLIST). SOLIST is a high-efficiency cryo-lift-out method that produces up to tenfold more lamellas per lift-out session, reduces sample contamination, and enables subnanometer resolution in tissues and organoids within a fraction of the time required by older methods. By bridging nanoscale resolution with mesoscale context, SOLIST makes complex, medically relevant systems accessible to cryo-ET on a routine basis. Here, we extend SOLIST with a 3D-correlative targeting pipeline to study BRD4-NUT-induced chromatin condensates, an oncogenic BMC model relevant to NUT carcinoma. Using high-pressure freezing and fiducial-guided fluorescence targeting, we selectively mill and image individual condensates in lamellas – a level of targeting not achievable before SOLIST. Tomograms revealed phase-separated droplets with compact chromatin

architecture. Notably, the phase transition is very sharp and different from previous reports with sponge-like structures, which we attribute to artifacts due to sample deformation while plunge freezing in these earlier studies. While our current resolution is still limited, our work is the first demonstration of molecular-resolution LLPS imaging with high-pressure freezing and targeted lift-out. These advances establish SOLIST as a versatile platform for a “biopsy at the nanoscale”. We combine tissue-scale reach with molecular detail and open the door to in situ structural studies of phase separation in both reconstituted systems and native patient-derived samples.

B450/P1016

Recent Advances in Cryo Electron Microscopy and Tomography

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In the recent years, cryo-electron microscopy (cryo-EM) single-particle analysis (SPA) has greatly benefited from a multitude of technological advances cumulating in breaking the atomic-resolution barrier for biological macromolecules. Since then, structural insights by cryo-EM have led to driving rational design of new vaccines and therapeutics due to high throughput and high-resolution screening and data collection technologies. Additionally, cryo-electron tomography (cryo-ET) has become an indispensable tool for structural cell biology. Here we elaborate on the latest Thermo Fisher Scientific innovations for 300kV cryo electron microscopy a.k.a. Krios 5 for SPA and cryo-ET to advance more workflow efficiency and performance. Our ongoing mission is to further improve the connectivity between the cryo-FIB and cryo-TEM for an even more streamlined tomography workflow that is safer, faster and cleaner. Data acquisition and processing can be a significant cryo-ET bottleneck. Our integrated solution, combining Thermo Scientific Tomography 5 and Tomo Live, automates pre-acquisition tasks, enhances productivity with predictive tracking and enables rapid quality assessment. Robust and optimized motion correction helps produce crisp tomograms and image quality needed for sub-tomogram averaging. Results are displayed with new quality metrics on a web platform, making the workflow user-friendly and facilitating structural biology advancements.

B452/P1018

Sisters, Not Twins: Unique Regulatory Mechanisms in P-Rex RhoGEFs Investigated Using Integrative Structural Biology

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Rho guanine-nucleotide exchange factors (RhoGEFs) accelerate the function of small GTPases to drive cell motility, proliferation, and growth. The phosphatidylinositol-3,4,5-trisphosphate (PIP3)-dependent Rac exchanger (P-Rex) subfamily of RhoGEFs contains P-Rex1 and P-Rex2 which, when misregulated, contribute to diseases like diabetes, heart disease, and cancer. Despite being attractive therapeutic targets, the mechanisms governing P-Rex regulation remain poorly understood, limiting the development of effective isoform-specific inhibitors. P-Rex proteins share conserved upstream signaling pathways, domain architecture, and high sequence similarity, suggesting that they may adopt similar structures and may have similar regulatory mechanisms. However, they differ in interaction with regulators such as PTEN and in substrate specificity, raising questions about why they engage different binding partners in ways that cannot be explained solely by tissue- or cell-type-specific expression. P-Rex protein activity is modulated by accessory domains which allow them to remain in a cytosolic, autoinhibited state until activated by membrane-bound factors PIP3 and Gβγ.

Although the P-Rex1 autoinhibited state is structurally and biochemically well-characterized, there is little such information regarding P-Rex2. Previously, our lab determined a moderate resolution cryo-EM structure of autoinhibited P-Rex1 which identified key sites that maintain autoinhibition. Here, we determined the cryo-EM structure of autoinhibited P-Rex2. To our surprise, our low-resolution structure and biochemical data allude to potentially divergent regulatory mechanisms in P-Rex2. This structure reveals that the N-terminal module, which includes the catalytic core, maintains a similar arrangement to that of P-Rex1, but is oriented differently with respect to the other accessory domains. Guided by P-Rex1 regulation within the N-terminal domains, we mutated homologous residues in P-Rex2 and contrasted which residues are required to maintain autoinhibition within the N-terminus. Hydrogen-deuterium exchange mass spectrometry showed that P-Rex2 is not stabilized by the PIP3 head group, IP4, like P-Rex1, suggesting that long-range interactions between distal accessory domains and the catalytic core may be less important in P-Rex2 autoinhibition. Together, these experiments uncover the fundamental differences in autoinhibition between P-Rex1 and P-Rex2 and provide the first evidence that P-Rex isoforms exhibit distinct conformations to mediate autoinhibition. Determining the molecular differences between P-Rex proteins will significantly advance the field by enabling rational design of isoform-specific inhibitors with therapeutic potential.

B449/P1015
Streamlining Cryo-Electron Tomography for Cell Biology
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Cryo-electron tomography (cryo-ET) enables visualization of organelles and cellular structures in their near-native state at a resolution that far surpasses the limits of light microscopy. Cryo-ET workflows have evolved to the point that even non-expert users can produce sufficient cryo-lamellae to generate statistically relevant data. Our latest advances have addressed key challenges such as sample contamination, data acquisition speeds and workflow efficiency, thereby improving data quality and throughput for cell biology. The vacuum capsule has been developed to seamlessly link the Arctis cryo-plasma-FIB instrument with the TEM, minimizing ice contamination during transfer. This innovation in lamella transfer is crucial for improving both the quality and throughput in tomography, as it allows the full surface area of the lamellae to be used. When combined with the Krios 5 cryo-TEM, the vacuum capsule ensures a streamlined workflow with efficient transfer of large numbers of lamellae. These developments, along with other advances such as the use of user-friendly software for lamella preparation, mean that cryo-ET is now more accessible to cell biologists The streamlined cryo-tomography workflow will enable new quantifiable insights into cell morphology and interactions, thereby advancing cell biology research.

B445/P1011
Structural Insights into Lysosomal Supercomplexes and Niemann-Pick Type C Pathology
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Lysosomes are central hubs for cellular recycling and metabolic signaling, functions that depend on specialized dynamic protein organization at the lysosomal membrane. A key player in both processes is the V-ATPase,

which is proposed to form higher-order supercomplexes with mTORC1, the cholesterol exporter NPC1, and other regulatory proteins. NPC1 is of particular interest, as loss-of-function mutations cause Niemann-Pick Type C, a devastating lysosomal storage disorder. How lysosomal protein complexes are organized at the molecular level, and how this organization underlies lysosome function and disease, remains a major open question. To probe lysosome function, we used fluorescence lifetime imaging microscopy and found that lysosomal pH dropped in NPC1 knockout cells, an unexpected result given reports of lysosomal leakage. Cryo-blockface imaging revealed large vesicular Lysotracker-positive aggregates in NPC1-deficient cells. Cryo-electron tomography (cryoET) showed that these aggregates consist of individual lysosomes enclosed within a larger membrane compartment, suggesting lysophagy. CryoET further identified mitochondria with altered membrane morphology, likely reflecting cholesterol imbalance and impaired lysosome-dependent mitophagy. To investigate protein organization directly, we developed an affinity-grid system that captures intact lysosomes from human cell lysates, enabling high-throughput cryoET. Combined with an automated pipeline, we now collect >2,000 high-quality tomograms per week, yielding a dataset of ~20,000 tomograms, likely the largest of its kind. To pinpoint protein complexes, we designed probes for cryo-correlative light and electron microscopy (cryoCLEM). This approach revealed V-ATPase and mTORC1 in multiple assembly states, including larger assemblies consistent with supercomplex formation. Dual labeling showed that V-ATPase, mTORC1, and likely additional proteins co-localize within the same supercomplexes, providing the first direct structural evidence for their existence. We are now developing automated computational workflows for subtomogram averaging, aiming to achieve high-resolution maps of lysosomal supercomplexes. Together, these advances establish a framework for analyzing protein organization at the organelle scale and uncovering molecular principles of lysosome function in health and disease.

B438/P1004

Unveiling structural heterogeneity in Leishmania flagella through cryo-electron tomography

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Introduction: Leishmania are unicellular parasites from the Excavate superkingdom that infect humans and other mammals through the bite of sandflies causing leishmaniasis, a tropical disease that leads to skin sores, ulcers, fever, low blood counts, and death. Protruding from the Leishmania cell body is a single motile flagellum with the conserved [9 + 2] arrangement of microtubules within its axonemal core structure. The Leishmania flagellum is essential for the parasite's life cycle and infectivity, yet its ultrastructure has not been studied in high resolution. Better understanding the structures within the Leishmania flagellum could reveal both evolutionary conserved and parasite-specific structures that could be targets of future therapeutic intervention. Methods: Leishmania flagella were isolated using Ca2+ shock and mechanical agitation before cryo-sample preparation by plunge freezing. We then performed cryo-electron tomography and subtomogram averaging on the isolated Leishmania flagella to characterize the 3D structure of the axonemal 96 nm repeat, central pair complex and axoneme-associated complexes with molecular resolution. Results: The flagellar structures of Leishmania overall resemble other organisms from across the eukaryotic tree of life that have previously studied. For example, the radial spoke heads appear broad and interconnected like in Tetrahymena (TSAR) cilia and Chlamydomonas (Archaeplastida) flagella [1, 2]. Leishmania flagella have two-headed outer dynein arms, similar to unicellular and multicellular Opisthokonta, which includes animals. Further, Leishmania displays a prevalent A-tubule hole like the doublet specific A-tubule hole found in Choanoflagellate flagella. The nexin-dynein regulatory complex structure also appears conserved. On the other hand, Leishmania flagella also exhibit several unique features such as asymmetric radial spoke heads, extra A- and B-tubule holes, and doublet-specific structures, such as connections to the extra-axonemal paraflagellar rod on doublets 4-7,

resembling other Euglenoids. Conclusion: Our detailed structural findings provide both new insights into Leishmania biology with practical implications for future therapeutic research, while simultaneously highlighting evolutionary conserved and derived features shedding light on cilia evolution throughout the eukaryotic tree of life. The unique doublet-specific complexes, the paraflagellar rod, and the asymmetric radial spoke heads suggest a divergent evolutionary step with unique flagellar characteristics of the Excavates. These distinct features not only deepen our understanding of eukaryotic evolution but could be exploited as novel therapeutic targets, offering new avenues for combating Leishmaniasis.

B447/P1013
Using VLP in Ecoli as a new benchmark for Cryo-ET
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Cryoelectron tomography is an incredibly valuable modality for acquiring cellular context at sub-nanometer resolution in a frozen state. However, in order to achieve high resolution structures of protein complexes, a series of tools are needed from sample preparation to data processing. This creates the need for full workflow benchmarking. An adequate sample for benchmarking requires 1. High symmetry structures 2. High-throughput and reproducibility of the sample 3. Relatively easy identification of cellular structures by a human for training models. Through a collaboration with the Finn Research Group at GeorgiaTech, we introduce virus-like-particles (VLPs) over-expressed in Escherichia coli as a new cryoET benchmark. VLPs are highly symmetrical with icosahedral structures being dominant, and very stable, which is ideal for benchmarking Cryo-ET processing pipelines. Inoculating the VLP into E.Coli creates a more challenging sample for machine learning applications like particle picking and processing pipelines like sub-tomogram averaging due to the cellular crowdedness . We aim to benchmark focused-ion beam milling (particularly using waffle protocol), data acquisition (i.e. tilt scheme/dosing), particle picking, sub-tomogram averaging, and phase plates.Moreover, these datasets will be publicly available on the cryoET Data Portal.

B439/P1005
Visualization of lysosomal membrane proteins by cryo electron tomography
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Lysosomes are essential organelles for cellular homeostasis and signaling, with dysfunction linked to neurological disorders, lysosomal storage diseases, and cancer. While proteomics has advanced our understanding of lysosomal composition, the structural characterization of lysosomal membrane proteins in their native environment remains a significant challenge. Here, we developed a cryo electron tomography workflow to visualize lysosomal membrane proteins within intact, native lysosomal membranes. We isolated endolysosomes by independently targeting two lysosomal membrane proteins, transient receptor potential mucolipin 1 and transmembrane protein 192, enriching organelles that exhibited the expected morphology and proteomic composition of the endolysosomal system. Sub-tomogram averaging enabled the structural refinement of key membrane and membrane-associated proteins, including V-ATPase, Flotillin, and Clathrin, directly within the lysosomal membrane, revealing their heterogeneous distribution across endolysosomal organelles. By integrating proteomics with structural biology, our workflow establishes a powerful platform for

studying lysosomal membrane protein function in health and disease, paving the way for future discoveries in membrane-associated lysosomal mechanisms.

Advances in Cell Biology Tools and Technologies - Emerging Model Systems

B538/P1102
Chlamydomonas Cellular Phenotypes Enable in vivo Validation of Computationally Designed Therapeutic ADA1 Variants
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Adenosine deaminase (ADA) deficiency causes severe combined immunodeficiency, and current treatments include enzyme replacement therapy with immunogenic bovine proteins. To develop improved therapeutic variants, robust model systems are needed for testing rationally designed enzymes in vivo. We used Zoogle (zoogle.arcadiascience.com), a computational dataset that selects model organisms based on conserved protein characteristics rather than sequence similarity, to identify Chlamydomonas reinhardtii as an optimal system for studying human ADA1 function. This approach can identify effective models that traditional phylogenetic methods might overlook. We characterized Chlamydomonas ADA1 mutants and found clear phenotypic defects in motility and cellular metabolism, particularly altered starch accumulation under nutrient stress. We established quantitative phenotyping approaches, including high-throughput motility tracking, metabolic profiling via Raman spectroscopy, and biochemical staining to assess cellular function. These multi-modal readouts provided robust, reproducible measures of ADA1 activity in living cells. We're validating this system using wild-type human ADA1 and candidate variants designed through machine learning approaches to enhance stability and improve therapeutic properties. Initial results demonstrate that the algal system can detect functional differences in ADA1 variants, establishing a platform for screening computationally designed proteins. This approach enables systematic evaluation of engineered enzymes in a physiologically relevant cellular context. Our work establishes Chlamydomonas as an effective model for human metabolic enzymes and demonstrates the power of protein characteristic-based organism selection over traditional phylogenetic approaches. This validation platform enables rapid, cost-effective screening of designed therapeutic proteins before advancing to mammalian studies, potentially accelerating the development of next-generation enzyme replacement therapies for genetic diseases.

B537/P1101
Establishment and characterization of a fetal fibroblast cell line for genetic modification in goats
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The objective of this study was to establish and characterize a fetal fibroblast cell line that can be used for introducing and verifying genetic modifications, prior to somatic cell nuclear transfer, in goats. The early stage (~35dpc) fetuses were procured from a commercial abattoir, washed twice with DMEM media supplemented with 10% fetal bovine serum, 50 units/mL of penicillin, 50 µg/mL of streptomycin and 2.5 µg/mL of fungizone. Fetal skin was chopped into 2-3 mm2 explants, adhered onto 60mm dishes, and cultured at 37°C, 5% CO2 in a humidified environment. Outgrowth of cells around the explants began to appear within 24 h of culture and reached 80-90% confluence by day 6 of culture. Morphologically, the outgrowing cells were fibroblast-like with occasional epithelial cell colonies. Outgrowth was observed in all (100%) of explants. Selected cultures were

established and purified by serial passaging using selective trypsinization (to remove epithelial cells). The purified fibroblast-like cells were cryopreserved at p3 level in multiple vials (1x105 cells/vial). Mean ± SD of post-freezing cell-viability was 95 ± 2.2 %. Immunofluorescence analysis of cultured cells labelled with anti-vimentin-FITC (fibroblast specific marker) antibodies revealed positive signals in 100% of cells. Growth curve generated from passage 4 cultures revealed standard lag, log and stationary phases of a typical mammalian cell with a calculated population doubling time of 32.4 ± 0.8 h ($p < 0.05$). Cytogenetic analysis performed on twenty G-banded metaphase cells revealed a normal karyotype (60, XX) without any genetic aberrations. Mean ± SD of transfection of cells using 1.0 µg of gmaxGFP plasmid and 4D nucleofector system was 80.6 ± 1.21 % ($p < 0.05$). In conclusion, we have established a highly proliferative fibroblast cell line from early-stage fetuses in goats, which is a potential tool to introduce and verify genetic modifications in goat genome prior to nuclear transfer.

B539/P1103

Human iPSC and 3D organoid models as next-generation in vitro platforms for functional and toxicological assessment

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The FDA Modernization Act 3.0 encourages the reduction of animal testing in drug discovery and the adoption of New Approach Methodologies (NAMs) such as human induced pluripotent stem cells (hiPSCs), and organoid systems. hiPSCs and their derived organoid models overcome key limitations of 2D cultures and animal studies by recapitulating human tissue architecture and patient-specific genetics, offering physiologically relevant platforms for disease modeling and drug assessment. At Greenstone Biosciences, we leverage a biobank of over 2500 patient-specific hiPSC lines encompassing diverse genotypes, disease backgrounds, and demographics. From these, we routinely generate cardiomyocytes, hepatocytes, pancreatic progenitors, neuronal progenitors, enterocytes, immune cells, and fibroblasts as well as multicellular organoids representing cardiac, hepatic, intestinal, and pancreatic tissues. These systems provide a versatile foundation for investigating cellular mechanisms across diverse tissues and for advancing translational research in inflammation, fibrosis, and metabolic regulation. Using this resource, we have established high-throughput screening (HTS) and high-content analysis platforms to identify and characterize anti-inflammatory and anti-fibrotic compounds. These efforts have led to two small-molecule candidates, GSB-D170 and GSB-D341, currently in IND-enabling preclinical development. Functional assays on iPSC-derived cells, include electrophysiology and contractility (cardiomyocytes), gut permeability (enterocytes), and glucose sensitivity (pancreatic cells). We integrate multi-omics (transcriptomics, proteomics, and metabolomics), AI/ ML-based analytics, and organ-on-chip technologies to enhance predictive accuracy for efficacy and toxicity endpoints. hiPSC-derived models retain native transporter expression, metabolic enzymes activity, and pharmacokinetic properties supporting ADMET (absorption, distribution, metabolism, and excretion and toxicity) evaluations. They reliably predict drug-induced cardiotoxicity (hERG/ProArrhythmia), hepatotoxicity, and genotoxicity – critical factors in pre-clinical decision making. Together, these platforms represent the next generation of human-relevant models that bridge pre-clinical and clinical translation, offering scalable path to reduce development costs, improve safety prediction, and accelerate the discovery of effective therapeutics.

B535/P1099

In vivo Time-resolved Chemical Labeling of Endogenous Proteins: Measuring Histone Synthesis Rates in Mice

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Background/Purpose: Targeting histone synthesis to impair cell cycle progression is a promising anti-cancer approach, but concerns remain about toxicity of targeting cell-essential histones. Tools for studying histone dynamics in vivo are lacking, leading to uncertainty about the therapeutic window of targeting histones in adult animals. To address this gap, we developed chemical labeling tools and engineered mice to express SNAP-tagged histone H4 from the endogenous loci. This model enables time-resolved, tissue-wide imaging of newly synthesized histones in animals that can be leveraged to evaluate histone synthesis as a druggable vulnerability in cancer. Methods: A SNAP tag was inserted immediately upstream of the coding region of the H4C3 and H4C1 genes to produce functional H4-SNAP fusion proteins. Expression was validated across multiple tissues by western blotting. For in vivo pulse-chase labeling, we synthesized and systemically delivered TMR-Star (fluorescent SNAP substrate) and BG-Nor1 (non-fluorescent blocking substrate) to label nascent histones while quenching pre-existing ones. Pharmacokinetics were determined, and labeling efficacy was tested across multiple timepoints and dosing conditions. Results: Fluorescent substrate labeling produced robust and widespread histone signal across all organs consistent with widespread histone expression for H4C3, but a more restricted expression of H4C1. A single dose of TMR-Star was sufficient to saturate SNAP tags, enabling simplified experimental workflows. Pulse-chase labeling with blocking reagent first followed by fluorescent label selectively marked newly synthesized histones, allowing us to track cell division dynamics across tissue types. This allowed assessment of histone synthesis rates across organs in vivo for the first time. Histone synthesis rates were strongly correlated with proliferative tissues with highest levels detected in the spleen and epithelial compartment of the intestine. In contrast, other organs composed of quiescent cell types (e.g. liver, lung, heart) had minimal detected histone synthesis over the 24-hour labeling period. Induction of liver damage with toxins caused a pronounced spike in histone synthesis rates during recovery periods. This SNAP-tagged H4 mouse model is a powerful tool for tracing histone synthesis and turnover in vivo. It enables precise labeling of nascent histones with temporal and spatial resolution, offering new opportunities to dissect histone biology in development, disease, and regeneration. Finally, this system can be generalized to measure in vivo protein synthesis rates for any protein.

B536/P1100

Motion-capture analyses of a zebrafish model of the neuro- and cardio-degenerative disorder friedreich's ataxia

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Friedreich’s Ataxia (FA) is an inherited neuro- and cardio-degenerative disorder caused by mutations in the gene encoding the protein frataxin. Frataxin localizes to mitochondria and functions in the biogenesis of iron-sulfur-clusters, which are prosthetic groups important for many proteins, including in the mitochondrial electron-transport chain. Signs and symptoms of FA include ataxia, hypertrophic cardiomyopathy, fatigue, and exercise intolerance. We used CRISPR/Cas9 to mutate the zebrafish (ZF) frataxin gene. Phenotypes of homozygous mutants include small size, an increased heart-to-body-weight ratio, and starvation sensitivity. In this study, we used motion-capture analyses to quantify movement abnormalities that might be used to test

therapeutics for reversal. For these motion-capture analyses we used a Basler Gen1Cam. After 15 minutes of acclimation, data was collected at 25 Hz while the ZF were subjected to three light/dark cycles (10 minutes per condition). Mutant and wildtype sibling pairs were studied concurrently to control for environmental variation. Measures of velocity (cm/s) and direction heading (degrees) were collected. To measure turn angles, direction change (degrees) was calculated as the smallest arc between adjacent direction headings. Data were separated by lighting conditions. Histograms of the data displayed frequency distributions of each measure from all time points. The experiment had n = 5 for both the wildtype and mutant groups; however, this included technical replicates. All analyses were done in R. Velocity histograms in the light condition revealed distinct active and resting states. Mutants spent more time in the resting state and had lower mean active-state velocities than wildtypes, possibly reflecting fatigue. Direction change histograms in the dark condition showed higher standard deviations among mutants and a greater frequency of larger magnitudes of direction change compared to wildtypes, possibly reflecting ataxia. Group level differences in summary statistics of these measures (percentage of time spent in the resting state for velocity and percentage of direction change values less than 8 degrees in magnitude) were statistically significant ($p < 0.05$). Limitations of this study include a small sample size and the use of technical replicates. If confirmed, these phenotypic differences would further validate our mutant zebrafish as a model for FA and could be used to test therapeutics.

B540/P1104

Phenotypically-relevant patient-derived 3D cell models to interrogate personalized medicine approaches at scale

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The complexity and heterogeneity of cancers both intra- and inter-patient have led to the precision medicine paradigm of cancer therapy where treatment is personalized to the individual patient. Recent clinical trials such as the I-PREDICT study [1] have underscored the benefits of approaches matching combination therapies to the patient's molecular alterations, leading to improved disease control and survival rates. Tumoroids are patient-derived cancer cells that grow as 3D self-organized, multicellular organoids that maintain key characteristics of the patient tumor of origin including molecular features such as genotype and gene expression, and biological behaviors. Whilst these models serve as valuable tools for studying tumor biology and responses of patient-derived cells to various anti-cancer therapies, the establishment and maintenance of tumoroids has historically been difficult and labor intensive, limiting more widespread use. Here, we utilize the Inventia RASTRUM™ platform and OncoPro™ colorectal cancer (CRC) Tumoroid Cell Lines (ThermoFisher Scientific [2]) to easily create plug and play patient-derived 3D cell models based on synthetic PEG-based hydrogel matrices, which were tuned to mimic the tissue stiffness and ECM composition of CRC tumors. We used this workflow to evaluate how these 3D models maintain the clinicopathological features of patient-derived tumoroid cells, as well as molecular features and cellular signaling native to the tumor and its microenvironment. We further demonstrate the utility of this approach for throughput drug screening and predicting personalized therapy responses. Overall, our approach provides a scalable framework for evaluating precision medicine approaches using clinically-relevant patient-derived tumoroids. Future work will focus on the development of processes to support direct dissociation and generation of patient-derived models from biopsies from a variety of cancer types and larger scale utility of the RASTRUM platform in workflows that could be leveraged to inform treatment decision making. References 1. Sicklick JK et al. Molecular profiling of cancer patients enables personalized combination therapy: the I-PREDICT study. Nat Med. 2019 May;25(5):744-750. 2. Paul, C.D. et al. Long-term maintenance of patient-specific characteristics in tumoroids from six cancer indications. Scientific Reports 2025, 15(1):3933.

B461/P1820
Simplifying Multigene Vector Design and Construction for Streamlined Investigation of Complex Cellular Pathways
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Many cellular functions involve complex regulatory pathways that require targeting multiple genes to understand these processes at the molecular level. However, constructing multigenic vectors is hindered by high costs, limited precision, off-target effects, and the difficulty of troubleshooting increasingly complex designs. In addition, the size and complexity of multigene vectors are challenging for modern DNA synthesis methods, necessitating labor-intensive and time-consuming cloning procedures. To overcome these barriers, a plasmid library was developed that simplifies the construction of multigene vectors by using standardized modular genetic elements and restriction enzyme-based cloning. In this study, we evaluated the feasibility of using this plasmid library and Zymo Research's cloning tools to build a four-gene reporter vector containing rLUC, fLUC, GFP, and RFP. Restriction-digested DNA inserts and plasmids from the library were purified using a gel extraction method with micro-elution spin columns, yielding concentrated, ultra-pure fragments ready for ligation. Recombinant plasmid was transformed into competent E. coli cells that do not require heat shock, enabling efficient plasmid propagation. Highly concentrated, endotoxin-free plasmid DNA was then isolated using a rapid spin-column method, ensuring suitability for downstream transfections. To evaluate functionality and cloning success, monogenic, bigenic, and quadgenic constructs were transfected into HEK293T cells. Fluorescence and luciferase assays revealed that all constructs exhibited successful reporter gene expression. Additionally, rLUC expression was consistent across mono-, bi- and quadgene constructs, and fluorescence microscopy verified colocalization of GFP and RFP expression. These results demonstrate the effectiveness and efficiency gained when using this platform for the construction of multigenic vectors. This streamlined approach for targeting multiple genes enables efficient analysis of complex regulatory pathways, facilitating advancements in cell biology.

Advances in Cell Biology Tools and Technologies - Emerging Tools and Technologies

B517/P1081
A 3d printed modular microscope for in-incubator time-lapse fluorescence imaging
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We aim to develop a low-cost fluorescence microscope capable of obtaining time-lapse images of cell biology processes in vivo within a cell incubator setting. The commercial systems for fluorescence microscopy are expensive and require extensive setup for use in incubators. In recent years, 3D printing technology has enabled affordable alternatives to traditional frame structures and rigs, based on which multiple open-source frameworks have emerged for building low-cost, laboratory-grade microscopes. We used the open-source UC2 framework to develop a modular microscope tailored to fluorescent imaging within an incubator. We adopted standardized modules for the motor-controlled objective z-stepper, the Raspi camera, and a mirror, and developed custom-built modules for the laser, neutral density filter, beam expander, dichroic beamsplitter, tube lens, and sample holder. The initial version was built for benchtop imaging in a minimalist approach

containing only a sample holder, objective, and Raspi camera module. This allowed us to optimize the software for controlling the motor to focus the objective. In subsequent iterations, we developed customized drop-in modules for fluorescent imaging. The current version features infinity optics, allowing placement of additional optical components between the camera and the objective. These modules can be combined in multiple ways based on the physical constraints of the incubator and the desired light path. The assembled microscope is controlled by a Raspberry Pi device. We developed Python code to control the objective z-stepper and the laser, as well as to coordinate between the two modules for automated time-lapse image capture. We are testing the system to image the cellular response to wounding in an ex vivo chick post-cataract surgery explant system, an established experimental model for studying the lens response to cataract surgery-induced wounding. We are visualizing cell boundaries using fluorescently conjugated Wheat Germ Agglutin, a lectin that demarcates cell membranes by binding GlcNAc and sialic acid residues. We are collecting images of the lens cellular response to wounding over 3 days, a typical period for wound closure. In summary, we demonstrate a modular, reconfigurable, 3D-printed microscope custom-built for in-incubator time-lapse fluorescence imaging for cell biology applications.

B515/P1079

A Method to Identify Direct Kinases for Orphan Phosphorylation Sites in Cancer

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Kinase signalling is critical for the underlying biology of the majority of cancers, and kinase inhibitors constitute a large proportion of targeted cancer therapies. However, the kinases responsible for the phosphorylation of most of the thousands of substrate phosphorylation sites in cells remain unknown. To better understand cancer biology, and to improve identification of therapeutic targets, we have developed a novel approach, KiPIK, to identify cellular kinases responsible for the phosphorylation of specific sites. The technique employs cell extract kinase assays, with peptide substrates, in the presence of a library of kinase inhibitors to obtain a “fingerprint” characteristic of the relevant kinase. This is then compared to the fingerprints of over 400 human kinases, derived from in vitro kinase inhibitor profiling data, to identify candidate direct upstream kinases for the orphan phosphorylation site. Currently, the method uses phospho-specific antibodies to measure substrate phosphorylation, which limits its application. This project aims to enhance the utility of KiPIK by employing mass spectrometry as a label-free method to broadly measure substrate phosphorylation. As a first example, we are investigating Mps1/TTK, a mitotic kinase required for accurate chromosome segregation that is the target of current drug trials in Acute Myeloid Leukemia (AML) and other cancers. Recent work has implicated Mps1 residues S436 and S821 as targets of the Akt signalling pathway in AML, but the direct kinases for these phosphorylation sites are unclear. We could not detect S436 phosphorylation in our system, but conventional KiPIK assays identified Cyclin B-Cdk1 as the likely kinase for Mps1 S821 in mitosis. Follow-up experiments confirm that Cyclin B-Cdk1 phosphorylates recombinant Mps1 in vitro, and that Cdk1 inhibition reduces S821 phosphorylation in cells. We are now developing assays suitable for mass spectrometry-based analysis to identify kinases responsible for phosphorylation of multiple sites on Mps1 using KiPIK.

B509/P1073

A plasmonic imaging method for real-time spatial profiling of extracellular signaling molecules during live-cell timelapse microscopy using light microscopes.

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The ability to directly observe cell-secreted proteins with spatial resolution has long been a barrier in cell biology and disease research. Conventional assays such as ELISA quantify secreted factors only in bulk, obscuring heterogeneity and preventing insight into how individual cells contribute to their microenvironment. Herein we present a nanoplasmonic imaging platform that enables real-time, label-free detection of secreted proteins with spatial resolution compatible with live-cell microscopy on standard light microscopes. Our approach employs patterned nanoplasmonic sensors integrated into a microscopy-compatible substrate, allowing direct visualization of secreted molecules as they interact with the surface using light microscopes common to life science laboratories. To reduce the data processing bottleneck associated with time-lapse microscopy, we developed two automated analysis pipelines that (1) determine the fractional occupancy of the detected analyte and (2) employ optical flow computer vision with machine learning for segmentation and morphometric parameterization of cells. Combined, this plasmonic imaging hardware and dual analysis approach enables secretion-phenotype multivariate correlation. By bridging the gap between bulk secretion assays and cellular imaging, nanoplasmonic imaging offers a versatile platform for both fundamental biology and translational research, with applications in drug discovery, regenerative medicine, and precision oncology.

B514/P1078

A universal platform for high-throughput and spatially controlled electrotaxis

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Bioelectricity plays a critical, but underexplored, role beyond neuromuscular systems as local ion fluxes can lead to electric fields that alter a range of cellular processes. The most well characterized of these is electrotaxis—directed cell migration along direct-current (DC) electric field gradients—which plays a key role in complex biological processes such as wound healing, development, and cancer metastasis. Unfortunately, such processes cannot be studied using any of the standard neuromuscular electrical apparatus as these generate toxic redox when used for DC stimulation. As a result, the field of DC electrobiology has often relied on hand-made, low-throughput equipment that makes reproducibility, throughput, and advanced control difficult. These issues, in turn, have substantially raised the barrier to entry and slowed progress. Here, we present SCHEPHERD – a reproducible and broadly accessible electrotaxis platform for high throughput, precise experiments. A key element of SCHEPHERD is that it is fully integrated and self-sufficient without requiring anything else to run. To achieve this, we combined shareable 3D printed parts with an open-hardware, stand-alone, custom electronics system to drive up to 8 simultaneous experiments under computer control all through a simple GUI interface. We have validated SCHEPHERD by reproducing nearly all previously published in vitro electrotaxis experiment with 2D engineered tissues, 3D cysts, and organotypic culture models. Beyond validation, SCHEPHERD has led to new insights into migration dynamics, highlighting how direction and cell migration speed seem to be independently controllable with electrical stimuli. SCHEPHERD also allows real-time confocal imaging of cytoskeletal dynamics, allowing us to observe the F-actin network reconfiguring in response to dynamically changing electric fields. Finally, SCHEPHERD allows creative shaping of the electric field shapes, allowing us to demonstrate surprisingly complex patterns of migration within single tissues. Our goal is to get SCHEPHERD into the hands of biology groups that take advantage of it to better understand and dissect the exciting roles of bioelectricity outside the neuromuscular system.

B526/P1090

Acute and reversible control of protein trafficking using biotinylated halotag ligands

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Protein dimerization systems enable manipulation of protein localization by tethering targets to “hooks” on organelle membranes. The Retention Using Selective Hooks (RUSH) assay is a key example: an ER-localized Streptavidin-KDEL hook retains secretory cargo fused to a Streptavidin-Binding Peptide (SBP). Upon addition of excess biotin, the secretory cargo is released from the hook and its trafficking is monitored as it exits the ER, traffics through the Golgi towards its downstream organelle or is secreted. We developed a new protein manipulation system, Biotin Operated Ligand Trapping (BOLT), and first tested if it could be used as an alternative to the conventional RUSH system. BOLT employs HaloTag-fusion proteins with HaloTag ligands containing biotin or desthiobiotin moieties and a fluorogenic Janelia Fluor (JF) dye. Upon labeling with these HaloTag ligands, the HaloTag-cargo fusion simultaneously becomes fluorescent and is retained in the ER by a trap, Streptavidin-KDEL. Addition of biotin then rapidly releases cargo, enabling visualization of trafficking through the secretory pathway. BOLT efficiently tracked multiple cargos, including exogenous TNFα-HaloTag, HaloTag-GPI, and Man2-HaloTag, demonstrating its compatibility with a variety of HaloTag-cargos. A major advantage of BOLT is its inducibility (add the ligand to induce trapping) and reversibility (add biotin to release), making it ideal for manipulating endogenous HaloTagged proteins without perturbing their steady-state function. We took advantage of this aspect to investigate bidirectional trafficking of an endogenous Golgi enzyme, MAN2A1-HaloTagKnockin. At steady state, the bulk population of MAN2A1-HaloTagKnockin localizes to the Golgi network, but we hypothesized MAN2A1-HaloTagKnockin recycles through the ER. By pulse labeling MAN2A1-HaloTagKnockin with desthiobiotin-JF646, we observed robust Golgi-to-ER redistribution as the majority of this enzyme became trapped by Streptavidin-KDEL. After subsequent biotin release, MAN2A1-HaloTagKnockin rapidly trafficked back to the Golgi, demonstrating completion of a full circle of retrograde and anterograde trafficking. Finally, we extended BOLT to cell cycle control. A cytosol-facing Streptavidin hook sequestered constitutively active Cyclin B-HaloTag or CDK1-HaloTag in the cytoplasm. Upon biotin addition, these nuclear factors were rapidly released, translocated to the nucleus, and in some cases triggered mitosis. Together, these results establish biotinylated HaloTag ligands as versatile tools for acute, reversible control of protein localization, with applications ranging from secretory trafficking to regulation of cell cycle progression.

B511/P1075

Advancements in TEM Automation and Acquisition for Large-Area Cell and Tissue Imaging: Enhancing Ease of Use for Cellular Structure and Organelle Visualization

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While transmission electron microscopy (TEM) is unparalleled in its ability to reveal ultrastructural details at the nanometer scale, this level of resolution comes at the cost of limited field of view, requiring high magnification and often making it difficult to contextualize observations within larger cellular or tissue architectures. For cell biology research, where both fine detail and broader spatial context are essential, this presents a significant challenge. In this poster, we present recent software developments in the new Thermo Scientfic Talos 12 TEM that focus on improving automation and ease of use for multi-scale imaging, enabling

broader accessibility to high-quality TEM imaging for users with varying levels of expertise and equipping proficient users to improve workflows and increase their productivity. We will share how recent advancements for an automated package within an easy-to-use platform provide high-throughput and un-attended acquisition of multi-scale data. Automated data acquisition can be extended into 3D through tilt series tomography, providing a richer spatial context. Additionally, observations from multiple sources can be linked, providing the necessary multi-scale and multi-modal insight for correlative examinations, including fluorescence microscopy data (CLEM). These software updates make TEM instruments more accessible to those with limited TEM experience, effectively expanding a possible user base and their contribution to projects, delivering results in less time.

B527/P1091
Ballistic Microscopy (BaM)
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Understanding how proteins and their interactomes function in living cells is fundamental to deciphering cellular homeostasis and the origins of disease. Conventional approaches for analysing unknown proteins often require fixed samples or whole-cell lysis, or are restricted to tracking known analytes in living cells. Meanwhile, light or electron microscopy provides structural resolution from cellular to atomic scales but lacks the ability to uncover the de novo molecular composition of unknown structures. Techniques such as mass spectrometry or sequencing excel at molecular identification but sacrifice live-cell and subcellular spatial context. Here, we introduce Ballistic Microscopy (BaM), a new approach that bombards millions of nanoparticles traveling at ultra-high speed (~1000 m/s) into living cells. Each particle penetrates and exits the cell while capturing "attoliters" of cytoplasm onto a capture substrate, preserving the spatial mapping of individual components. Because thousands of particles can traverse a single cell simultaneously, BaM achieves high spatial resolution, enabling comparison of materials from distinct subcellular regions. Extracted materials can be imaged on a gel to preserve spatial distribution or collected for downstream biochemical and structural analysis. We demonstrate the versatility of BaM by integrating it with fluorescence microscopy, TEM, Cryo-ET, mass spectrometry, confocal imaging, and DNA amplification. In live cells, BaM enabled spatially mapped extraction of membrane components, cytoplasm, and phase-separated condensates. Applied to the microtubule plus-end tracking protein CLIP-170, BaM revealed the protein composition of CLIP-170 condensates for the first time, identifying ~641 associated proteins within a strong protein-protein interaction network. This included experimentally validated keratin-18 intermediate filaments. These findings establish BaM as a novel platform for live-cell molecular sampling, offering multiple detection modalities when combined with existing methods. Looking ahead, BaM could be applied to investigate diverse subcellular structures such as cilia, spindles, filopodia, organelles, membranes, and disease-associated protein condensates or aggregates across a broad range of animal, plant, and other cell types.

B518/P1082
Cell Biology at the Scale of the Organism: Whole Body Cellular Activity Imaging in Vertebrates
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Multicellular life depends on the dynamic coordination of cells distributed across the body. Yet current technologies force trade-offs: focusing on small tissue subsets, producing static whole-organism snapshots, or achieving organismal coverage at the cost of cellular resolution. Without whole-organism context, the ultimate goals and consequences of physiological processes are difficult to infer; without temporal dynamics, the algorithm is hidden; without cellular resolution, the implementation is inaccessible. To overcome these barriers, we developed WHOLISTIC: a platform for imaging cellular activity throughout the entire body of live zebrafish at single-cell resolution. WHOLISTIC extends functional imaging beyond the nervous system to all organs and tissues—including gut, skin, liver, pancreas, and more—by combining pan-cellular calcium reporter lines, rapid volumetric fluorescence imaging, new machine-learning pipelines for whole-body registration and activity analysis, and complementary whole-body expansion microscopy. Using this approach, we uncover a host of new cellular properties and inter-organ interactions, including state-dependent oscillatory ependymal-cell dynamics during sleep-like states, meningeal responses to ketamine, and pulsating waves along kidney nephrons. At the whole-organism level, we captured rapid brainstem-controlled blood flow redistribution during hypoxia, followed by decreased enteric activity, suggesting a novel mechanism of cellular activity regulation via controlled oxygenation. Integration with optogenetics enables all-optical causal mapping of brain–body interactions, revealing a topological correspondence between central and peripheral activity. Together, these advances establish a new paradigm for systems biology, bridging cellular and organismal physiology with implications for fundamental biology, systems neuroscience, and drug discovery.

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Conditional Localization Pharmacology Manipulates Cell Cycle with Spatiotemporal Precision

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To overcome traditional pharmacology's limited control of drug activity and localization in space and time, we describe an approach for kinase regulation using conditional localization pharmacology (CLP), where an inactive caged inhibitor is localized to a site of interest in a dormant state using intracellular protein tethering. The activity of the inhibitor can be regulated with spatial and temporal precision in a live cellular environment using light. As a proof of concept, a photocaged MPS1 kinase inhibitor (reversine) bearing a Halo-tag ligand tether was designed to manipulate the cell cycle. We demonstrate that this new caged reversine halo probe (CRH) strategy is capable of efficient localization and exceptional spatiotemporal control over spindle assembly checkpoint (SAC) silencing and mitotic exit. Using CRH, we studied the cytokinesis function of a kinesin, KIF18A, whose inhibition triggers mitotic arrest via the SAC. We show that KIF18A inhibition increases the rate of spindle elongation after SAC silencing, suggesting that the microtubule depolymerase activity of this motor regulates spindle length during cytokinesis.

B529/P1093

CZ CELLxGENE Discover: Analytical platform and the largest repository of standardized single-cell expression data

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<div selected"="" id="eng-abstract"> CZ CELLxGENE Discover (cellxgene.cziscience.com) is a free-to-use online data portal hosting a growing corpus of more than 1800 single-cell datasets comprising over 115 million unique cells from the major human and mouse tissues. All data have been curated to include consistent cell metadata, such as cell type, tissue, and donor age and disease, each standardized to a community ontology. The metadata schema and required raw counts indexed by Ensembl gene identifiers enables searchability and reusability of the data. Recent updates to the schema has allowed for support of ATAC-seq, Visium, and Slide-seq assays.The CELLxGENE Explorer displays an interactive 2-dimensional representation of cells in a dataset and allows users to color cells by metadata (e.g. cell type, disease, metadata features etc.) or gene activity. Users can also subset and analyze subgroups of cells, perform differential gene expression and create scatter plots of gene expression. The Gene Expression feature allows querying the expression of any gene across all human and mouse cell types available in the portal, and enables lookup of differentially enriched genes for any cell type. The Cell Guide feature allows users to explore an interactive encyclopedia of 900+ cell types that provides detailed definitions, marker genes, lineage, and relevant datasets in one place. The Differential Expression tool allows for calculation of differentially expressed genes between custom groups of cells across the CELLxGENE corpus.The CELLxGENE Census is a cloud based platform that allows for data access, efficient cell-based queries and analysis of cells regardless of the dataset of origin. Standardized metadata allows for subsetting and slicing of the data in AnnData, Seurat, and SingleCellExperiment objects. The Census also hosts pre-calculated embeddings and pretrained models, enabling cell type prediction and data projection of new query data.CELLxGENE is a tool intended for community use and contributions. By supporting multiple modalities and data generated by labs around the world, the CELLxGENE suite of tools and data aims to maximize rapid use of high quality data describing the phenotypes of cells and tissues. To date, CELLxGENE supports data sharing from worldwide labs as well as consortia such as the CZ Biohub Tabula projects, LungMap, BICCN, Allen Institute for Brain Science, KPMP, HTAN and the Human Cell Atlas. Groups interested in submitting their own data can inquire about the inclusion of your data and the submission process by contacting the CELLxGENE team at cellxgene@chanzuckerberg.com.

B513/P1077

Detection of submicroscopic G3BP1 assemblies enables sensitive and dynamic monitoring of cell stress

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The Integrated Stress Response (ISR) is a highly conserved mammalian pathway that activates in response to a broad range of cellular stressors. ISR activation is found throughout human pathologies; mutations in ISR components are associated with cognitive diseases, ISR activation is closely associated with age-related neurodegenerative diseases, and the ISR is thought to play a role in the pathogenesis of cancer and metabolic disorders. Inhibition of translation upon ISR activation frees cellular RNA from polysomes, leading to the formation of large condensates of protein and RNA, termed Stress Granules (SGs). Significant study has uncovered the components, molecular regulators, and environmental conditions that give rise to SGs. Despite direct study, the exact function of SGs themselves within the network of stress responsive signaling remains unclear. Furthermore it is unclear the extent to which SG formation is a sensitive and reliable marker of ISR activation. Recent evidence in Saccharomyces cerevisiae suggests that RNA begins condensing in response to low doses of stress, giving rise to stress assemblies that are sub-microscopic, but may nevertheless constitute a molecularly relevant stress response. Here we present a real-time direct readout of sub-microscopic

oligomerization of endogenous G3BP1, a core SG component, in live single cells using CluMPS (Clusters Magnified by Phase Separation), a genetically encodable fluorescent probe which forms large condensates upon binding to an oligomeric target. We find that oxidative stress, osmotic stress, and temperature stress all have low-dose regimes where treatment does not produce SGs, but does give rise to G3BP1 assemblies detectable by CluMPS. This low-dose stress is sufficient to inhibit translation, a hallmark of a stress response, suggesting that G3BP1 assembly at these doses is a bona fide indicator of ISR activation in cells. While CluMPS can detect sub-microscopic assemblies of G3BP1 in the absence of SGs, it minimally perturbs SG size, shape, density, constituents, or dynamics under acute stress doses where SGs are present. Downstream outcomes following cell stress including translation inhibition and cell death (at high stress doses) are also unperturbed by CluMPS. CluMPS is also able to detect chronic stress as ISR activation persists despite dissolution of SGs, even at high stress doses which eventually cause cell death. We leverage this approach to track G3BP1 assembly in iPSC cells, showing similar low-dose regimes of oxidative and osmotic stress where G3BP1 oligomers form but do not coalesce into SGs. CluMPS provides a method for single-cell real-time tracking of sub-microscopic transformations in protein organization caused by ISR activation in live cells with high fidelity and sensitivity.

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Engineering clickable fluorophores for dynamic visualization of oxidative stress in cancer cells

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Click chemistry has been used as a powerful tool for developing advanced cellular imaging technologies, enabling the investigation of specific biological pathways and the site-specific labeling of intracellular organelles via fluorescence microscopy. Effective imaging of organelles in biological systems particularly requires stable imaging probes with key spectroscopic properties, including high signal-to-noise ratios, fast kinetics, and tunability. Although several commercially available antibodies and fluorophores exist, many fall short in critical aspects such as size, kinetics, solubility, photostability, and Stokes shifts. To overcome these limitations, our main objective is to introduce a straightforward click chemistry approach for designing small-molecule probes to study oxidative stress in cells. This strategy offers a promising solution for creating probes with the necessary chemical and photophysical properties to enhance imaging performance. In this study, we developed small-molecule probes with broad spectral ranges, designed to rapidly detect carbonyl species and metal ions in oxidative stress-induced cellular systems. Spectroscopic analysis, fluorescence and confocal microscopy show that that our newly synthesized probes effectively react with carbonyl species to produce a high fluorescence and track oxidative stress labeling in different cancer cell lines. Our findings revealed a red shift in both the absorption and emission maxima of the fluorescent probes upon binding to their target molecules, accompanied by a substantial Stokes shift—highlighting their sensitivity and specificity. Notably, one of the newly developed probes demonstrated metal ion chelation, triggering a "turn-on" fluorescence response that enhances its potential for dynamic biological imaging. These advances underscore the promise of our small-molecule fluorescent probes as powerful tools for visualizing disease processes. We envision this innovative fluorescence platform driving the development of next-generation imaging technologies and accelerating the discovery of therapeutics for cancer and other diseases linked to oxidative stress.

B519/P1083

Expanding the Cell Biologist's Toolkit: From Nanoscale Imaging to AI-Driven Predictive Assays

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I will discuss our Magnify expansion microscopy (ExM) technology and its latest developments, demonstrating their versatility for both fundamental cell biology and pathology applications. Our lab has recently developed Magnify and microMagnify that physically expand cells and tissues up to 11-fold. These techniques are useful cell biology tools that enable nanoscale 3D imaging of complex structures, organelles, and even host-pathogen interactions using standard confocal microscopes. We have built upon this foundation to create new applications. First, we developed HistoMagnify, a variant chemically optimized for bright-field imaging of conventionally stained cells and tissues. Our results demonstrate that HistoMagnify successfully resolves subcellular features, achieving an effective resolution of ~80 nm with just a standard 40x air objective. Second, we created an AI-driven predictive assay by applying multiplexed fluorescence Magnify to pre-treatment bladder cancer biopsies from patients with known immunotherapy outcomes. We then trained a deep learning model to correlate the resulting high-content, nanoscale spatial features of the tumor microenvironment with the clinical response. The Magnify-based AI model accurately predicted treatment responders versus non-responders, reaching an Area Under the Curve as high as 88% during five-fold cross-validation by identifying complex cellular patterns invisible to traditional pathology. In conclusion, these developments showcase the broad applicability of our Magnify ExM platforms. Magnify and its variants are robust tools for fundamental cell biology investigations, while new adaptations like HistoMagnify has broaden the choice for researchers and AI-driven nanopathology are paving the way for future clinical and diagnostic applications.

B530/P1094

Fluorescent cross-correlation spectroscopy reveals the affinity of cyclin-CDK complexes using green and near-infrared fluorescent proteins

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Protein–protein interactions are central to diverse biological processes, including cell cycle progression, differentiation, and signal transduction. However, quantifying the strength of specific interactions in living cells remains technically challenging. Fluorescence cross-correlation spectroscopy (FCCS) enables direct measurement of molecular interactions in living cells by tagging two proteins with spectrally distinct fluorescent proteins and simultaneously monitoring their fluorescence fluctuations. Because photobleaching reduces measurement accuracy, photostability of fluorescent proteins is critical for FCCS. While the green fluorescent protein mNeonGreen (mNG) is sufficiently photostable, a comparably stable red fluorescent protein has been lacking. Here, we demonstrate the advantage of the near-infrared fluorescent protein miRFP670 for FCCS. Compared with red fluorescent proteins such as mCherry2 and mScarlet-I, miRFP670 exhibited markedly higher photostability, enabling more reliable FCCS measurements in living cells. Using the mNG and miRFP670 pair, we quantified binding affinities between cyclins and cyclin-dependent kinases (CDKs) in living HeLa cells. CDK is a master regulator of the cell cycle, which is activated through cyclin binding. In mammals, multiple cyclins and CDKs form specific combination of complexes during each cell cycle phase, which is an essential mechanism ensuring cell cycle phase–specific regulation. However, the extent of these interactions in living cells has remained elusive. We measured the dissociation constants of 36 cyclin–CDK complexes. As expected, conventional complexes such as cyclin D–CDK4/6, cyclin E–CDK2, cyclin A–CDK1/2, and cyclin B–CDK1 showed high affinities. Intriguingly, we also detected unconventional interactions, including cyclin D1/D3–CDK2 and cyclin B1–CDK2, suggesting that the cyclin–CDK interaction network is broader than previously appreciated.

B520/P1084

Gentle Media Exchange on an Automated Platform: High Cell Retention, Imaging-Compatible Workflow

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Gentle media exchange is a persistent bottleneck for imaging assays that rely on suspension and semi-adherent cells, where even brief shear or over-aspiration disrupts morphology, causes cell loss, and degrades segmentation fidelity. We introduce a centrifuge-less, flow-controlled exchange on the Curiox C-FREETM Pluto workstation that preserves culture integrity while achieving meaningful refresh of spent media. In feasibility and verification exercises across 24- and 96-well formats, the approach delivered targeted partial exchanges (e.g., ~50% and ~87.9% dilutions) while maintaining high cell/bead retention (≈95.9% and ≈92.9%, respectively), indicating minimal physical disturbance of loosely adherent and suspension populations. The workflow scales to multi-well batch operations and supports multi-cycle refresh when deeper exchange is needed, without compounding loss - an advantage for longitudinal imaging where repeated manipulations are common. By replacing spin/decant steps with defined, low-shear wash/mix routines and tunable exchange volumes, the method reduces operator variability, shortens hands-on time, and sustains plate-to-plate consistency. Crucially for imaging, cells remain in place and viable, improving downstream focus stability, feature extraction, and reproducibility. Together, these results position centrifuge-less, automated media exchange as a practical route to more reliable live or fixed imaging of fragile cell types, and as a foundation for higher-throughput assay designs, including future extensions to perfusion-like refresh on Pluto HT.

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Hydrazone chemistry for cellular carbonylation detection and visualization

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Hydrazone-based bioorthogonal chemistry offers a highly selective approach for detecting biomolecule carbonylation, a marker of oxidative stress, which is associated with a variety of diseases. The standard detection methodologies, and therefore commercial kits, rely on the chemistry of dinitrophenylhydrazine (DNPH). Unfortunately, the low chemical reactivity of DNPH requires strong acid catalysis, which subjects the sample to harsh conditions. Moreover, a large excess of DNPH must be removed with multiple washing steps, making it a lengthy process. Here, we present a straightforward and robust fluorescence labeling approach for carbonylated biomolecules. Our approach employs both a commercially available and a synthetically produced hydrazide-functionalized fluorophore, 7-diethylaminocoumarin-3-carbohydrazide (DCCH) and julolidine coumarin hydrazide (JCH), respectively. Of note, JCH's absorption band is red shifted from DCCH and can therefore be observed using lower energy excitation. These probes react specifically with cellular biomolecule carbonyls to generate a strong, quantifiable fluorescence signal. Importantly, this assay can be performed with low micromolar concentrations of the fluorophore using mild reaction conditions. This methodology is easy to implement, resulting in a chemically stable product that does not require subsequent chemical stabilization steps. Reproducible results are obtained using fluorescence plate readers and fluorescence microscopy, making the method accessible to most research labs. Validation of the method is demonstrated using A549 cells exposed to oxidative stress. Overall, this approach addresses key limitations of current detection techniques by combining bioorthogonality, chemical stability, commercial accessibility, and ease of integration into common laboratory workflows, supporting broader applications in cell biology and disease research.

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Imaging Molecular Tension in Live Tissues with WHaloForce

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Cells interact with their surroundings not only through biochemical signals but also through mechanical forces that shape development, physiology, and disease. However, tools for visualizing molecular tension in living tissues have remained limited. We developed WHaloForce, a compact, fluorescence-lifetime-based molecular tension sensor that overcomes key limitations of existing approaches. Building on a reversible dye-quenching strategy, WHaloForce converts mechanical tension into predictable changes in fluorescence intensity and lifetime. By integrating the sensor intramolecularly into force-bearing proteins at cell–matrix adhesions, cell–cell junctions, and within the extracellular matrix, we detected fluorescence lifetime shifts that report cell-generated mechanical tension in tissue culture. Through CRISPR knock-ins of E-cadherin, vinculin, and laminin in C. elegans, WHaloForce enables quantitative imaging of molecular tension in vivo. The sensor reveals directional tension at epithelial junctions, tissue-specific tension patterns, and adaptive mechanical behavior of the basement membrane during repeated deformation. Together, these results establish WHaloForce as a broadly applicable and quantitative platform for imaging molecular tension in live tissues, opening new opportunities to study how mechanical forces organize and maintain living systems.

B521/P1085

Imaging-PHD: Empowering data reuse and reproducibility through microscopy-community-defined Persistent Hardware Descriptors

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Accurate documentation of an instrument's hardware configuration is essential for the interpretation, reproducibility, and FAIR-sharing of scientific data. Existing registries such as RRIDs for instrument models and PIDINST for individual devices do not capture the detailed configuration needed to ensure data quality across studies. To address this gap, we have launched Persistent Hardware Descriptors (PHDs) for light-microscopy—a standardized framework that records comprehensive hardware metadata alongside experimental results. Our work, Imaging-PHD, delivers three key contributions: (1) a vendor-friendly schema for describing microscope configurations that can be linked to publications via a DOI; (2) a collaborative platform with a graphical user interface that enables researchers to author, review, and edit hardware descriptors when vendor software lacks automated support; and (3) a coordinated outreach program that educates vendors and users, provides hands-on training, and builds a curated corpus of hardware descriptions. This corpus will facilitate quality-control improvements and enable community-wide large-language-model applications through high-quality training data. All components are designed to be interoperable and extensible, allowing deployment across multiple sites and scientific domains. By standardizing hardware metadata capture, Imaging-PHD enhances the reliability of microscopy-based research, promotes accessibility of advanced technologies, and supports long-term data preservation—advancing reproducible scientific practice for the broader life-science community

B522/P1086
Insights into Aberrations and Usage of Imaging Phantoms
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Glioblastomas, whether imaged in situ or in vitro, present a laundry list of aberrations and complications. One cellular line, glioblastoma multiforme 39 (GBM-39), was used to initially look at the components of tunneling nanotube (TNT) in addition to how these transient tubes operate and grow. This study transitioned into an investigation of how to overcome and document the process to image the tumor made aggregates and TNTs and movement of mitochondria, if possible, in a 2-D and 3-D environment. Widefield images were obtained on an epifluorescence microscope of the aggregates and network of TNTs created within along with gliospheres created and mounted on a silicone insulator. Micrographs were also taken of gliospheres to highlight the lack of clarity or structural resolution that optical instruments currently lack without proper standards. Current polymer bead standards, like the TetraSpeck beads, cannot properly account for the density of cells or density of light scatter exhibited amongst the GBM-39 aggregates or gliospheres. Through trial and error of calibrations, adaptive optics (AO) iterations, alignments, and troubleshooting, the pollen grain sample is a prime testbed for an adaptive optic multiphoton fluorescence microscope (AO-MPFM) especially when designed for 3-D imaging and analysis of 3-D tumor models such as the GBM-39. Pollen grains allow an AO-MPFM to recognize multiple types of sample scenarios, including density, size, depth, and optical aberration effects in a singular environment that vary or increase with a change in scanning depth during testing and calibration procedures. In combination with the multiphoton fluorescence microscope (MPFM), the AO unit only further enhances the positive outcomes of using an MPFM system. Research partially supported by grants to ER-M from the National Institute of Standards and Technology (NIST) of the U.S. Department of Commerce (USDOC) and by the University of Kansas Comprehensive Cancer Center/National Cancer Institute Cancer Center Support Grant P30 CA168524..

B532/P1096
Integrating molecular dynamics and high-speed single-molecule tracking to link protein structure and function
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Multi-color, high-speed single-molecule tracking enables quantitative analysis of protein–protein interactions and dynamics in living cells. However, while trajectories reveal how proteins move, they rarely capture how underlying structural features govern these behaviors. To bridge this gap, we developed a general pipeline that integrates computational structural bioinformatics, molecular dynamics (MD) simulations, and correlative single-molecule tracking to directly link protein structure to motion in cells. We apply this approach to two model systems central to metabolic regulation. First, we examine VAMP-associated protein B (VAPB), an ER-resident tether that mediates contacts with mitochondria, endosomes, and the Golgi. Our analysis identifies specific structural elements in VAPB and its binding partners that regulate the kinetics and stability of inter-organelle contact sites. Second, we investigate Protein Kinase A (PKA), a major signaling hub, and demonstrate how structural features influence its dynamic behavior in live cells. Together, this work establishes a broadly applicable framework for integrating structural and dynamic measurements of proteins, providing new insight into the molecular regulation of organelle communication and signaling.

B523/P1087

ISG-Timer: a universal live-imaging reporter to dissect spatio-temporal interferon dynamics at the single-cell level

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The interferon (IFN) response is a central pillar of antiviral defence, yet its spatio-temporal regulation at the single-cell level remains poorly understood. Respiratory viruses such as human metapneumovirus (hMPV) and measles virus (MeV) exemplify this challenge: both rely on evading IFN responses to spread, while only a fraction of infected or exposed epithelial cells initiate IFN production. This heterogeneity in IFN and interferon stimulated gene (ISG) induction remains elusive, largely due to the absence of tools capable of resolving dynamic activation in real time. Here, we developed and validated the ISG-timer, a live-imaging reporter designed to analyse type I and III IFN dynamics at the single-cell level. It combines two fluorescent proteins with different half-lives expressed from an ISG promoter, allowing simultaneous detection of active responders (currently transcribing ISGs) and past responders (previously activated but now silent). By overcoming the limits of transient reporters, the ISG-timer enables retrospective tracing of ISG responses across time and space. Using the ISG-timer in air-liquid interface (ALI) airway epithelial models, we defined key kinetic parameters of the IFN response. Unlike previous reports, IFN-β stimulation did not lead to digital responses but to a homogeneous, progressive activation across the cell population. Coupling experimental data with mechanistic mathematical modelling, we began reconstructing key steps of the IFN pathway, from viral sensing and IFN secretion to paracrine amplification and ISG induction. When calibrated with ISG-timer data, the model should capture both single-cell and population-level variability and provide insights into how sentinel cells may protect epithelial domains through IFN diffusion, and how feedback mechanisms likely contribute to resolution of the response. Relatively to hMPV and MeV infections, the ISG-timer revealed distinct IFN signatures that are virus-specific. While hMPV induced a quick and potent IFN response, MeV caused a delayed ISG activation, associated with its cytopathic effects and mitochondrial damage. In both cases, viral replication outcomes were shaped by timing and magnitude of ISG induction. In conclusion, the ISG-timer provides a universal platform for observing transcriptional dynamics in living cells. Future applications could go beyond the interferon biology. It opens perspectives for studying host-pathogen interactions in epithelial-immune co-cultures, antiviral drug screening, and personalized strategies for patients with dysregulated IFN responses. More generally, it enables the dissection of spatio-temporal transcriptional regulation in any process where single-cell dynamics shape outcomes at population level.

B510/P1074

May the Force Be Measured: DNA probes reveal the biophysical forces in immune activation

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Our immune system relies on T cells to recognize and eliminate infected or abnormal cells. But how do these tiny sentinels know when to respond? Surprisingly, it turns out that T cells don’t just see their targets—they feel them. T cells are triggered when the T-cell receptor (TCR) encounters its antigenic ligand, the peptide-major histocompatibility complex (pMHC), on the surface of target cells. Because T cells are highly migratory

and immune recognition occurs at a dynamic junction where the T cell physically touches the target cell, the TCR as well as its many co-receptors must experience some level of molecular force. Indeed, the elegant work of Lang and Reinherz indicated an important role for mechanics in modulating TCR-antigen bond lifetime and T cell response. Hence, a long-standing question in the field is whether T cells transmit defined forces to the TCR and whether biophysical forces influence immune function. In this talk, I will focus on the development of DNA-based probes to visualize and manipulate TCR-pMHC forces during T-cell activation. I will first detail how DNA probes are engineered to measure and manipulate the magnitude of TCR-pMHC forces with piconewton force resolution. I will show that naïve OT-1 (CD8+) T cells transmit 12–19 pN forces to the TCR-antigen complex within seconds of ligand binding and preceding initial calcium signaling. Next, I will describe our work using DNA "locking" to measure the lifetime of highly transient TCR forces and especially with single amino acid mutants of the pMHC (altered peptide ligands). Next we employ advanced photonic methods to measure the orientation of TCR-pMHC forces. These experiments aimed to test the hypothesis that the TCR is an anisotropic mechanosensor. Finally, we show that T cells display a dampened and poorly specific response to antigen agonists when TCR forces are chemically abolished or physically "filtered" to a level below ∼12 pN using mechanically labile DNA tethers. Altogether, the data suggests that T cells harness pN forces to boost the fidelity of immune recognition. These findings reveal that T cells act not only as biochemical sensors but as physical sensor—using force to sharpen their responses and avoid costly mistakes. This points to a more general principal of how physical forces could be harnessed in biology.

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Novel CellCage™ technology integrates image-based phenotyping and single-cell transcriptomics to study dynamic behaviors of living cells

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Imaging-based technologies are widely used to study cellular phenotypes in vitro because they provide multi-modal readouts of morphological, molecular, and functional cell states. However, integrated technologies which can directly link dynamic behaviors of living cells with high quality transcriptomic data at single-cell resolution are lacking. To address these limitations, we developed a novel CellCage™ Enclosure (CCE) technology: biocompatible and degradable hydrogel microenvironments formed by light-guided polymerization around individual cells or defined groups. CCEs enable long term culture of tens of thousands of cells, longitudinal imaging of dynamic cell behaviors, and direct mapping of image-based phenotypes to transcriptomic profiles on Cellanome's R3200 platform. In contrast to standard well plate, nanowell, or droplet-based methods, the properties of CCEs (i.e., size, shape, location, porosity, substrate) can be configured depending on the cell and assay type. CCEs can be formed on the order of seconds and selectively degraded on the order of minutes using cell-compatible chemistry. Permeable CCE walls allow diffusion of nutrients, waste, and fluorescence reagents, enabling the culture of suspension or adherent cells for weeks. To demonstrate the utility of CCEs, we selectively retained single NK cells from a PBMC cell mix to study NK cytokine secretion. PBMCs were loaded into flow cells and enclosed in CCEs. CD56+ NK cells were identified via receptor staining. Cells can then be selectively retained by forming a second CCE around the first with a second gel type, followed by degrading the first gel to remove CD56- cells. To quantify cytokine secretion, activation (PMA) and detection reagents were serially diffused. The percent of cells secreting IFNγ and GZB after 6 hours is consistent with literature. CCE geometry can also be tuned to study cell-cell interactions over time. For example, neurons enclosed in CCEs with microscale openings formed neurite outgrowths and interacted across CCEs, enabling calcium imaging data to be linked to transcriptomics from single somas without disrupting neural networks.

Together, these results demonstrate that CCE technology enables novel imaging-based phenotypes to be studied and directly linked to single-cell sequencing data.

B533/P1097

Novel GTPase Biosensor Designs Preserve Upstream and Downstream Protein Interactions

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GTPases are key signaling molecules that regulate cell proliferation, cytoskeletal organization, migration and gene expression. They act as molecular switches, with an active and inactive conformation under the regulation of GEFs, GAPs and GDI. The active conformation is capable of interacting with effector proteins that modulate downstream events. The spatio-temporal dynamics of the activating conformational change are critically important for localized control of signaling, leading to extensive study with fluorescent biosensors in live cells. Most current biosensor designs employ an "affinity reagent" (AR) that binds to the active state of the GTPase, and generates a fluorescence change upon binding (e.g. FRET between the AR and GTPase). These AR are derived from naturally occurring downstream effectors, so they block the GTPase effector binding site. This precludes the use of biosensors to study feedback loops operating through downstream effector binding. Here we describe two biosensor designs that leave both effector and upstream interactions intact. We are building biosensors for the GTPases Rac1, Cdc42 and K-Ras. Both designs are based on an allosteric loop, away from known binding sites, that changes conformation in concert with the active site. This loop was identified using a pipeline previously applied for optogenetic analog. In one approach (AlloRac1 and AlloCdc42), a circularly permuted green fluorescent protein (cpGFP) is inserted in the allosteric loop such that fluorescence intensity depends on the GTPase activation states. In another design (Giraffe K-Ras), a short peptide (SsrA) is inserted in the allosteric loop such that it interacts with a small binder protein (SspB) only when K-Ras is in a specific conformation. The binder is attached to the K-Ras via a chain containing a pair of fluorescent proteins, such that FRET is affected by binding of SspB to SsrA in the allosteric loop. Ongoing assays show that upstream and downstream interactions of AlloRac1 and AlloCdc42 are maintained. Applications to dissect the role of effector feedback in GTPase activation using multiplexed imaging will be described. The Giraffe K-Ras biosensor shows good fluorescence response to conformational change, and ligand binding studies are underway. Allosteric loops could be harnessed to produce biosensors for a wide range of protein structures.

B525/P1089

Optimization and validation of 3D printing materials and designs for cell biological research with applications in cell motility and electrotaxis

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Recent functional genomics studies from our group required the invention and development of a screening device for isolating migratory cells in response to chemical and electrical cues. Fused filament fabrication (FFF) 3D printing provided an accessible and affordable approach that enabled rapid prototyping and optimization. However, limited cytotoxicity data on the plastics and adhesives required further characterization prior to performing experiments with cultured cells. Using HL60 cells, a human promyelocytic leukemia cell line derived from granulocyte-macrophage progenitor cells, we determined that many commonly available plastics used in FFF are indeed biocompatible. In addition to custom-designed parts, our device required permanent and

temporary adhesives. Assessing cell toxicity to an array of adhesives, we found that silicone-based adhesives were largely biocompatible, along with many tapes with acrylic or silicone adhesive. Importantly, we note that the designation of “medical grade” did not guarantee that an adhesive was nontoxic to cultured mammalian cells. For long-term studies, we also developed and validated a technique to sterilize 3D printed parts utilizing vapor phase paraformaldehyde at 37°C. This sterilization technique can be easily monitored with readily available test strips and is nontoxic in most cases. However, we found that parts made from the plastics can become toxic in the cases when the polymer or adhesive formulation contains an amine group. The plastics TPU and nylon are two such plastics that are nontoxic when used without sterilization but which become irreversibly toxic after exposure to paraformaldehyde vapors. We present toxicity analysis of the sterilized parts along with a method to reduce toxicity of some materials by exposure to ammonia vapor. Given the broad utility of FFF in new technology development, these results provide an important resource for applications in cell biological research.

B534/P1098
Using Double Helix-Point Spread Function Engineering to Map Tissue Deformation At High Strain Rate
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Sustaining mechanical load is a normal part of physiology for mammalian tissues. However, high strain rate loading associated with acute injury can lead to cell and tissue damage. Investigating the mechanical response of tissues subjected to high strain rates, can help provide insight into the mechanisms of disease and tissue injury. However, accurately mapping the deformation of a tissue in response to high strain rate loading is technically challenging and there are currently no tools that are well suited to address this gap. We have developed a multifunctional optical system that integrates Double Helix Point Spread Function (DH-PSF) Engineering that can be used to accurately and rapidly track the displacement of fiducial markers in tissues in 3D. By integrating this system with a translational sample stage and inverted fluorescent microscope, we can subject simple tissues to an indentation and track fluorescent markers at high speeds and simultaneously map tissue deformation and measure the mechanical response. The use of this system can further our understanding of the strain fields created when mechanical stimuli is induced, characterizing the mechanical properties of simple tissues, which can lead to a better understanding of diseases of tissue fragility.

Advances in Cell Biology Tools and Technologies - Gene Editing

B487/P1053
AfterLife GOLDFISH: Combining live-cell, DNA repair tracking with high-resolution genomic mapping
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Understanding DNA repair in a locus-specific manner requires both precise timing and accurate spatial mapping. Tracking a synchronized repair response to a single double-strand break (DSB), while keeping protein recruitment linked to its genomic address remains challenging. Current fluorescent tagging approaches are limited to highly repetitive DNA arrays, and conventional DNA-FISH requires harsh denaturation that disrupts chromatin structure. To address these limitations, we developed AfterLife, an integrated imaging platform that unites live-cell tracking of endogenous repair proteins with post-fixation specific mapping of genomic loci. AfterLife combines very fast CRISPR (vfCRISPR) for real-time visualization of DNA repair factors, and GOLDFISH-

X for mapping specific loci after fixation. To assess spatial correlation between live-cell vfCRISPR imaging and post-fixation GOLDFISH-X mapping, we applied AfterLife to the PPP1R2 locus and quantified 2D distances between paired foci in matched nuclei. These results demonstrate that AfterLife's combination of live monitoring and fixed-cell mapping can be used for site-specific analysis of DNA damage responses. Moving forward, we will apply AfterLife to compare PPP1R2 with ACTB and examine how timing, spatial mobility, and nuclear organization vary across genomic loci. This approach establishes a foundation for spatially resolved studies of DNA repair and other nuclear processes, such as transcriptional regulation or chromatin remodeling, where spatiotemporal coordination is critical.

B492/P1058

An In Vitro Method for Base Editor Outcome Determination to Facilitate Experimental Design

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Base editing technologies enable the precise installation of defined substitutions into genomic sequences, which is useful for applications such as the creation of cell lines and animal models, as well as agricultural engineering. Many variants of base editors exist, with differences in characteristics such as editing windows, sequence context preferences, and sensitivities to methylation. Because of this broad range of variation, it can be difficult to predict the most likely outcomes of an editing experiment for a given editor:guide RNA combination. This can be particularly problematic for systems with long experimental times or significant expense, including plants, mouse zygote microinjection, large scale screens, and rare cell types, such as patient-derived cells. We have developed a method to use in vitro editing and DNA sequencing to determine base editing outcomes without the need for cell culture or transfection. This approach allows for quick (as little as 1-2 days to result) screening of multiple base editor proteins with different characteristics and/or multiple guide RNAs to determine which editor:guide complexes are most likely to yield a successful editing outcome. The use of in vitro transcribed crRNA reduces the cost associated with screening multiple targets or multiple guides per target. Additionally, editing outcomes can be evaluated using Sanger-based sequencing to determine editing patterns or using NGS to determine the frequency of specific allele outcomes. Finally, the option to use genomic DNA as a substrate yields data that takes into account the native methylation status of a target, while the option to use PCR-amplified DNA as a substrate enables the identification of rarer outcomes. This predictive method provides a tool to aid the prioritization and experimental design of base editor experiments.

B490/P1056

Enhanced Cellular Uptake and Gene Silencing Efficiency of AUMsilence sdASOs in diverse Hard-to-Transfect Cell Types

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Conventional RNA silencing tools including siRNAs and ASOs and gene editing platforms like CRISPR have shown promise for gene modulation, but their translational application has been limited by poor cellular uptake and inconsistent knockdown across different hard-to-transfect cell types. Our goal was to evaluate the cellular uptake and gene silencing efficiency of chemically modified self-delivering AUMsilence sdASOs in cancer cell lines, primary cells, and in vivo models. We incubated far-red fluorescent-labeled AUMsilence

sdASOs (1.5 µM) with murine splenocytes, A549 lung carcinoma cells, and HEp-2 cells for time-course analysis. For functional studies, we treated human PBMC and cancer patient samples with FOXP3 targeting AUMsilencesdASOs (1.5 µM, 5 days) or AUMscramble controls, and measured FOXP3 mRNA expression by qPCR and Treg numbers by flow cytometry. AUMsilence sdASOs showed rapid cellular uptake without transfection reagents. In murine splenocytes, 80% of primary cells had intracellular ASO fluorescence within 15 minutes, with uptake observed in monocytes/macrophages, lymphocytes, and neutrophils. Both A549 and HEp-2 cell lines achieved 100% ASO uptake at 15 minutes post-incubation. We observed significant FOXP3 mRNA reduction and decreased FOXP3 protein expression in PBMC and in cancer samples (all $p<0.0001$). Intratumoral Tregs were more sensitive to AUMsilence sdASOs treatment than peripheral blood Tregs. Isolated human Tregs, incubated with AUMsilence sdASOs for 3.5 hours, had significantly impaired suppressive function (66.4%) versus scramble control. In murine studies, we observed significant inhibition of tumor growth, while 13.6% (MC38) to 22% (TC1) of tumors were completely resorbed. Intratumoral Foxp3 mRNA was reduced with decreased numbers of intratumoral Tregs, while there were no changes in draining lymph nodes and spleens, confirming localized effects. ASO treatment had no effects on cell viability or cell division. We report that AUMsilence sdASOs achieve efficient cellular uptake and potent gene silencing across diverse cell types, including difficult-to-transfect primary cells, demonstrating consistency and reproducibility of data for cell biology research and translational applications.

B493/P1059

Gene editing in "cell villages" enables exploring disease-relevant mutations in many genetic backgrounds

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The effects of a single gene mutation in polygenic human disorders can vary depending on individual genetic background. We developed a novel, scalable “village editing” approach to assess how background modifies the impact of disease risk mutations. Using pooled editing across 15 iPSC lines with diverse schizophrenia (SCZ) polygenic risk scores, we efficiently generated homozygous and heterozygous knockouts in two SCZ-associated genes: LRP1, a cholesterol import gene, and NRXN1, a presynaptic adhesion gene. By mixing all lines prior to editing and de-multiplexing them afterward, knockout lines were produced with high efficiency. Transcriptional profiling of derived neurons revealed that the same mutations produced variable effects across genetic backgrounds. Knockout of NRXN1 or LRP1 altered both expected and novel pathways, including neurotransmitter transport and cholesterol biosynthesis. These findings establish village editing as a robust platform for scalable, multi-donor iPSC disease modeling, providing new opportunities to dissect polygenic disease mechanisms.

B488/P1054

Optimizing lentiviral vector design and production for cell and gene therapies

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Lentivirus has become one of the most widely used viral vectors for gene delivery, owing to its high transduction efficiency, ability to infect both dividing and non-dividing cells, and capacity for stable, long-term transgene expression. Lentiviral vectors are used across a broad range of applications, from basic research to clinical settings, particularly for ex vivo modifications, with several FDA-approved therapies already in use. However, clinical translation remains challenging, with approximately 90% of cell and gene therapy trials failing, often due to suboptimal vector design that compromises efficacy, safety, and scalability. Although the gene of interest is crucial for therapeutic efficacy, optimization of the transfer plasmid backbone and helper plasmids is often overlooked, despite their significant impact on both efficacy and manufacturability. We demonstrate that rational vector design, coupled with optimized packaging protocols, can markedly enhance lentiviral vector production. Additionally, we demonstrate functional CAR expression in T cells using an optimized lentiviral vector, highlighting the potential of this platform to support the development of cell and gene therapies.

B491/P1057
Optimizing non-viral genome editing workflows for iPSC with advanced electroporation technology
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Induced pluripotent stem cells (iPSCs) have emerged as a crucial platform for studying human diseases, with significant potential in disease modeling, drug discovery, and cell therapy applications. Electroporation is a crucial technology in this field, utilizing short, high-voltage electrical pulses to create temporary pores in the cell membrane. This method facilitates the delivery of DNA, RNA, or protein payloads into cell types that are difficult to transfect, without the complexity and limitations associated with viral systems. In our workflow, we utilized the Neon™ NxT Electroporation System with the 8-Channel Pipette to rapidly optimize electroporation parameters for editing iPSCs. This process is crucial for reliable and reproducible genome editing results and is facilitated by the pipette's user-friendly interface. Using the Neon™ NxT Electroporation System, we generated chimeric antigen receptor (CAR)-iPSCs by delivering CD38-loci targeted anti-Meso3 donor DNA, CTS™ HiFi™ Cas9, and TrueGuide™ Synthetic gRNA to iPSCs. Editing efficiency and cell viability were assessed using flow cytometry and Next Generation Sequencing. Our results highlight the ability to simultaneously test multiple electroporation conditions, significantly reducing the time required for optimization experiments. Additionally, we evaluated different electroporation buffers and achieved up to 30% CAR knock-in efficiency in iPSCs. Through systematic screening of voltage, pulse duration, and pulse number, we optimized payload delivery while maintaining high cell viability with the CultureCEPT™ supplement. The use of CultureCEPT™ supplement for post-genome editing iPSC recovery resulted in improved cell viability and a higher total number of CAR-iPSCs. In summary, the Neon™ NxT 8-Channel Electroporation System has proven to be an effective tool for the rapid and efficient optimization of non-viral based genome editing and engineered iPSC manufacturing workflows. This capability significantly advances iPSC-based research and cell therapy applications, addressing scalability challenges and improving patient outcomes.

Advances in Cell Biology Tools and Technologies - High-Throughput Screening Approaches

B568/P1830
A microscopy-based CRISPR screening platform enables organellar functional genomics and illuminates ciliary

biology

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Microscopy offers an indispensable technique for visualizing diverse biological processes and for defining cytological abnormalities characteristic of disease. However, combining microscopy with the power of pooled CRISPR screening presents considerable technical challenges, hindering application of systematic genetic analysis to imaging-defined phenotypes. Here, we present a microscopy-based CRISPR screening platform that uniquely combines ease of implementation and compatibility with live-cell or antibody-based molecular markers, including post-translational modifications. Illustrating the value of this methodology, we systematically identify regulators of primary cilium structure and function in human cells through targeted and genome-wide screens. We further show that integration of screens focused on complementary but distinct phenotypes yields multi-dimensional profiles that delineate precise gene functions. Among newly identified hits, we identify a micro-protein at the ciliary transition zone that is required for ciliogenesis in human cells and for ciliary function in Xenopus embryos. Ongoing development of this technology aims to offer increased throughput, more facile application in diverse cellular models, and compatibility with complementary functional genomic assays. More broadly, our approach provides a technological and conceptual strategy for microscopy-based functional genomics with wide applicability.

B566/P1690

A multiplexed approach for genetic screening of human cells by electron microscopy uncovers an intermembrane space protein impacting mitochondrial cristae-shape

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Mitochondria form complex membrane-architectures essential for their diverse functions across physiological contexts. While much is known about the core proteins sculpting mitochondrial membranes, many components remain unidentified, and their orchestration unclear. A major limitation for functional discovery is technological–visualizing ultrastructure (US) diversity relies on low-throughput, high-resolution methods, such as electron-microscopy (EM). We here describe a scalable pipeline for screening human cells by EM. Our method, hMultiCLEM, works by fluorescently barcoding human cells enabling Multiplexing of samples and ultra-structure phenotyping by Correlative Light and EM. This unique approach for systematic US visualization permits routine screening of dozens of samples across a few EM experiments, yielding thousands of images. To showcase the power of hMultiCLEM we performed a genetic screen exploring mitochondrial US – systematically and at scale. hMultiCLEM robustly confirmed proposed cristae modulators and uncovered additional ones. Biochemical and computational analyses for validation of candidates highlighted an uncharacterized intermembrane space (IMS) protein linked to familial Ménière's disease, which we named MISHA (Mitochondrial-IMS membrane-SHApe-impacting protein). hMultiCLEM thus illuminates the protein networks driving cristae organization. More broadly, hMultiCLEM propels the EM field towards an era of genetic/chemical screening in basic research and medical contexts.

B563/P1687

Advanced panel design with new iQue® HTS platform to streamline immune cell profiling

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As research for novel immunotherapies for cancer treatment continues to expand, there is an increasing need for efficient and rapid phenotyping of potential cell therapy products. This involves characterizing a wide range of surface markers including markers for cell type, as well as therapeutic biomarkers. To address this demand, it is crucial that scientists have access to advanced panel designing tools, and high throughput instrument for cell interrogation. This study highlights the application of the iQue® Cytometry Panel Builder, a Sartorius panel design tool to streamline the assay building process, and iQue® HTS Cytometer Platform with expanded channel coverage in immune cell profiling. A comprehensive T cell panel was developed using the panel design tool, incorporating phenotype markers (CD3, CD4, CD8), activation markers (CD69, CD25), exhaustion markers (LAG3, TIM-3, PD-1), memory markers (CCR7, CD62L), and a cell viability indicator. Two panels were developed, one for iQue® 3 and a more extensive panel for the new iQue® HTS platform utilizing the additional channels. Compensation particles were used to automatically calculate spillover values using iQue Forecyt® software. Human PBMCs were cultured in RPMI 1640 media with 10% serum and IL-2, with or without CD3/CD28 T cell activation beads (1 bead per cell) to activate the T cell population. Cells were collected in a 96-well plate at 48 hrs, and 72 hrs post activation and data analysis were done using the panels. CD3+, CD4+ and CD8+ T cell populations were gated and their activation and exhaustion markers characterized on either platform. The extra capability of the new iQue® HTS allowed for an increased number of activation and exhaustion markers to be analysed in addition to memory markers. An increased expression of activation markers, and exhaustion marker over time was observed. The new iQue® analysis provided extra data on T cell exhaustion and memory profiles in the various T cell populations. In addition, the new iQue® Platfrom captured more events per second and provided consistent analysis of high throughput experiments. In summary, the integration of the iQue® Cytometry Panel Builder and the iQue® HTS Platform offers a streamlined approach for designing flexible panels to profile immune cell populations. The panel can be further enhanced by incorporating cytokine profiling using the iQue Qbead® immunoassay portfolio, building on the ability of the iQue® Platform to independently track both cell and bead populations within the same sample.

B567/P1691

Development of ERK biosensor using circularly permuted fluorescent proteins

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The ERK MAPK pathway is essential for regulating cell proliferation, differentiation, and survival, and aberrant ERK activity is frequently associated with developmental disorders and tumorigenesis. These context-dependent cellular responses are mainly caused by its temporal and spatial ERK activation dynamics. It is still challenging to understand the encoding and decoding mechanisms of ERK activation dynamics due to its complex network of dynamic interactions, crosstalk, and feedback loops. To untangle these complexities, multiplex imaging is a promising approach. Although Förster resonance energy transfer (FRET)-based biosensors have provided powerful tools to visualize ERK activity in living cells, they require two fluorescent

proteins per target, limiting their compatibility with multicolor imaging due to spectral overlap. Kinase translocation reporters (KTRs) represent an alternative strategy but cannot resolve localized activity within subcellular compartments. To overcome these limitations, we aim to develop a monochromatic ERK activity biosensor suitable for multiplex imaging using circularly permuted fluorescent proteins (cpFPs) such as cpEGFP and cpmApple. These sensors contain an ERK substrate domain and a phosphorylation recognition domain (FHA1). Upon ERK activation, the substrate domain is phosphorylated and FHA1 binds to this phosphorylated site. This intramolecular binding induces conformational change of the cpFP, thereby altering fluorescence intensity. To guide sensor engineering, we employed AlphaFold2-based structural prediction to evaluate domain orientation and linker configuration, followed by structure-guided optimization. These efforts yielded prototype sensors that reproducibly exhibit enhanced fluorescence signals upon ERK activation. As the initial screening step, we performed saturation mutagenesis on the linker region and expressed the mutant sensor library in E. coli. From this library, we identified variants with low basal fluorescence intensity. In the second step, we evaluated these variants in vitro under ERK activation conditions and isolated those showing increased fluorescence intensity relative to basal levels for further optimization. These results demonstrate the feasibility of engineering single-fluorescent protein ERK biosensors with improved dynamic range and reduced background. Our study provides a foundation for multiplex live-cell imaging of ERK signaling and establishes a framework for expanding this strategy to other kinases.

B564/P1688

Efficient Imaging of Airway Epithelium: Advancing Research Through Whole Transwell Membrane Scanning

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Airway epithelial remodeling is a hallmark of respiratory diseases such as asthma and COPD. To support research efforts in this area, we modeled goblet and squamous cell metaplasia in human large airway epithelial air-liquid interface (ALI) cultures treated with IL-13 or EGF. Our key objective was to establish a high-throughput imaging strategy capable of scanning entire ALI membranes, overcoming limitations of traditional Regions of Interest (ROI)-based confocal imaging. We compared whole membrane tile scanning using an open pinhole approach to conventional Z-stack confocal imaging. The open pinhole method increases signal intensity by allowing more light from out-of-focus planes to reach the detector. Despite this, the confocal laser's narrow spectral range preserves a high signal-to-noise ratio enabling efficient full-membrane coverage and producing enhanced, high-quality images. Open pinhole imaging reduced acquisition time to 4 minutes per sample, compared to 75 minutes for Z-stack imaging, resulting in a nearly 20-fold increase in imaging speed. This translated into approximately 95% reduction in imaging time. Quantification of MUC5AC+ goblet cells and KRT13+ squamous cells was robust across entire membranes, and open pinhole imaging yielded results comparable to Z-stack imaging for both markers. This approach provides a scalable, time-efficient solution for ALI culture phenotyping. The streamlined imaging methodology supports translational research across multiple therapeutic areas – from airway diseases to ophthalmology – in which similar culture systems are employed. This innovative technique has the potential to significantly accelerate research in airway remodeling and related fields.

B562/P1686

Enhancements in the utility of benchtop cell counters for cell viability and functional assays

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Since 1907, trypan blue has been the gold standard for cell viability staining. However, significant drawbacks, including potential carcinogenicity and toxicity, have led to its ban in the EU since 2019. Additionally, trypan blue precipitates rapidly, complicating accurate cell counting and viability assessment. To address these issues, we developed SafeCountTM, a new cell viability reagent. SafeCount is non-carcinogenic and remains stable in solution for years at room temperature. SafeCount works upon the same principle of trypan blue, which is dye-exclusion by intact cell membranes, resulting in specific staining of dead cells in brightfield imaging. Benchmarking against trypan blue across seven cell types, SafeCount demonstrated equivalent performance and superior stability. SafeCount may also exhibit lower cell toxicity, offering stable cell viability measurements for up to 1 hour post-addition when using the Countess 3FL. In addition to the new brightfield reagent, we demonstrate the compatibility of CellEventTM Caspase-3/7 fluorescent reagents with the Countess 3FL, facilitating quick assessments prior to downstream applications such as flow cytometry or imaging. Apoptosis was induced in U397 cells using camptothecin for 4 hours. The caspase 3/7 (CellEvent green) dye exhibited 45% positivity on the Countess 3FL and 53% on the Attune Flow Cytometer, while the caspase 3/7 (CellEvent red) dye showed 51% positivity on the Countess and 45% on the Attune. A similar study with CellROXTM verified the rapid and accurate oxidative stress assessment on the Countess, benchmarked against flow cytometry data. Utilizing these reagents on the Countess 3FL provides scientists with a quick overview of cell cycle status and treatment effects, thereby saving time before proceeding to more detailed analyses. All reagents are intended for Research Use Only (RUO). Not for use in diagnostic procedures.

B559/P1683

Exploring dynamic remodeling of the embryonic zebrafish heart by high-throughput light sheet imaging of ischemia-reperfusion injury

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Cardiovascular disease remains the leading cause of mortality worldwide, with ischemia–reperfusion injury (IRI) representing a critical yet incompletely understood driver of heart failure. To resolve the dynamic morphological and cellular processes underlying IRI, we are using a zebrafish (Danio rerio) embryo model that integrates genetic accessibility with cutting-edge imaging platforms. Using a transgenic fish line expressing green fluorescent protein (EGFP) in cardiomyocytes, we are utilizing a workflow for direct visualization of cardiac muscle architecture using fluorescence imaging during and after IRI. This system permits precise assessment of tissue-level injury and remodeling, while preserving the optical clarity and regenerative potential unique to zebrafish embryos. To scale analysis and reduce imaging bias, we are implementing next-generation high-throughput light sheet fluorescence microscopy optimized for high-content screening. This platform has potential for parallel imaging of hundreds of embryos with minimal phototoxicity, generating multidimensional datasets that capture cardiac morphology, contractility, and recovery trajectories in real time. By extracting structural predictors of survival outcomes, we aim to identify phenotypic signatures that distinguish resilient from vulnerable hearts. Together, these advances establish a versatile model that bridges developmental biology, imaging technology, and translational cardiovascular research. Beyond providing mechanistic insight into how the embryonic heart responds to IRI, this approach opens avenues for high-throughput screening of genetic and pharmacological modifiers of cardiac injury. Our work underscores the zebrafish as a uniquely powerful platform to interrogate the interplay between cellular architecture and functional resilience, and to accelerate discovery of strategies to prevent or reverse heart failure.

B561/P1685

High-Throughput Screening for Cysteine-Reactive Compounds Uncovers New Anticancer Drug Candidates Acting in Early Mitosis

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Cysteine residues play a central role in protein function through catalysis, disulfide bond formation, and post-translational modifications, making them attractive targets for covalent drug design. To identify compounds that selectively impair cancer cell proliferation, we performed a high-throughput screen of ~4,000 cysteine-reactive small molecules. We developed a HeLa cell–based synchronized arrest-and-release assay to specifically monitor the early stages of mitosis, enabling us to capture compounds that induce cell cycle arrest and apoptosis at this critical timepoint. Confocal microscopy was used to visualize mitotic phenotypes, and an IN Carta AI-trained model quantitatively analyzed large-scale imaging data. From this screen, we identified 17 candidate compounds that triggered early mitotic arrest with variable phenotypes. These hits represent promising candidates for the development of covalent inhibitors with enhanced target affinity and extended pharmacological activity. Our study demonstrates the power of combining synchronized cell cycle assays with AI-assisted imaging analysis to accelerate the discovery of cysteine-targeted anticancer agents.

B565/P1689

Leveraging automation for live virus vaccine potency assay development

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Live virus vaccine manufacturing is a complex, multi-step process that requires a variety of analytical tools to control quality attributes at key process steps. As vaccine production technology evolves, the need to expand process characterization capabilities grows with it. Potency of pre-clinical candidates is a critical, universally monitored attribute that is used to inform dosing, stability and efficacy of the final product that goes to the clinic, or to determine whether it goes to the clinic at all. In the field of live virus vaccines (LVV), potency is typically measured via cell-based infectivity assays, where virus test articles are allowed to infect a model cell population, and the effects resulting from the infection are then quantified as either phenotypic changes in the cells or as detectable levels of expression of viral replication products. Adaptation of cell-based assays to high-throughput platforms is a valuable tool given the accuracy and reproducibility of automation and the labor-intensive nature of manual versions, and while the principles behind all cell-based automation assays may be similar, successful implementation depends on multiple key factors such as the specific biology of the virus, the choice of cell lines, the target protein, and quantification logic. Using these factors as guide, a novel, hybrid infectivity-based assay format for a current LVV pipeline was successfully adapted for end-to-end automation, based on detection and measurement of localized infected cell clusters (foci) expressing various viral proteins of interest. Complete with a data management workflow, the high-throughput focus-forming assay (FFA) is a flexible tool that can be adapted to various viral candidates based on image segmentation and analysis. Significantly, the hybrid infectivity is performed without the simultaneous need for infection and quantification of a known reference material, which makes this an ideal tool for characterization of novel LVV candidates or for process optimization workflows that lack an established reference standard.

B560/P1684
Recovery assessment of intra- and extracellular antibiotic resistance genes in water and wastewater
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Antibiotic resistance genes (ARGs) in the environment can be found in two forms: intracellular (iARGs) and extracellular (eARGs). The emergence and dissemination of these genes in the environment pose serious threats to both public health and ecological balance. This study assesses various methods for extracting iARGs and eARGs followed by comparing the recovery efficacy using quantitative PCR from water bodies. For intracellular DNA (including iARGs and intracellular 16S rDNA), the E.Z.N.A.® Bacterial DNA kit (Omega) achieved a higher recovery rate (23%) compared to the traditional Phenol-Chloroform-Isoamyl Alcohol method (14%) and the boiling method (15%). In contrast, recovery of extracellular DNA (eARGs and extracellular 16S rDNA) was low when using direct extraction via the Phenol-Chloroform-Isoamyl Alcohol method (0.16%) or two commercial kits (4%), prompting the need for a sample concentration step prior to extraction. Three concentration-extraction approaches were tested: 1) concentration using Amicon filter with DNA extraction via E.Z.N.A.® Bacterial DNA kit (Omega), 2) concentration using 3M Sodium Acetate with DNA extraction via ethanol precipitation, and 3) concentration using Nucleic Acid Adsorption Particles (NAAPs) with DNA extraction via isopropanol precipitation. The highest recovery rate was obtained using the 3M sodium acetate method with ethanol precipitation of almost 90%, whereas the NAAP approach yielded an 80% recovery rate. The Amicon filter method yielded the lowest recovery rate of 7%. These findings indicate that concentrating water samples with 3M Sodium Acetate followed by ethanol precipitation is the most effective method for eARG recovery. The validated extraction methods were further applied to environmental water samples to detect and quantify iARGs, eARGs, and 16S rDNA. The results provide insights into the distribution and abundance of ARGs in aquatic environments, aiding in efforts to monitor and mitigate the spread of antibiotic resistance through water systems.

Advances in Cell Biology Tools and Technologies - Image-Based Phenotyping

B571/P1693
A whole-cell view through correlative light and soft X-ray 3D cryo-imaging
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Advancements in high-resolution imaging of the cellular world have revolutionised high-content volume data acquisition, enhancing our understanding of subcellular organisation and behaviour. One notable development in cellular imaging is the emergence of a cutting-edge 3D imaging technique—cryo-soft X-ray tomography (cryoSXT) of vitrified samples compatible with cells and tissues up to 12 µm. cryoSXT operates without relying on chemical fixation, sectioning, milling, or using contrast-enhancing agents. Under cryogenic conditions, samples are imaged in the 'water-window' energy range, achieving a lateral resolution of cellular structures up to 25 nm1. The cryoSXT platform can be paired with other techniques, such as electron tomography, FIB-SEM, spectroscopy, and X-ray fluorescence microscopy, to achieve a high level of throughput, thereby obtaining

multimodal information from specimens2. At beamline B24, we have developed a custom-built 3D cryoSIM super-resolution fluorescence microscope that allows us to align fluorescent markers with high-resolution structural data obtained from cryoSXT. This set-up provides a unique 3D Correlative Light and X-ray Tomography (CLXT) platform for analysing the same sample in cryogenic conditions. The CLXT experimental workflow is adaptable for a broad range of biological specimens, including multi-species adherent or suspension cell cultures (primary cells and immortalised cell lines), bacteria, algae, fungi, and viruses3. We will detail recent methodological advancements and applications, and discoveries in cellular biology enabled by cryoSXT and CLXT platforms. We will showcase representative data from various projects, including insights into the human herpesvirus and reovirus biology3. Additionally, we will delve into the ultrastructure of the mammalian primary cilium in the context of cell signalling, investigating the sporulation and protein crystallisation processes in Bacillus thuringiensis, and the establishment of a cellular model for studying Candida infections. This 3D biological cryoimaging method offers a novel approach to investigating cellular structures in a near-native condition, complementing electron microscopy and fluorescence imaging techniques. Reference: Harkiolaki, M., et al. "Cryo-soft X-ray tomography: using soft X-rays to explore the ultrastructure of whole cells." Emerging topics in life sciences 2.1 (2018): 81-92. Mendonça, L., et al. "Correlative multi-scale cryo-imaging unveils SARS-CoV-2 assembly and egress." Nature Communications 12.1 (2021): 4629. Kounatidis, I., et al. "3D correlative cryo-structured illumination fluorescence and soft X-ray microscopy elucidates reovirus intracellular release pathway." Cell 182.2 (2020): 515-530.

B574/P1696

Decoding dynamic remodeling of organelles from time-lapse datasets using self-supervised contrastive learning

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Decoding how organelles dynamically remodel under perturbations is essential for understanding cellular responses to perturbations. DynaCLR is a self-supervised contrastive learning framework that captures organelle remodeling dynamics from time-lapse microscopy with minimal human annotations. Trained on images from Organelle Box—a community resource of cell lines fluorescently labeled with distinct organelle markers—DynaCLR learns temporally consistent mapping from images to organelle morphology without requiring manual feature engineering. This approach reveals large-scale remodeling patterns in cell populations that are difficult to detect by inspecting individual images. To capture coherent patterns of organelle remodeling across heterogeneous single-cell responses, we apply Dynamic Time Warping (DTW) to align individual morphological trajectories along a common progression axis. This approach enables robust quantification of dynamic trends and reveals condition-specific differences independent of absolute timing. Applying DynaCLR to label-free phase imaging, we also show that the learned representations accurately predict the infection state of individual cells. DynaCLR and Organelle Box provide a generalizable, annotation-efficient platform for probing cellular dynamics and systematically mapping organelle responses to stress, drugs, and infection.

B573/P1695

Inspecting magnetosensitive autofluorescence in live and fixed cells

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Flavoproteins, a family of proteins that contain flavin cofactors, have been shown to exhibit magnetosensitivity. Previously, it was observed that in cancer cells (HeLa), the magnetosensitivity of endogenous flavoproteins is manifested in autofluorescence. Because the triplet-born radical pairs (RPs) in the endogenous flavoprotein tend to be trapped in the metastable triplet state in the presence of external magnetic fields, decreasing the possibility of radiative decay with photon emission, the intensity of the autofluorescence varies depending on the strength of the externally applied magnetic field at the order of mT. In this study, we explored whether this observation could be extended to other cell lines, especially the ones representing normal cells, by examining normal lung fibroblasts (IMR90). By comparing the ratio of autofluorescence detected at 528 nm (green) to that at 645 nm (red) upon the blue light illumination, we found that indeed the ratio decreased monotonically when subjected to magnetic fields from 0, to 11, to 20, and to 50 mT. Next, we reasoned that the expression and composition of flavoproteins, especially those residing in mitochondria, differ between normal and cancer cells, and such a difference might serve as a biomarker to distinguish normal and diseased cells in fixed tissue sections from biopsy. To test this hypothesis, we measured the ratio of green to red autofluorescence in formalin-fixed cells. In the fixed cells, we observed increased green signals without colocalized red signals, a signature of autofluorescence emitted from molecules other than flavoproteins. We quantified the green to red ratio after excluding the autofluorescence not emitted from flavoproteins. We found no trend of decreasing autofluorescence intensity as the magnetic field strength increased. Two possible explanations exist. First, flavoprotein magnetosensitivity might be completely impaired during the fixation process. Second, flavoprotein magnetosensitivity might be attenuated during the fixation process, and our current measurement and analysis framework is not sensitive enough to detect the attenuated magnetosensitivity. Further investigation is required to identify the true reason.

B572/P1694

Quantitative label-free holotomography identifies biophysical signatures of hepatic stellate cell activation

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Hepatic stellate cell (HSC) activation is a central driver of liver fibrosis, characterized by extracellular matrix (ECM) production and hallmark morphological changes such as increased spreading, stress-fiber formation, and loss of lipid droplets. The objective of this study was to identify quantitative, label-free biophysical signatures of HSC activation using holotomography. We applied the HT-X1 Plus holotomography (HT) system with the FLX100 fluorescence module to investigate HSC activation under live-cell conditions. LX-2 cells were starved and treated with TGF-β to promote a pro-fibrogenic phenotype. Holotomographic imaging revealed clear morphological differences between control and activated cells. To validate the activation process, vimentin and α-smooth muscle actin (α-SMA) expression were confirmed by fluorescence imaging acquired simultaneously with refractive index (RI)-based HT. Furthermore, time-lapse HT enabled continuous observation of phenotypic changes during activation. Quantitative analysis of RI tomograms was performed using AI-assisted segmentation. Activated HSCs displayed significant biophysical alterations compared to controls, including increased projected area, surface area, and cell volume, along with decreased sphericity. Notably, intracellular protein concentration and mean RI values were also decreased, highlighting distinct biophysical signatures of the activated state. In conclusion, our findings demonstrate that HT provides a powerful label-free approach to distinguish quiescent and activated HSCs in real time. By eliminating the need for fluorescent labeling, HT offers a non-invasive and efficient method for assessing HSC activation. This

capability holds strong potential for phenotypic drug-screening applications in liver fibrosis research, enabling high-throughput evaluation of candidate compounds without additional staining.

B570/P1692

Real-Time Visualization of Platelet Dynamics and Thrombogenesis in a Photothrombosis-Induced Cremaster Arteriole Injury Mouse Model.

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There remains an unmet need for clear and comprehensive evidence to explain platelet accumulation and thrombus formation under various physiological and pathological conditions. The cremaster arteriole laser-induced injury model provides a powerful approach for studying thrombus formation, enabling precise induction of thrombosis and real-time visualization of platelet accumulation. Integrating intravital microscopy (IVM) into this model further allows dynamic, real-time assessment of platelet activation and thrombus formation, which can enhance our understanding of platelet dynamics in thrombogenesis. This study aimed to investigate the relationship between platelet accumulation and thrombogenesis using an intravital microscopy-based photothrombosis-induced cremaster arteriole injury mouse model. A photothrombosis-induced cremaster arteriole injury model was established in mice using Rose Bengal injection followed by laser activation. Intravital microscopy (IVM) was employed to monitor, in real time, platelet activation, platelet aggregation, and vascular morphological changes during thrombus formation. Using IVM, we observed that photothrombosis induced by Rose Bengal triggered rapid platelet activation, followed by abnormal platelet aggregation and progressive vascular narrowing over time. These findings provide direct visual evidence that photothrombosis-induced platelet accumulation can drive thrombus formation. Furthermore, excessive or abnormal platelet aggregation was shown to contribute to vascular stenosis. Real-time observation of physiological vascular changes caused by platelet activation and abnormal thrombus formation using an IVM-based cremaster arteriole injury model offers a deeper understanding of the relationship between platelets and thrombosis. This approach represents a valuable experimental platform for elucidating novel mechanisms underlying thrombus formation and has potential applications in the evaluation of anti-thrombotic therapies.

B575/P1697

The organelle box: an open-access library of fluorescently labeled cell lines to study organelle dynamics

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Cells undergo extensive molecular remodeling in response to perturbations, including changes in gene expression and the rewiring of molecular networks which shape adaptation and survival. While transcriptional and signaling responses are well studied, less is known about the remodeling of subcellular architecture at a global scale, and how these changes influence function. To address this gap, we developed the Organelle Box, a library of A549 lung carcinoma cell lines, engineered with endogenous GFP-3X-HA tags in 23 proteins that represent 17 distinct organellar compartments. This open-source resource enables high-resolution analysis of organelle dynamics in living cells. As a proof-of-concept, we are using the Organelle Box to characterize organelle remodeling during infection by two medically relevant and related viruses, Zika Virus and Dengue Virus. Correlative live-cell imaging combined with self-supervised deep learning has revealed conservation and

differences in the patterns and kinetics of remodeling of mitochondria, ER, and stress granules between these closely related flaviviruses. We're making the Organelle Box available to the community, aiming to provide a shared toolkit to accelerate research. Beyond viral infection biology, this resource enables systematic exploration of how organelles are reorganized across a wide range of stresses and stimuli, offering opportunities to connect cellular architecture with molecular state and function.

Advances in Cell Biology Tools and Technologies - Microfluidic and 3D culture systems

B486/P1052
High resolution neural activity mapping in human-iPSC derived guided cortical organoids via high-density multi-electrode array technology
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iPSC-derived guided cortical organoids (gCOs) are a significant advancement for studying neuropsychiatric disorders providing spatially organized neural networks that retain patient-specific genetic characteristics. These models recapitulate key aspects of such conditions through complex cell-cell interactions and three-dimensional tissue architecture [1]. The integration of high-density multi-electrode array (HD-MEA) technology, particularly 3D HD-MEA, with organoid models enables comprehensive electrophysiological characterization of human neural networks at single-cell resolution [2]. Human iPSC lines, previously generated and characterized in our laboratory [3], were differentiated into gCOs [4]. Samples were collected from day 15 to day 150. Temporal expression of neural markers was quantified by qPCR. Spatial distribution of neuronal subtypes was assessed by immunofluorescence and two-photon microscopy (Zeiss LSM880). HD-MEA recordings enabled single-cell spike detection, network connectivity mapping, and profiling of neural activity. Electrophysiological studies were performed using the 3Brain AG platform, comprising CorePlateTM 1W or CorePlateTM 1W 3D, the BioCAM DupleX acquisition system and BrainWave 6 analysis software. Neural network activity was recorded on day 150 for 10 minute sessions in a controlled recording chamber maintained at 37°C and 5% CO2. At day 15 of differentiation, neural precursor identity was confirmed through qPCR detection of early markers SOX2, PAX6, and FOXG1. By day 25, successful cortical specification was validated by qPCR expression of patterning markers OTX2 and TBR2. From day 50 onward, cortical maturation was demonstrated through TBR1 and MAP2 expression via qPCR and immunofluorescence analysis. Immunofluorescence revealed co-expression of CTIP2+/MAP2+ neurons, indicating deep-layer cortical neuron development. At day 90, detection of VGLUT1+/MAP2+ neurons confirmed glutamatergic projection and neuron maturation. HD-MEA recordings at day 150 demonstrated a high profile of electrical activity with complex network burst patterns. Functional connectivity was tested by applying electrical stimulation or via pharmacological modulation with BIC (25mM), AMPA (400nM), or CTZ (400nM). Our gCOs successfully recapitulated developmental milestones and demonstrated robust electrophysiological maturation. MEA integration enabled comprehensive neural network characterization at unprecedented resolution. This platform provides a powerful foundation for neuropsychiatric disorder modelling and drug screening.
References: [1] Whiteley J.T. et al. Stem Cell Reports 2022 [2] Yoon S.J.et al. Nat Methods. 2019 [3] Mapelli L. et al, PLoS One. 2025 [4] Collo G. et al, Neural Plast. 2018

B485/P1051

Microphysiological Systems (MPS) for the Evaluation of Anti-Bacterial Agents

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Microphysiological systems (MPS) have arisen as alternative platforms to traditional animal models and conventional two-dimensional in vitro platforms for evaluating chemical and biological threats within the Department of Defense (DoD). Recent advances in organ-on-a-chip technology have allowed the potential of connecting multiple organ systems together through microfluidic engineering. The objective of this study was to examine the ability of the HUMMIC Chip3+ platform (TissUse GmbH) containing lung, liver, and intestinal tissues to effectively model a pneumonic Yersinia pestis infection. In addition, we examined the response of this system to antibiotic treatment, levofloxacin. Endpoint analysis included the evaluation of cell viability/cytotoxicity, albumin production, and histology. For these experiments, the BSL-2 Y. pestis strain (A1122) was chosen as the model organism. Initially, infections were performed on static MatTek EpiAirway tissues to evaluate conditions for a bacterial infection. Different conditions were tested on the MatTek EpiAirway tissues – untreated, scratch, and puncture after 24 and 96 hours. Various concentrations of Y. pestis cultured in [broth] were tested for an infection. Media was collected to measure OD600 concentrations and tissues were processed for histology (hematoxylin & eosin staining). This preliminary work was crucial for determining bacterial concentration and time points for advancing this infection into the HUMIMIC Chip3+ platform.

B484/P1050

Tissue Engineering of the World's Thickest Artificial Human Skin reveals Immunogenic Responses to mRNA Vaccines

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mRNA vaccines hold great promise in disease modulation and were widely used during the COVID pandemic. However, immune response upon skin injection, particularly during the early stages, has been difficult to evaluate, leaving a gap in patient safety. We have created the deepest artificial bioprinted skin model available, including elements of the epidermis, dermis, hypodermis and muscle layer, with added immune cells, in order to attempt to bridge this gap in vaccine safety assessment. Primary human skin donations were obtained with national and local ethical consent, disaggregated into component cells and placed in cell culture. Keratinocytes, fibroblasts, adipocytes, myoblasts and monocytes (blood derived) were pre-expanded at low passage number. A 4-layer skin model was designed using SketchUp and Slic3r to generate G-code for the bioprinter. Bioink with nanofibrillar cellulose, fibrinogen and alginate were combined with different cell populations and bioprinted in a hierarchical 4-layer skin model and matured (5% CO2 37℃ for up to <4 weeks) with GM-CSF M1 macrophage differentiation in-situ. Injections of test mRNA vaccines or PBS were made and control of topical LPS, TGFβ, GM-CSF, & PBS. Live-Dead, histology, cytokine bead array and metabolic activity were evaluated. A full-thickness epidermal-dermal-hypodermal-muscle skin was achieved with living M1 viable macrophages, Nile Red positive adipocytes and Myogenin-Myo-G expressing muscle layers. Topical treatment showed M1 macrophage response, but also adipose and muscle irritation. IL-1β, IL8 and IL10 were elevated compared to

controls of untreated, PBS, TGFβ and GM-CSF conditions. Interestingly of the mRNA vaccine formulations created, dose-response and differences were found in immune and layer stimulation in the artificial skin model (IL-1β, IL-6, IL8 and IL10). Since we were able to print 100's of skin models in one afternoon, the prospect of routine vaccine testing for immunogenic and skin reactivity is possible. Further, since the skin can be maintained for several 3 weeks, longer-term studies can be made to evaluate effects that mRNA changes that give significant advantage over historical safety analyses.

Advances in Cell Biology Tools and Technologies - Multi-omics

B578/P1700

A Multiomics and Functional Testing Pipeline for Solid Tumor Precision Medicine

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The emergence of tumoroid technology has transformed the ability to culture patient-derived cancer cells in vitro while maintaining the morphological, genetic, and transcriptomic hallmarks of the original tumor. Numerous studies have shown that tumoroid responses to therapy often parallel clinical outcomes, underscoring their value in functional precision oncology. In this context, patient-derived tumor cells can serve as ex vivo models to assess therapeutic sensitivity and potentially inform individualized treatment strategies. In this study, we evaluated an integrated workflow combining multiomics analyses with next generation sequencing to advance precision medicine applications in colorectal cancer. Tissue material from six colorectal cancer donors was sectioned, stained, and processed for nucleic acid extraction using an automated purification platform. DNA and RNA were analyzed on the Genexus™ Integrated Sequencer with the Oncomine™ Comprehensive Assay v3, enabling targeted detection of single-nucleotide variants, copy number variants, indels, and gene fusions. Purified RNA also underwent bulk RNA sequencing to generate transcriptomic profiles for each donor. Tumoroids were exposed to chemotherapy drugs commonly used in standard-of-care colorectal cancer treatment, and cell responses were quantified. Dissociated Tumor Cells (DTCs) were used for untargeted proteomic and metabolomic profiling. Multiomics analyses revealed heterogeneity in genomic alterations, transcriptomic signatures, proteomic and metabolomic landscapes, as well as differential drug response profiles across donors. Comparative pathway enrichment analysis also captured key biological processes central to immune function, RNA metabolism, and cell division. In comparing late-stage colon cancer (IIIC) to early stages (I, IIC, IIA), IPA revealed highly consistent molecular, and pathway overlaps (~80–90%), particularly in neutrophil degranulation, RNA processing, and cell survival/proliferation functions. Cancer-associated molecules remained similar in number (~7,400–7,500), but key regulators such as MYC showed stage-dependent differences, being activated in IIIC vs IIC but inhibited in IIIC vs I and IIA. These findings suggest that while core biological programs are conserved across stages, regulatory shifts may drive progression to advanced disease. PCA clustering of the metabolomics profiles distinctly separated the tumor cell samples, revealing clear donor-specific metabolic differences. Our findings demonstrate the feasibility of a unified workflow that leverages genomic, transcriptomic, proteomic, metabolomics and functional assays to inform patient-specific treatment decisions to advance tumoroid technology.

B576/P1698

High-Resolution Size Proteomics Dissects Organelle Diversity and Protein Complex Heterogeneity in Brain

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Living systems are organized into ordered molecular structures in which each component is compartmentalized and maintained in its proper form and size to sustain cellular and tissue functions. Disruption of this order—through altered localization or abnormal size changes such as dimerization, polymerization, and aggregation—is a central driver of disease pathogenesis. Conventional molecular analyses have relied on microscopy or targeted biochemical purification, but these approaches lack the resolution and coverage needed to quantify molecular localization and size at scale. To overcome these limitations, we developed High-Resolution Size Proteomics, an integrated platform combining stepwise ultracentrifugation, density-gradient fractionation, size-exclusion chromatography, precise recovery of all molecular fractions (international patent WO 2024/067890), and ultrasensitive mass spectrometry. This approach generated proteome-wide maps of brain tissue under physiological conditions, resolving organelle size distributions, particle densities, molecular compositions, and elemental contents. Analyses of membrane fractions revealed distinct size spectra for mitochondria, endoplasmic reticulum, Golgi, lysosomes, and synaptic vesicles. For example, while conventional fractionation confirmed mitochondrial enrichment in the P2 fraction, our profiling resolved greater heterogeneity: marker proteins UQCRC2, COX2, and TOMM70 delineated distinct populations of large, medium, and small mitochondria, each with characteristic abundances and co-fractionating protein groups. Several mitochondrial-associated proteins were also detected in the cytosolic fraction, suggesting release or redistribution beyond the organelle membrane. Analyses of soluble fractions further resolved the sizes and compositions of protein complexes. Tandem size-exclusion columns provided extended separation, and quantitative proteomics generated distribution profiles that distinguished proteins incorporated into higher-order assemblies from those present predominantly as monomers. Together, this high-resolution size proteomics framework enables seamless localization- and size-dependent molecular profiling, offering a powerful strategy to decode physiological organization and pathogenic mechanisms in complex biological systems.

B577/P1699

Mass spectrometry for the masses: accurate quantitative proteomics on a cost effective instrument

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Quantitative proteomics has become a powerful approach to assay cellular states in health and disease, but access remains limited. Most workflows depend on complex hybrid instruments that combine quadrupoles, ion traps, Orbitraps, or time-of-flight analyzers, with substantial purchase price and extensive expertise required for maintenance. In contrast, standalone ion trap instruments are far simpler, more affordable, and robust, but they have traditionally been limited to qualitative applications, such as identifying proteins from gel bands, and are not considered suitable for quantitative proteomic analyses. Here we demonstrate the repurposed use of an ion-trap only mass spectrometer (LTQ Velos) - available for ~ 10% of the cost of typical high-end platforms - for quantitative multiplexed proteomics. We show that the ion trap can perform MS1 scans, precursor isolation, fragmentation, and MS2 readout with sufficient resolution and mass accuracy to support TMTpro complement reporter ion quantification (iTMTproC). Using yeast-human interference standards, we obtain accurate nine-condition multiplexed measurements with good quantitative accuracy and precision. While the method requires longer instrument time than high-end systems, we quantified approximately ~7k proteins (~70% of state-of-the-art coverage) from a HeLa standard. Ongoing work is applying this approach to complex biological samples to further assess performance in routine cell biology experiments. These results demonstrate that modest ion-trap instruments can provide robust quantitative

proteomic data, lowering barriers to adoption and opening up the possibility to bring mass spectrometry-based proteomics into many more biology laboratories.

B579/P1831
Telomere to Telomere References and Long-Read Genomics Unlocks a New Era of Functional Cell Biology
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Reference genomes are the scaffolds on which aspects of modern cell and molecular biology are built. Yet for decades, a single human reference genome has been available that has remained incomplete, composite, and far removed from the cells we actually study. Here, I present our telomere-to-telomere assembly of the human RPE1 cell line, the first reference genome for a widely used model in cell biology, generated using high-fidelity long reads, ultra-long nanopore data, and advanced chromosome conformation capturing phasing of the parental haplotypes. This assembly resolves centromeres and structural variants at single base resolution, enabling accurate mapping of regulatory and structural elements that were previously inaccessible. I'll present how isogenomic reference frameworks transform our ability to connect genome structure with transcriptional and epigenomic states, enabling high precision genome engineering and multi-omics analyses. Using this appraoch, we reveal cell line–specific rearrangements, epigenetic landscapes, and structural variation with functional consequences for chromosome segregation, and higher-order modes gene regulation along chromosome arms. Together with our novel discovery of the centeny map of the human genome, we'll showcase an innovative computational suite to study centromeres that unveiled a chromosome-specific genome architecture conserved across individuals, populations and species. Altogether, our work outlines a roadmap toward isogenomic reference sets for key model systems, and offers proof-of-concept on how their application is set to unlock the next wave of functional genomics. Cell-type-specific telomere-to-telomere references, anchored in real experimental systems, can redefine precision, reproducibility, interpretability, and discovery in the omics era.

Advances in Cell Biology Tools and Technologies - Optogenetics

B480/P1046
Development of an artificial cargo system to study the regulation of microtubule-based transport in living cells
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Microtubule based transport is mediated by various motor proteins that can attach to intracellular cargo and move them along microtubule tracks. It is tightly regulated to influence cell growth and survival. This regulation is especially pertinent in neurons, where the formation, function, and maintenance of elaborate neurites requires long distance transport of key cargos to different compartments. While numerous biochemical and biophysical studies have revealed different mechanisms underlying transport regulation, less is understood about the role of these mechanisms in living cells. To directly assay the cellular context that influences motor function, we have developed an artificial cargo system that uses blue light to rapidly form inert fluorescent protein clusters based on a CRY2 mutant (Park et al., Nat Comm 2017) and simultaneously recruit optogenetic motors to the cluster based on the iLID/SSPB system (Nijenhuis et al. JCB 2020). To validate our system, we tested two kinesin motors with known preference for axons vs dendrites and found that in immature cortical neurons, artificial cargo bound by KIF1/kinesin-3 (Opto-KIF1A) were transported into all neurites, whereas those bound by KIF5/kinesin-1 (Opto-KIF5C) selectively entered the axon. We next used the

system to study how microtubule associated proteins (MAPs) regulate transport of different motors in embryonic sensory neurons. The current study focuses on MAP7, a unique MAP that has been shown in single molecule in vitro assays to inhibit KIF1 motility but promote KIF5 motility by recruiting KIF5 via its interaction with the motor's stalk region (Monroy et al., Nat Comm 2018). In axons overexpressing MAP7, artificial cargo transport driven by Opto-KIF5C (K560) motors that contain the MAP7 binding region showed increased transport events but decreased velocity, consistent with the biphasic regulation previously observed in vitro (Ferro et al., Science 2022). For comparison, artificial cargos driven by truncated Opto-KIF5C motors (K385) lacking the MAP7-binding region had reduced transport events and reduced velocity in the presence of MAP7, providing further evidence of biphasic regulation. Interestingly, artificial cargo transport mediated by two different versions of Opto-KIF1A (K365 and K393) was largely unaffected at sites of MAP7 overexpression in sensory axons, raising several interesting explanations. Finally, we are using this system to probe the microtubule landscape in different axonal compartments. In summary, the artificial cargo system will allow us to interrogate known regulatory mechanisms and identify new ones across different cellular contexts.

B481/P1047

FilaBuster: A Photochemical Strategy for Acute and Precise Intermediate Filament Disassembly

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Intermediate filaments (IFs) are essential to cellular architecture, force transmission, and organelle positioning, and their dysregulation is linked to diverse diseases. Yet, despite decades of study, IFs remain the only major cytoskeletal system without a method for rapid, selective disassembly—a long-standing gap that has limited functional insight. We present FilaBuster, a photochemical strategy for acute, spatiotemporally precise IF disruption in living cells. The method uses a three-step workflow: (1) HaloTag targeting to IFs, (2) covalent labeling with a photosensitizer ligand, and (3) light-induced reactive oxygen species generation at the filament surface. This modular design applies across multiple IF subtypes—including vimentin, GFAP, desmin, peripherin, and keratin 18—and is compatible with diverse dyes and imaging platforms. Using vimentin as a model, we establish rapid, specific disassembly with strikingly low light doses and then extend the approach with nanobody-based targeting, expanded photosensitizer options, and irradiation schemes that enable both global and focal control. Together, these findings position FilaBuster as the first robust platform for acute and tunable IF disassembly, with broad utility for probing IF function in cytoskeletal biology, development, and disease.

B479/P1045

Intraprotein domain competition dictates pulsatile response of photoreceptors shaped by temperature and evolution

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Stimulation of sensory systems commonly shows pulsatory responses that are generated by networks of molecular interactions. Here we find that pulsatory activation patterns can also be encoded by individual proteins in response to multiple concurrent environmental inputs, and that such unimolecular dynamics have been conserved over 100s of millions of years of evolution. BcLOV4 is a fungal photoreceptor that can generate

a temperature-dependent pulse of activity in response to light in mammalian cells, though the molecular nature of pulse generation and thermosensitivity were not understood. We combined live cell imaging, in vitro biochemistry, and simulations to find that activity pulses result from intraprotein competition between distinct light- and temperature-sensitive protein fragments. This competition generates 3 temperature-dependent classes of activity in response to a constant light input: constant (low T), pulsatile (intermediate T), or silent (high T). These schemes occur because light stimulation exposes an activation region that is subsequently caged by a C-terminal temperature-sensing domain (TSD) whose caging strength increases with increasing temperature. The TSD implements thermosensing through conformational changes in two co-evolved loop regions , and mutations or deletions within these loops tuned the amplitude and dynamics of BcLOV4 activity. Finally, we found that the 3-class photo-thermal response has been conserved in fungi that diverged over 300 million years ago, and that the temperature of switching between classes adapted to match the ecological niche of the host, including in extreme environments like Antarctica and in thermal ponds. Collectively our study finds 8 novel proteins that can be actuated with light and temperature in heterologous cells, enabling development of new light- or temperature-responsive molecular probes and deeper understanding of how proteins can sense small changes in temperature. More broadly our study uncovers how time-varying activation dynamics can be compactly encoded within individual proteins and implies the functional importance of such response dynamics throughout evolution.

B482/P1048
Optogenetic Control of Cytosolic Protein Transport
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The active transport of proteins along the microtubule cytoskeleton is important for many cellular processes such as cell migration, establishment of cell polarity, cell division, and protein homeostasis, while defective transport can be associated with cancer and neurodegenerative diseases. Active transport involves coordination between molecular motors, which hydrolyze ATP to transport cargo, and adaptor proteins that link cargo to the motor proteins. While it is well-established how proteins are actively trafficked in multiprotein assemblies (such as organelles, vesicles, or RNA granules), it remains an outstanding question whether and how soluble cytosolic proteins are trafficked on microtubules. In this study, we demonstrate that induced oligomerization of a motor adaptor protein leads to processive transport of soluble proteins along the cell's cytoskeleton. We fused the light-sensitive oligomerizer, CRY2olig, to different motor adaptor proteins, which results in light-dependent cumulative relocalization of protein to plus- or minus- ends of microtubules. Our study provides evidence that cytosolic proteins, on their own, can undergo progressive transport by assembling into condensates or multivalent assemblies with multiple binding sites for motor proteins, and develops a unique method for spatial and temporal control of protein transport using light.

B483/P1049
Optogenetic control of p53 signaling pathway
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The tumor suppressor p53 functions as a hub protein regulating a variety of stress-related signaling pathways. Various stresses activate p53, and activated p53 regulates the transcription of multiple target genes. Accumulating evidence indicates that p53 functions not only in tumor suppression but also in a wide variety of

biological processes such as senescence induction, metabolic regulation, and stem cell differentiation. However, since these diverse outcomes are induced in concert with various factors, it is difficult to characterize the context-dependent role of p53 itself. Therefore, the technology that can specifically manipulate the p53 pathway is highly desirable. Here, we report the optogenetic systems allowing control of the p53 signaling pathway. p53 activation is mainly mediated by a feedback loop with its negative regulators, MDM2/MDMX. Therefore, we focused on a short peptide (PMI) that inhibits the p53-MDM2/MDMX interaction and developed two types of optogenetic systems. First, we used a light-dependent dissociation system, LOVTRAP, to induce the translocation of PMI from the mitochondrial membrane to the nucleus in a light-dependent manner and observed p53 activation. Second, we designed a system in which PMI is exposed to the protein surface in a light-dependent manner by fusing PMI to the photoreceptor domain LOV2. We screened various substitution/deletion mutants of the LOV2-PMI fusion module and obtained several mutants that can induce light-dependent p53 activation. By combining these two technologies, we have successfully developed an optogenetic system with both low basal p53 activity and efficient p53 activation upon light illumination. We will present the detailed design of our system and demonstrate manipulation of downstream gene expression and cell cycle progression.

Advances in Cell Biology Tools and Technologies - Protein structure prediction (e.g., AlphaFold)

B508/P1072
Statistical Optimization for Production of Bioactive Peptides from Food Isolated Bacterial Cells for Sustainable Applications
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Introduction: Small protein fragments known as bioactive peptides have antibacterial, antibiofilm, and antioxidant properties that may be used therapeutically. Though new research indicates that some bacterial strains isolated from fermented foods naturally create or release these peptides during growth, they are traditionally acquired by the enzymatic degradation of dietary proteins. Using these microorganisms offers a low-cost, sustainable path to useful proteins while advancing the ideas of the circular bioeconomy. Methodology: Bacterial isolates were obtained from various foods such as curd, kimchi, and yogurt. Pure cultures were screened for proteolytic activity using skim milk agar and casein hydrolysis assays. High-activity strains were cultured in protein-rich media optimized for peptide secretion. Cell-free supernatants were collected, and peptides were partially purified using ammonium sulfate precipitation and dialysis. Antioxidant potential was evaluated by DPPH radical scavenging assay, while antimicrobial activity was tested against common foodborne pathogens (E. coli, S. aureus). Protein profiles were visualized using SDS-PAGE to confirm peptide presence and molecular weight range. Sustainable applications of these peptides in antibiofilm activity against harmful pathogens were also observed. Results: To create bioactives from different dietary samples, different bacterial strains were isolated. Production conditions and medium formulation were optimized for yield enhancement using response surface methodology (RSM), which optimized substrates into carbon source (xylose), nitrogen source (peptone), and inoculum size (7%v/v). Following optimization using the Box Behnken design of RSM, the yield was 7.31g/L, confirmed by analysis of variance (ANOVA) and determined to be significant ($p<0.05$). Using bioanalytical techniques, the bioactive structural analysis indicated that it was a glycopeptide. It was a cationic and thermostable biomolecule with a critical micelle concentration of 900 mg/L after further functional evaluation. The final product was also tested for emulsification against vegetable oils for the food industry and evaluated for antibacterial and antibiofilm properties against typical Gram +ve and -ve pathogens for the medical applications. Conclusion: Bacterial isolates obtained from food are a valuable

natural source for the synthesis of bioactive peptides. Their cell biology can be used to generate sustainable, useful biomolecules that are useful in the pharmaceutical and nutraceutical sectors, particularly in relation to protease secretion and stress adaptability. Keywords: Bioactive peptides, sustainability, antibacterial, antibiofilm, optimization, functional molecules.

Advances in Cell Biology Tools and Technologies - Spatial omics

B581/P1701
Combining single-cell and high-resolution spatial transcriptomics to uncover developmental trajectories in the embryonic lung
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The mature mammalian lung contains ~50 different cell types organized in precise spatial positions along the airway epithelial tree. High-throughput single-cell RNA-sequencing (scRNA-seq) has enabled the discovery of many of those cell types but suffers from a lack of spatial context. It therefore remains unclear how the precise spatial patterns of different cell types arise during development. Here we optimized a microfluidics-enabled, deterministic barcoding-based sequencing (DBiT-seq) workflow to provide spatial context and achieve high-resolution and highly sensitive spatial mapping of embryonic lung tissue sections at near-single-cell level. The embryonic lung epithelium differentiates into proximal Sox2-expressing airway epithelial cells and distal Sox9-expressing alveolar epithelial cells. We mapped the epithelial subclusters, carried out pseudotime analysis, and generated the spatial differentiation trajectory at each stage of lung development. This analysis confirmed epithelial differentiation along the distal-proximal axis of the organ but unexpectedly revealed that conventional markers of distal epithelial progenitors are expressed in proximal regions of the epithelium at early stages of development. This pattern contrasts with traditional conceptual models of lung development, which have assumed an initial proximal-distal patterning of the epithelial tree. We used a similar approach to explore the notoriously heterogeneous mesenchyme of the embryonic lung. We identified a novel mesenchymal cluster, which we termed as Matrix Mesenchyme, spatially localized around the trachea and the medial boundary of the lung lobes. Bioinformatics analysis predicted that the Matrix Mesenchyme interacts with neurons, which we confirmed experimentally. Integrating single-cell RNA-seq and unbiased microfluidic-enabled spatial transcriptomics thus allowed us to uncover previously unrecognized cell types and differentiation patterns.

B582/P1702
Optimizing Fluorescence Microscopy through Informed Mounting Media Selection
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Fluorescence microscopy is fundamental for visualizing biological targets in cultured cells and tissue sections, yet the impact of mounting media on imaging performance is often underappreciated. Mounting media (mountants) are the final reagents applied to fixed, fluorescently labeled samples before imaging; beyond securing the specimen between a coverslip and slide, fluorophore performance is enhanced, long-term preservation is enabled, and the refractive index (RI) is adjusted to improve image quality. The objective of this study is to provide practical guidance for selecting mounting media to optimize fluorescence imaging, with emphasis placed on refractive index matching and fast-curing technologies. Fluorophore compatibility in cultured cells was evaluated, preservation of labeled specimens using archived rodent brain tissue in 2D and

3D was assessed, and the influence of RI matching on image quality in thick rat brain sections was demonstrated. Strategies to reduce the delay between mounting and imaging through comparative 3D imaging workflows incorporating rapid-curing mountants were tested. Signal intensity was maintained and photobleaching was reduced by appropriate mountant–fluorophore pairing; contrast and resolution in thick tissues were improved by RI matching; archival stability was enhanced by suitable formulations; and turnaround time was shortened while high-quality 3D acquisitions were enabled by fast-curing approaches. It is concluded that informed mounting media selection—integrating compatibility, RI matching, and fast-curing technologies—improves image quality, preserves specimens, and streamlines fluorescence microscopy workflows.

B580/P1832
SpatialCell AI: Morphology-guided computational enhancement for single-cell resolution spatial transcriptomics
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Study Objective: We developed SpatialCell AI to achieve true single-cell resolution from spot-based spatial transcriptomics through morphology-guided computational enhancement, addressing fundamental limitations of existing deconvolution methods. Methods: SpatialCell AI integrates AI-powered cell segmentation from histological images with spatial gene expression data, eliminating reference dependencies that constrain all existing computational approaches. The framework processes tissue images to identify individual cell boundaries, extracts morphological features, and integrates spot-level expression with cell-level spatial information through distance-based weighting and biological constraints. Results: Rigorous validation using matched colorectal cancer samples across Visium (55µm), Visium HD (8µm, 16µm), and Xenium (single-cell ground truth) demonstrated strong performance. SpatialCell AI achieved expression correlation of r=0.791 with Xenium ground truth, representing 7.82-fold improvement in expression accuracy and 5.30-fold enhancement in gene detection compared to spot-based measurements. The framework showed robust performance across gene expression ranges, with highest accuracy for marker genes (r=0.870) and substantial correlation for lowly expressed genes (r=0.672). Comprehensive benchmarking against 28+ existing methods revealed universal limitations including reference dependency (25/28 methods), platform effects (30-50% accuracy drops), and the complete neglect of morphological information. Conclusions: SpatialCell AI represents a paradigm shift from computational inference to morphology-guided direct measurement, converting standard Visium outputs to true single-cell resolution (Cell_1, Cell_2, Cell_3 format). This approach democratizes access to single-cell spatial analysis, enabling precise cellular interaction mapping and rare cell type identification for research and clinical applications.

Bioengineering and Synthetic Cell Biology - Cell Engineering

B420/P0986
Delivery of Peptide Coacervates to Form Stable Interactive Hubs in Cells
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Cells compartmentalize biochemical processes to enhance the rate and fidelity of these reactions. Many of these are membraneless organelles which form through self-assembly and condensation of intrinsically disordered proteins (IDPs) in mesoscale subcellular compartments. Previously, our lab designed synthetic

membraneless organelles to rewire cellular signaling and control cell function through transduction of key IDP scaffold-encoding genes. However, to date, such micro-size hubs have not been deliverable to non-phagocytic cells. Here, I sought to assemble and deliver membraneless compartments to a variety of cell types and to use nanobodies to interface with native cellular targets. I demonstrate formation of micron-size particles from the self-assembly of HBpep, a 26-amino-acid disordered peptide, which serves as the scaffold to construct deliverable membraneless hubs. I show these particles can be delivered with high efficiency to a wide variety of cell types and are stable intracellular for least 5 days. Uptake readily occurs through macropinocytosis related mechanism. By loading them with nanobodies, I demonstrate selective recruitment of a target protein both in vitro and in cells. The topology and efficiency of recruitment can be controlled to create internal or surface binding. In cells, I demonstrate it feasible for hubs to escape endosomal compartments using TAT-HA2 or Chloroquine. Furthermore, by co-assembly with a bioPROTAC, these synthetic hubs can function as degradation centers. These findings demonstrate the potential for delivering pre-formed condensates to target native cellular pathways, providing a foundation for future efforts to rewire signaling circuits via deliverable organelles.

B422/P0988

Engineering Mesenchymal stem cells for Inflammation Control without Immunosuppressive Support

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The objective of this study is to engineer mesenchymal stem cell (MSC) spheroids to control local inflammation at the target disease site. Neurodegenerative diseases are debilitating brain diseases that are associated with specific neuronal degenerations and present significant unmet medical needs. Stem-cell therapies offer a promising pathway to treat neurodegenerative diseases. However, persistent overactive neuroinflammation in degenerative brains creates a destructive environment unfavorable for transplanted stem cells. Thus, there is a critical need to develop a new strategy to protect donor cells from the attack of recipient's immune system and meanwhile alter surrounding environment. To address this challenge, we engineered MSCs at both the intracellular and extracellular levels. After engineering, the cells could secret a key anti-inflammatory cytokine interleukin-10 (IL-10) and acquire a biomimetic coating for protection. Methods: The lentiviral vector expressing IL-10 and packaging plasmids were co-transfected into HEK203T cells. Lentiviral particles in the medium were collected 24-48 hours after transfection, and concentrated by ultrafiltration. MSCs were infected with high tier of lentiviruses expressing IL-10 for 48 hours and the stable IL-10-secreating MSC line was selected for expansion. Total cumulative release of IL-10 was analyzed by ELISA. Stability of cell line and stemness was confirmed through MTS assay and surface markers for MSCs. To protect MSCs from unfavorable degenerative microenvironment, MSC spheroids were encapsulated within a biomimetic hydrogel, and IL-10 secretion was analyzed compared to control spheroids. To examine the immunomodulatory ability of IL-10 expressing MSCs, TNF-alpha release induced by LPS stimulation was measured in vitro. Results: Our study successfully established the stable MSC lines secreting anti-inflammatory cytokine IL-10. Compared to control MSCs, IL-10 secretion level is increased by three orders of magnitude in IL-10-expressing MSCs. IL-10 expression doesn't affect the multipotency measured by MSC markers. Moreover, after IL-10-secreating MSCs were encapsulated into spheroids, LPS-induced inflammatory cytokine TNF-alpha release was significantly suppressed. These results support that IL-10-secreating MSCs can provide anti-inflammation function benefiting future stem cell therapy. Conclusion: Our findings demonstrate the stability of an IL-10 secreting MSC cell line while retaining multipotency and viability after transduction and encapsulation. Spheroids enhanced immusunomodulatory capacity in vitro, supporting their potential for further therapeutic tests in vivo.

B425/P0991

Gene Expression Changes Induced by MAHS Expression in Human Cells Include Stress-Associated Genes

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Tardigrades are microscopic animals known for their ability to survive extreme conditions, including but not limited to extreme temperatures, radiation, and desiccation. This resilience is incompletely understood but has been attributed in part to genes encoding intrinsically disordered proteins, such as the mitochondrial abundant heat-soluble (MAHS) protein. Although transgene expression of MAHS has been shown to increase stress tolerance of HEp-2 cells and adipose-derived stem cells following various stressors, the mechanisms by which it reprograms cellular states to confer resilience remain unclear. To address this, we used lentiviral transduction to generate U87 cells stably expressing MAHS and performed triplicate RNA sequencing followed by gene ontology (GO) analysis. MAHS expressing cells had 754 upregulated and 221 downregulated genes with respect to control cells (expressing AcGFP). Key findings of the enrichment analysis include differentially expressed genes clustered in transcriptional regulation, including GO:0000978. Notably, this enrichment was largely driven by changes in Cys2(C2) His2(H2) zinc finger transcription factors. Given zinc fingers are implicated in neurological disorders in humans, upregulation of neurogenesis-related genes such as LRFN5 (log2FC: +2.4) and NEGR1 (log2FC: +2.8), suggests that MAHS-induced transcriptional changes may intersect with neuronal pathways. In addition to transcriptional regulators, we observed differential expressions of genes involved in mechanotransduction such as upregulation of integrins (ITGA1, ITGA2, ITGA6, ITGA10, ITGA11) and collagens (COL7A1, COL13A1, COL4A3, COL12A1). Data also shows upregulation of several G protein–coupled receptors (GPR1, GPR37, GPR135, GPR155), PIK3AP1, a catalyzer for PIP3 production as a lipid second messenger, and several genes encoding calcium voltage-gated ion channels (CACNG7, CACNG8); Pointing to MAHS interfering with signal transduction pathways. Expectedly, several stress associated genes (E2F7, GAS1, SENS3) were also regulated in MAHS group. These findings suggest that MAHS expression, potentially mediated by zinc finger transcription factors, broadly reprograms cellular signaling and stress response in the mammalian context.

B424/P0990

Investigation of ligand recognition in cAMP GPCRs of Dictyostelium using AI- Based Computational Approaches

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Dictyostelium discoideum (Dicty), a cellular slime mold, exhibits a unique life cycle encompassing both unicellular and multicellular stages. Its ability to respond to extracellular signals is mediated largely by G protein-coupled receptors (GPCRs). Among the five GPCR classes identified in Dicty (Classes A–E), Class E receptors (cAR1–4) are particularly well-studied for their roles in mediating chemotactic aggregation through recognition of extracellular cyclic AMP (cAMP). Although a homology-modeled structure of the cAMP–cAR1 complex has been reported (Greenhalgh JC et al., 2020), no experimental structures are currently available for any of the cAR subtypes. Leveraging recent advances in AI-based structural biology, including AlphaFold3 (Google DeepMind) and Boltz-1 (MIT), we predicted high-confidence 3D structures of the cAR family and analyzed their interactions with known cAMP analogs. Molecular modeling uncovered a shared pharmacophoric signature for cAMP binding across cAR subtypes and identified key selectivity hotspots within

the binding pocket. Additionally, we computationally predicted point mutations capable of reprogramming ligand specificity toward alternative adenosine derivatives. This study lays a structural and functional foundation for understanding cAMP–cAR interactions and represents a first step toward engineering Dictyostelium as a programmable biosensor, potentially enabling a novel, Dicty-based drug screening platform.

B421/P0987
Quantum Actuation Enables Magnetic Control of Cellular Behavior
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Cryptochromes (CRYs) are flavoproteins that sense magnetic fields (MF) through spin-correlated radical pairs generated by blue light excitation. One member of this family, Arabidopsis CRY2, has been engineered into versatile optogenetic tools for controlling cell signaling. These tools exploit the facts that CRY2 oligomerizes upon blue light excitation and that many cell signaling molecules are activated by changes in their oligomerization state. However, the magnetic field effects on CRY2 and CRY2-tagged molecules in cells have not been established. In this study, we investigated whether weak MF in the tens of milli Tesla range could regulate CRY2 oligomerization. We first expressed a recombinant protein in which CRY2 and mCherry were fused to the cytosolic side of lysosomal-associated membrane protein 1 (LAMP). mCherry fluorescence was observed both at the cell periphery and in the perinuclear region, where late endosomes and lysosomes are located. We observed that when blue light excitation of CRY2 was applied in the presence of MF, the mCherry signal at the cell periphery decreased by 40% compared to the ambient (geomagnetic) field control. Furthermore, we found that CRY2 oligomerization occurred almost immediately after blue light excitation, whereas endolysosomal fusion was delayed by about 6 minutes. These results suggest that CRY2 oligomerization was increased under MF and drove the subsequent endolysosomal fusion. We then continued to explore the possibility of simultaneously triggering different signaling pathways via multiplexing different CRY2-facilitated oligomerizations. To test this, we co-expressed LAMP-CRY2 and cytoplasmic CRY2 in mammalian cells. In the co-expression system, CRY2 in different subcellular compartments were found to oligomerize in an orthogonal manner, independent of each other. Moreover, we found there might be a critical oligomer size of CRY2, above which a steric hindrance will be imposed on the oligomerization of the orthogonal CRY2-tagged molecules. Together, our results highlight the potential of CRY2-based recombinant proteins to enable quantum control of cellular behavior via MF regulation.

B423/P0989
Using charged biosensors for electrotaxis
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The role of electricity in biology has been studied for centuries, dating back to Emil du Bois-Reymond's discovery in the early 1800s that skin wounds generate endogenous fields. Today, it is clear that the body is full of non-neurological bioelectric cues, driven by natural ion gradients, that drive directed cell migration (electrotaxis) in development, healing, and disease. These gradients create direct current (DC) electric fields in the microenvironment (~1 V/cm), sustained by active ion transport or by "leaks" from disrupted tissue integrity, such as in skin injury. Cells have evolved multiple mechanisms to sense and respond to these fields through changes in membrane potential, ion channel activation, electrokinetic shear flow, and electrophoresis-redistribution of charged membrane proteins. While evidence supports electrophoresis as the main way

proteins respond to an electric field, how this signal is transduced to drive electrotaxis or gene expression remains an open question. Recently, TMEM154 was identified as a transmembrane protein required for electrotaxis in neutrophil-like HL60s. TMEM154 localizes to the anode during cathodal migration, and this is consistent with the large negative charge of the ectodomain of the protein. This finding emphasizes the electrophoreses hypothesis, and we helped with a study led by the Theriot and Belliveau groups to show that constitutive expression of TMEM154 in cells that do not normally carry it can subtly enhance electrotaxis. Building on this, we are now hoping to use TMEM154 as a biosensor, specifically as a proxy for when and where cells sense the electric field and align its relocalization with general downstream events that we already observe upon electrotaxis such as cytoskeletal remodeling, migration speed and directedness, and morphological changes. Additionally, we are interested in how TMEM154 redistributes across apical and basal surfaces in a polarized epithelium, especially within collective systems at the bulk and leading/lagging edges. Overall, this project seeks to establish TMEM154 as an electric field biosensor, to map its dynamics relative to other pathways involved in the sensing and responding of electrotaxis, and to uncover how its abundance tunes cellular responsiveness to electrical cues.

Bioengineering and Synthetic Cell Biology - Genome Engineering

B418/P0984
Investigating the role of protein kinase R's loop between ß-strands ß4 and ß5 in discriminating against poxvirus pseudosubstrate inhibitors
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Host-pathogen interactions are dynamic genetic spaces where conserved functionally critical host residues are vulnerable to viral pseudosubstrate mimics. Protein kinase R, part of the vertebrate innate immune system involved in antiviral stress response, is susceptible to this mimicry. When PKR binds to double-stranded RNA, a byproduct of viral replication, it co-localizes resulting in homodimerization and autophosphorylation. With its kinase activity stimulated, PKR phosphorylates eIF2α, a translation initiation factor, inhibiting translation and consequently viral protein production. Many poxviruses encode pseudosubstrate mimics of eIF2α that inhibit PKR and aid viral replication in the host cell. Therefore, there is an evolutionary pressure for PKR to discriminate between its natural substrate and pseudosubstrate antagonists. Previous research examining vertebrate PKR homologs has shown they have different capacities for discriminating against poxvirus pseudosubstrates. Previous research has also examined potential sites involved in PKR's discriminatory capability. PKR's loop between β-strands β4 and β5 is a highly variable region across vertebrates that is overlooked. We are interested in characterizing the role of the loop in discriminating against poxvirus pseudosubstrates. To study this, we combine computational analysis with a high-throughput yeast model to screen PKR homologs against poxvirus pseudosubstrates. Analysis was performed to isolate 624 vertebrate PKR β4-β5 loop homolog sequences from 581 unique species. Loop lengths ranged from 0 to 153 bp and had a median length of 48 bp. The most common loop length among the species studied was 57 bp. The extent of length variation further suggests that PKR tolerates high diversity of the loop. Preliminary data suggest that some loop homolog sequences may either (1) render human PKR nonfunctional or (2) result in a functional PKR with increased sensitivity to the poxvirus antagonist K3. Ultimately, this study informs how PKR influences poxvirus host range across vertebrates.

B419/P0985
Secretion of gut epithelial-penetrating protein domains by engineered probiotics
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Historically, protein-based therapeutics struggle to efficiently transport across the gastrointestinal epithelial barrier, whether it be from varying pH levels, lysosomal degradation, or the protective mucosal layer. Thus, medications are commonly delivered subcutaneously or intravenously. To address the difficulties of oral-based administration, this project utilizes cellular transcytosis, an endosomal pathway that cells use to absorb larger molecules. As compared to paracellular transportation, which occurs between cell junctions, transcytosis tolerates larger protein deliveries such as antibodies. Two unique cell-penetrating domains have been identified to bind extracellular receptors, initiate endosomal engulfment, and traffic to the basolateral membrane of the epithelial cell. Through this research, we aim to engineer E. coli Nissle 1917 (EcN) to secrete cell-penetrating peptides conjugated with fluorescent proteins that travel through mammalian intestinal cells. Cholix I domain (Chx) from Vibrio Cholerae and neonatal FcRn-receptor binding peptides (FcRn-BP) composed our candidate library due to their established receptor binding and trafficking properties. The protein sequences were cloned into a plasmid vector fused to secretion tags, histidine tag, and fluorescence marker. Vectors were transformed into EcN and grown under induced conditions. Endpoint assays determined 48-60% of recombinant protein was secreted compared to wildtype protein production. Subsequent optical density experiments showed non-significant impairment of bacterial proliferation. This suggests the engineered vectors did not limit EcN's growth, while maintaining roughly 50% secretion capacity. To establish an in vitro model of the gastrointestinal barrier for testing transcytosis, T84 colon cancer cells were seeded on transwell membranes and grown until a polarized monolayer was formed. Human serum albumin conjugated with fluorescein isothiocyanate (FITC) served as a positive control alongside the tested cell-penetrating proteins. Among the tested environmental conditions, starvation under acidic (pH 6) vs. neutral and 90-minute vs 20-hour incubation did not increase protein uptake by epithelial cells. Future studies will evaluate the cell-penetrating domains' in vitro performance to inform replacement of the fluorescent reporter with therapeutic proteins such as anti-inflammatory cytokines IL-10 and IL-22. In conclusion, engineering probiotic bacteria to secrete cell-penetrating peptides represents a promising strategy for oral delivery of protein-based therapeutics by exploiting the transcytotic pathway across intestinal epithelial barriers

Bioengineering and Synthetic Cell Biology - Minimal and Artificial Cells

B432/P0998
Decision making without central nervous systems or processors: how cells and robots navigate complex landscapes?"
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Decades of extensive studies combining approaches of genetics, cell biology, biochemistry, imaging, and computational modeling have built up a knowledgebase regarding the biomolecules, signaling pathways, and regulation associated with cell migration. However, important questions remain unanswered, including how cells navigate the extremely heterogenous mechanical environment in vivo, how they change migration modes in response to environmental cues, and how they process conflicting cues. Here we explore whether

robophysical modeling can be used to understand how cells traverse the diverse tissue landscapes with such versatility and efficiency. Robophysics is an emerging field, where robots are utilized as active physical models of organisms to provide insights otherwise difficult to obtain in biological systems. This approach has proven useful for macroscopic organismal locomotion, especially in cases where equations of motion are too complex to solve. Because robots enact real physical interactions, they are guaranteed to be governed by physical laws no matter how complex the system and interaction are. We envision the approach of robophysical modeling will complement other methods to accelerate the process of characterization, validation, and discovery. In this work, we evaluated whether it is feasible to construct robots recapitulating migratory behaviors of cells. Our first-of-its-kind, cell-mimic robot consisted of connected segments, with each segment independently adhering to the substrate and creep outward away from the center, emulating cellular locomotion. The robot design allowed us to vary the ligand density to create a gradient across the landscape, mimicking the scenario in which cells undergo haptotaxis. We could also vary the power of the motor to emulate the cellular traction force which increases as actomyosin contractility increases. Moreover, we could vary the stiffness of the inter-segmental connection to evaluate how cell body axial stiffness affects migration speed. Our robot was not equipped with any computing unit to process the mechanical cues and make locomotive decisions. Importantly, we ensured that the robophysical model is dynamically similar to cell migration conditions, traction forces from solid substrates is much larger than fluid drag. When subjecting the robot to conditions equivalent to a substrate stiffness gradient, the robot underwent haptotaxis as cells do. Furthermore, the robot recapitulated the oscillatory patterns of traction forces and stress fiber elongation observed in cells. In summary, we demonstrated the feasibility of studying cell migration by robophysical modeling.

B430/P0996

Lacticaseibacillus casei ATCC 393 genome minimisation by directed evolution

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Lacticaseibacillus casei ATCC 393 is a probiotic with potential industrial and therapeutic applications. The number of highly efficient strains for biotechnological production is still low due to strain-specific variations in genetic, metabolic, and functional properties as well as challenges in optimising growth conditions and metabolic pathways for industrial-scale applications. There is a growing interest in strain improvement through genetic engineering, adaptive evolution, and metabolic optimisation to enhance L. casei's capabilities for targeted applications in industrial and therapeutic applications. Genome minimisation can be achieved through progressive removal of non-essential genes using natural evolution or adaptive laboratory evolution (directed evolution), replacement of the original genome with synthetic fragments containing only putative essential genes, or deletion of genes using genome editing tools. In this study, a genome-minimised strain of L. casei ATCC 393 was developed for use as a probiotic chassis by adaptive laboratory evolution with differences in fitness of the minimal genome L. casei ATCC 393 and the wild-type L. casei ATCC 393 determined. The directed evolution by serial passage was initiated by streaking L. casei ATCC 393 from glycerol stock onto MRS followed by incubation at 37°C for 48 hours until individual colonies became visible. After incubation, a single colony was picked and streaked onto a fresh MRS agar plate, followed by a 48-hour incubation at 37°C. A random colony was selected and streaked onto another fresh MRS agar plate every 48 hours from day 1 to day 50 of the experiment, with a single colony selected each day to maintain clonal propagation and prevent lateral gene transfer. At regular intervals, every 10 passages, colonies were selected to prepare glycerol stocks and stored at -80°C for future genetic analysis. The NaCl evolution protocol was modified from the adapted evolution by serial passage and colony purification protocols previously developed. The survival rate data was calculated from mean 0D600 nm readings. Survival rates for directed evolution experiments 1 (E1) and 2 (E2) across

various NaCl concentrations over time were calculated from mean optical density readings at 600 nm (OD600) for each treatment group. The data revealed a significant difference in survival rates between E1 and E2 from day 1 to day 38 (V = 224, p = 0.00745), indicating that factors, possibly inherent to the isolates themselves or subtle variations in the environment, may be influencing the results. Overall, the significant differences indicate that survival rates were affected by specific NaCl concentrations. Lower survival rates were observed at 50 g/L and 71 g/L compared to other concentrations.

B431/P0997
Light-induced assembly in Ca2+ -driven chemomechanical protein networks
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Programming ultrafast, reversible motions in soft materials has remained a challenge in active matter and biomimetic design. Here, we present a light-controlled chemomechanical network based on Tetrahymena thermophila calcium-binding protein 2 (Tcb2), a Ca2+-sensitive contractile protein. These networks, driven by Ca2+-triggered structural rearrangements, exhibit dynamic self-assembly, spatiotemporal growth, and contraction rates up to tenfold faster than ATP-driven actomyosin systems with non-muscle myosin II motors. By coupling light-sensitive chelators for optically triggered Ca2+ release, we achieve precise, reversible growth and contractility of Tcb2 networks, revealing emergent phenomena such as boundary-localized active regions and density gradient-driven reversals in motion. A coupled reaction-diffusion and elastic model explains these dynamics, highlighting the interplay between chemical network assembly and mechanical response. We further demonstrate active transport of particles via network-mediated forces in vitro and implement reinforcement learning to program sub-second, spatiotemporal actuation in silico. These results establish a platform for designing responsive active materials with ultrafast chemomechanical dynamics and tunable optical control, with applications in synthetic cells, sub-cellular force generation, and programmable biomaterials.

Bioengineering and Synthetic Cell Biology - Synthetic Circuits and Systems

B426/P0992
Construction and phenotypic classification of synthetic dual-pole Escherichia coli cells
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Macromolecular complexes anchored at one cell pole have previously been utilized to achieve synthetic asymmetric cell division in Escherichia coli. In nature, however, robust asymmetric cell division often relies on gradients established between two opposing poles. Here, we present a second-generation synthetic genetic circuit that enables two distinct scaffolding proteins to localize at opposite poles in E. coli. By engineering a PodJ fragment as the second scaffold and integrating SpmX, a negative regulator of PodJ condensation, as a direct chimera with PopZ, we effectively confined PopZ and PodJ to opposite poles within E. coli. Additionally, we developed a neural network-based classification method to identify these synthetic dual-pole cells, further advancing the synthetic biology toolkit for programmable cell differentiation.

B429/P0995
Phase separation and fiber formation by Spirostomum centrin and Sfi1 centrin-binding proteins.
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The ciliated protozoan Spirostomum exhibits one of the fastest known cellular contractions, driven by a specialized calcium-responsive contractile system. This mechanism depends on a supramolecular assembly composed of two principal proteins: a large, extended α-helical scaffold distantly related to the yeast Sfi1 spindle pole body protein, and the calcium-binding protein centrin. Understanding the molecular basis of this contractility requires elucidating how these two proteins interact in the presence of calcium ions to generate force, which was the goal of this study. The unusually large size of the Spirostomum Sfi1 homolog posed challenges for direct study, prompting the design of a simplified construct consisting of tandem Sfi1 repeats that assemble head-to-tail into elongated polymers. When Spirostomum centrin and Sfi1 proteins are co-expressed in Escherichia coli, most of these proteins aggregate; however, they can be efficiently solubilized under high-salt conditions, enabling biochemical characterization. In vitro reconstitution experiments revealed that mixtures of centrin and the simplified Sfi1 construct undergo phase separation into fluid-like condensates. Upon adding calcium, these condensates transformed into a variety of gel-like structures. Remarkably, calcium frequently initiates the formation of long, linear fibers resembling the myonemes observed in the cortex of Spirostomum cells. Earlier experiments showed that centrin alone can undergo calcium-enhanced phase separation. When both proteins were present, the resulting phase-separated droplets often aligned to form linear structures upon adding calcium, suggesting that phase separation promotes the assembly of myoneme-like structures. These findings indicate that the essential principles underlying myoneme structure and function can be reconstituted from just two proteins, encoded by their intrinsic self-assembly and calcium responsiveness. This work presents a minimal model for analyzing the molecular mechanisms of ultrafast cellular contraction.

B428/P0994
Recapitulating hierarchical organization of the brain with directoids
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Three-dimensional stem cell-derived neural organoids provide a promising platform to investigate early stages of brain development, circuit formation, and interregional communication. While co-culturing region-specific organoids into assembloids has enabled the study of cortical and subcortical interactions, these models lack directional specificity and spatial controlim, liting their ability to recapitulate canonical circuit formation. To address this, we propose a novel microfluidic platform that enables the construction of directional and tunable interregional circuits while preserving anatomical distinction. This system, which we refer to as “directoids,” incorporates micropatterned polydimethylsiloxane (PDMS) structures to control uni- and bidirectional axonal growth between cortical and thalamic organoids. Our time lapse data demonstrates a fourfold decrease in speed for axons traveling in the inhibitory direction as compared to control and permissive channels. All microstructures confirmed a robust, directionally biased axon outgrowth with 100% successful biasing, which establishes a scalable and reproducible method for modeling structured and asymmetric connectivity in vitro.

This approach offers a powerful tool to investigate the mechanisms underlying hierarchical circuit formation, regional specification, and functional integration in the developing brain.

Bioengineering and Synthetic Cell Biology - Tissue Engineering

B436/P1002
Engineering High Fidelity Tissue Aggregates for Scalable Biomanufacturing via Vertical Wheel Bioreactor System
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Background: 3D culture systems are widely used for both basic and applied research across mammalian, plant, and fungal cell systems to better understand cell behavior and dynamics within a more mimetic tissue microenvironment. However the translation of these bench-scale technologies to deliver consistent and reproducible cell therapies and cell-expressed bioproducts alike, at scale remains a challenge for their future commercial success. Specifically for plant cell suspension cultures, traditional vessels and bioreactors generate heterogeneous tissue aggregates, resulting in variable growth (sub-populations observed within a single cell line) and different batch-to-batch metabolite yields. Objective: Our hypothesis was biomass growth and metabolite yield can be controlled through optimization of tissue aggregate size. To do this, we investigated whether a vertical-wheel bioreactor system (PBS Biotech) could provide a tunable and more uniform hydrodynamic environment compared to conventional Erlenmeyer flasks, thereby improving culture consistency and product output. Methods: Plant cell suspension cultures derived from a Solanum sp. cell line were grown in both a mini-vertical wheel bioreactors and traditional shake flasks and monitored at multiple timepoints up to 24 days in vitro. Aggregate morphology and size distribution were analyzed using image-based metrics; biomass accumulation was quantified via sedimentation and dry weight evaluation; and secondary metabolite production was evaluated using total phenolics assays. Results: The vertical-wheel bioreactor supports a narrower aggregate size distribution, higher reproducibility of biomass expansion, and enhanced bioactive yields relative to flask-grown controls. Conclusion: These findings highlight the potential of low-shear vertical-wheel bioreactor systems as an enabling technology for scalable, reproducible plant cell fermentation, advancing plant cell agriculture from bench to industrial consumer and pharma applications. Although this is the first study to evaluate plant cell cultures in a vertical-wheel bioreactor, we add to an emerging body of literature that this culture system can provide a finer level of control and more favorable economics for scaling other cell technologies beyond biomanufacturing, including hIPSC's and hMSC's for cell therapies.

B433/P0999
Engineering optogenetic control of anisotropic strain for sculpting epithelial morphogenesis
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Organoid technologies hold tremendous promise for modeling complex developmental processes and disease, yet controlling large-scale tissue architecture remains a formidable challenge. Engineering control over anisotropic strain in cell collectives could enable us to sculpt complex epithelial shapes such as tubule trees and tissue interfaces in next-generation organoids, helping to overcome the self-organization barrier that often limits long-range structure formation. We believe these strategies can be informed by developmental mechanisms in vertebrate embryos, where dramatic shape changes involve the lengthening and narrowing of

cell fields during convergent extension, a process essential for epithelial morphogenesis and regulated by Planar Cell Polarity (PCP). Despite progress in understanding native PCP signaling pathways, engineering approaches are lacking. Here we develop optogenetic strategies to synthetically reconstitute the outcomes of PCP-driven epithelial remodeling, focusing on technologies with direct implications for improving organoid morphogenesis. Our approach combines two key innovations: (1) We have hijacked polarized cellular components (from native PCP proteins to microtubule-associated structures) to establish cell geometry-dependent molecular asymmetries and are in the process of (2) using light-controlled RhoA activation to target actomyosin contractility to sites of asymmetry and induce T1 transitions. By recruiting optogenetic actuators to cell junctions orthogonal to predefined polarity axes, we aim to generate coordinated tissue rearrangements mirroring developmental convergent extension. This synthetic biology framework advances organoid engineering by enabling precise spatiotemporal control over collective cell behaviors. While we continue to explore strategies to reconstitute these behaviors, advances in this area have the potential to significantly enhance our ability to model developmental defects in organoid systems and hold promise for applications in regenerative medicine.

B435/P1001

Optimization of fdm parameters for flexible 3D-printed scaffolds for mesenchymal stem cell culture for regenerative purposes

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Fused deposition modeling (FDM) is a widely adopted additive manufacturing technique for fabricating polymeric scaffolds, with polylactic acid (PLA) being one of the most commonly used raw materials due to its printability and biocompatibility. However, conventional grid-like or hexagonal designs often produce rigid scaffolds with limited adaptability to cell culture conditions, leading to suboptimal adhesion of mesenchymal stem cells (MSCs) and thereby restricting their applications in regenerative medicine. The aim of this study was to optimize FDM parameters to fabricate flexible PLA scaffolds using exclusively open-source slicing software and to evaluate their capacity to support osteogenic differentiation of mesenchymal stem cells (MSCs) in comparison with conventional PLA grid scaffolds with the aim of enhancing and expanding their potential use as osteogenic precursor grafts. MSCs were induced to differentiate into osteoblasts on both scaffold types, and the expression of osteogenic markers BMP2 and RUNX2 was quantified by RT-qPCR. Mineral deposition was evaluated through Alizarin Red staining, while cell viability and proliferation were determined using MTT assays. Preliminary results indicate that optimized PLA scaffolds supported improved MSC adhesion and proliferation compared to conventional grid scaffolds, while both scaffold types-maintained expression of key osteogenic markers and evidence of mineralization. These findings suggest that FDM parameter optimization enhances the biological performance of PLA scaffolds without requiring complex CAD modeling. In conclusion, this study demonstrates that flexible PLA scaffolds fabricated through open-source FDM parameter optimization represent an innovative and cost-effective strategy for bone tissue engineering applications for therapeutical purposes, combining structural adaptability with osteogenic potential.

B434/P1000

Programmable progenitor cell patterning enables tunable kidney organoid architecture

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The kidney is essential for maintaining the homeostatic balance of fluids and electrolytes in the body through blood filtration and metabolic waste removal. However, its limited regenerative potential and the global burden of renal disease highlight a critical need for in vitro tissue models. Kidney organoids derived from induced pluripotent stem cells (iPSCs) hold promise for disease modeling and future renal replacement tissue, yet, their dependence on the intrinsic capacity of stem cells to self-organize leads to variability and limited functional maturity, which restrict their opportunities for translation. To achieve greater control over the microenvironment and, therefore, organoid morphogenesis, we pattern iPSCs using photolithographic DNA-programmed assembly of cells (pDPAC), a high resolution cell patterning technology that programs cell adhesion through base pairing between cell and substrate bound complementary single stranded DNA. This approach enables independent spatial arrangement of multiple progenitor cell types with precise control over seeding composition, ratio, and geometry. We leverage this programmable assembly to mimic the nephrogenic niche in vitro by positioning ureteric bud (UB) and nephron progenitor (NP) cells in defined configurations that mimic tissue arrangements observed during kidney development in vivo. We show that the proximal nephron segment in NP-only organoids scales with initial spheroid size, demonstrating a predictable relationship between initial seeding conditions and final organoid architecture. In mosaic organoids, initial NP:UB ratio tunes organoid composition including nephron segment identity and the proportion of ureteric epithelial cell types. In ongoing work, we are further quantifying the characteristic length scale of self-organization and how initial patterning geometry shapes organoid morphogenesis, including effects on functional maturity like tubule elongation, network connectivity, and distal nephron-ureteric bud fusion. By controlling the initial seeding conditions, we establish an engineering handle to direct organoid morphogenesis. This work provides the foundation for building more mature, functional, and reproducible kidney models to advance disease modeling, drug testing, and translational applications.

B437/P1003

Regulating Transepithelial Transport with Electric Fields

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The kidneys are fluid pumping machines that transport ~180 L of water every day. This is facilitated by kidney epithelial cells that surround fluid-filled lumens in individual tubules. These cells transport water by sensing and regulating transepithelial osmotic gradients through specialized ion channels/pumps that localize at the apical or basal surface of a single cell. Water flux also plays a key role in lumen-formation during morphogenesis by inducing tissue deformation through pressure generation. Previously, we showed that bioelectric stimulation of kidney cysts and intestinal organoids triggered rapid, sustained water influx into the lumen causing massive inflation. This ability to regulate the transport function of kidney epithelia with electric fields presents the opportunity to use this system to study morphogenetic forces, develop medical devices, and unique organ-on-chip systems. This works aims to develop an input-output function between electric field strength and fluid transport to then employ the kidney epithelium as a material in bio-hybrid systems. For this, we engineered MDCK-II renal epithelial monolayers on small blocks of hydrogel. Without electrical stimulation, the cells pump fluid apical-to-basal causing the hydrogel to swell. But upon applying electric fields parallel to the apical surface of the monolayer, we instantly reprogram the cells to pump fluid basal-to-apical causing the hydrogel to rapidly de-swell (within 60 minutes). The rate and extent of hydrogel deformation depends on field strength and the hydrogel re-swells only when the tight junctions of the epithelium are perturbed. Moreover, de-swelling only occurs in regions of the hydrogel that are covered by cells, enabling us to spatially pattern hydrogel deformation in 3D with a uniform electric field input. We further aim to identify

the key ion channels that regulate this process, and investigate how various electric fields such as alternating currents, basal stimulation, and perpendicular electric fields affect pumping behavior. This study demonstrates that the kidney epithelium is an active, programmable material that can actuate hydrogels through fluid transport.

Biophysics, Structural Biology, and Mechanobiology - Biophysical Properties of Tissues

B342/P0331
Adherent hiPSC lumenoids: A robust 3D platform for bridging cellular dynamics and multicellular organization
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Over the past decade, the Allen Institute for Cell Science has developed and distributed tools to study human induced pluripotent stem cells (hiPSCs), including fluorescently tagged lines, images of 2D hiPSC colonies, and image analysis workflows. However, these 2D cultures cannot replicate the complex multicellular interactions seen in 3D environments. To address this, we have optimized the adherent hiPSC lumenoid as a simple and tractable system for investigating cellular dynamics in a physiologically relevant 3D context. Lumenoids are hollow, acinar structures with a single central lumen surrounded by a coherent, edgeless, apical-in/basal-out, monolayered, pluripotent epithelium. Lumenoids form when dilute extracellular matrix (Matrigel) is added to 2D hiPSC colonies. Using live imaging, we have observed that this triggers a cascade of events, including apical contraction, colony edge lifting, and epithelial closure via a purse-string-like mechanism, creating a topologically closed lumen within ~12-24 hours. Lumenoid formation is accompanied by de novo basal secretion of basement membrane (BM) and fully formed lumenoids are encased in a BM shell. Once formed, lumenoids grow into large, often spherical 3D structures as the lumen inflates. Inflation appears to be driven by luminal pressure, and preliminary evidence suggests a robust scaling relationship between lumen diameter and overall lumenoid size. The simple geometry of lumenoids, their genetic tractability, the fact that they are a single cell type, and their reproducibility offer significant advantages over other 3D culture models. Importantly, lumenoids remain stably anchored to the coverslip, allowing for long-term live imaging in high throughput. They are also retained through media changes, fixation, and immunolabeling, enabling multimodal data acquisition. Moving forward, this assay will enable integration of cell dynamics, molecular features, and local cellular context in a reproducible, experimentally tractable, physiologically relevant 3D system.

B341/P0330
Cholesterol-containing lipid crystals directly stiffen the steatotic liver
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Lipid accumulation changes tissue mechanics and may contribute to fibrogenesis in steatotic liver diseases such as metabolic dysfunction associated steatotic liver disease (MASLD). We have shown that amorphous lipid

droplets (LDs) soften liver tissue; however, increases in cholesterol and cholesterol esters (CE) are known to cause ordering of lipid aggregates, and their mechanical impact on tissues is not known. We hypothesized that increased cholesterol storage induces formation of intrahepatic lipid crystals, which exert a direct stiffening effect on tissue. We fed rats either a high fat (HF) or high fat high cholesterol (HFHC) diet and measured liver stiffness by rheometry and magnetic resonance elastography. We found that HFHC (but not HF) livers stiffened compared to control, and this stiffening preceded the development of fibrosis. Lipidomics showed marked increases in cholesterol and CEs in the HFHC liver, with highly reflective, birefringent lipid crystals observed by polarized light and confocal reflection microscopy. Cryo-scanning electron microscopy showed both solid and liquid crystals in HFHC livers while HF livers contained only amorphous LDs. Microindentation with visualization showed that increased crystal content was associated with local tissue stiffening, while amorphous LDs were associated with softening. Lipid extracts with crystals from HFHC livers stiffened tissue-mimicking fibrous matrices, while HF lipid extracts did not. Cholesterol depletion from HFHC livers by perfusion with methyl-β-cyclodextrin (MβCD) reduced both crystal content and tissue stiffness despite having no effect on inflammation. MβCD perfusion did not change the stiffness of normal livers. These findings show that cholesterol storage leads to the ordering of lipids into crystals, and that these crystals exert a direct tissue stiffening effect independent of inflammation and fibrosis. Taken together, our results suggest a novel metabolic-mechanics pathway in which cholesterol-containing lipid crystals can directly stiffen the liver and drive disease progression in MASLD. Understanding this pathway may lead to new diagnostics and treatments for liver diseases, and may also be relevant to other tissues.

B345/P0334

Classifying biophysical subpopulations of insulin secretory granules using quantitative whole cell structure analysis

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Pancreatic β-cells regulate blood glucose homeostasis by secreting insulin, which is processed and stored within secretory organelles called insulin secretory granules (ISGs). As insulin matures from proinsulin, ISGs undergo extensive biochemical and structural remodeling, leading to a heterogenous mixture of subpopulations that vary in protein and lipid content, maturity, and release competence. Defects in ISG maturation and alterations in subpopulation composition are implicated in both type 1 and type 2 diabetes, yet systematic methods for defining ISG subpopulations remain limited. To provide a new perspective on ISG heterogeneity, we employ soft x-ray tomography (SXT), which can quantitatively measure the biochemical density of ISGs within whole β-cells. Within these tomograms, we apply unsupervised clustering to classify subpopulations based on molecular density, size, and spatial positioning. This approach reveals distinct ISG subpopulations corresponding to different maturation states and secretion readiness. Across several different insulin secretory stimuli conditions, we observe shifts towards more condensed and mature subtypes, demonstrating that exogenous signals can dynamically remodel ISG subpopulation distributions. To broaden this analytical framework, we extend this methodology to three-dimensional datasets collected using volume electron microscopy (vEM). Integrating vEM subpopulations with SXT subpopulations from primary β-cells uncovers insights inaccessible by a single method in isolation. This holistic strategy allows us to define ISG subpopulations from whole-cell structures, providing a basis for therapeutic approaches that can enrich physiologically beneficial ISG subpopulations.

B338/P0327
Cytokine control of immune tissue fluidity
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Unlike most solid-like tissues, immune tissue is protean and liquid-like—enabling it to patrol vast territories, find rare cellular partners, and dynamically self-organize into functional structures like germinal centers or tertiary lymphoid structures. While this fluidity is essential for adaptive immunity, the principles governing its control, and whether they can be chemically programmed, remain unknown. Here, inspired by minimal components that enable germinal center (GC) dynamics, we developed an agent-based model that predicted that efficiency of antibody evolution is governed by a tissue's 'effective temperature'. This motivated our search for biological rheostats capable of tuning this material property. Using a tractable in vitro model, we found that CD40L-stimulated human B cells self-organize into active, liquid-like condensates that round, fuse, and internally mix. GC cytokines IL-4 and IL-21 tuned this effective temperature, driving phase transitions from cohesive, liquid-like states to dispersed, gas-like morphologies. These findings demonstrate cytokine control of tissue fluidity, suggesting a previously unrecognized axis of biophysical immune regulation.

B343/P0332
Melanin composites demonstrate resistance to point impact energy
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Melanin is a black pigment found in most living creatures, and is best known for it's absorbance of ultraviolet light. Additionally, it has substantial and well documented extraordinary physical properties, which are currently being actively investigated for practical biotechnology applications by many researchers. We were intrigued by the fact that melanin is often found combined with other substances in living organisms, and that some available data suggest that these natural composites have greater physical properties than melanin alone. We hypothesized that combining synthetic polymers with melanin would have a similar effect in enhancing its physical properties. We here report that composites of cuttlefish melanin with the polymer polyvinyl alcohol demonstrate a surprising degree of physical resistance to point impact energy. We developed custom composites of several percentages of polyvinyl alcohol and melanin, and subjected them to testing according to a rigorous U.S. national standard. We found that for several formulations, the composite was not penetrated at all, even though the national standard permits several mm of penetration for each defined energy penetration test, including the most rigorous of 65 Joules. There are many potential applications for this sort of new biotechnology material.

B337/P0326
Physical Confinement Alters Cellular Dynamics from the Multi-Cellular to the Single-Cell Scale
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Physical confinement refers to the mechanical forces that constrain dynamic cellular processes. We propose that physical confinement is defined by the compressive stresses exerted on cells during processes like migration and growth. Compressive stresses act on multiple length-scales--from single immune cells migrating through epithelial junctions to multi-cellular tissues forming complex ductal networks. Compressive stresses change as tissues remodel, transmitting mechanical information that aids in organ size specification, apicobasal polarization, and epithelial barrier function. We focus on two distinct in vitro models where we study confinement 1) of multi-cellular pancreatic ductal organoids grown in 3D, engineered hydrogels and 2) of single macrophages migrating through ductal cell junctions. During pancreatic ductal adenocarcinoma disease progression, cancer cells recruit fibroblasts that promote tissue fibrosis, increasing local tissue stiffness and subjecting healthy epithelial cells and immune cells to increased compressive forces. Recently, we identified the differential effects of confinement on healthy and tumor-derived ductal organoids. By challenging organoid growth with a confining, engineered 3D hydrogel, we discovered two emergent collective cell behaviors in murine pancreatic ductal organoid models. Tumor-derived organoids exhibited a collective "pulsing" behavior that increased in frequency when confined and generated radially persistent forces that locally rearranged the microenvironment in the direction of organoid growth. In contrast, healthy organoids exhibit unidirectional rotation, generating more subtle rotational movements in the microenvironment orthogonal to the direction of growth. When confined, we found tumor-derived organoids grew at similar rates as in less confined conditions while normal organoid grew much more slowly and failed to establish proper apicobasal polarity. Our current work seeks to deepen our understanding of immune cell migration within densely packed tissues by live imaging pancreatic ductal organoid and macrophage co-cultures. Though these efforts, we focus specifically on how epithelial adhesion strength and membrane mechanics predict macrophage infiltration sites. Using live and fixed imaging, we are mapping how cellular rearrangements and division generate mechanical gradients on the surface of epithelial cells that guide macrophage infiltration. More broadly, we hope to provide a new framework for enhancing infiltration efficacy of cellular immunotherapies for solid tumors by demonstrating the non-negligible role of compressive stresses imparted by the microenvironment and epithelial membranes.

B344/P0333

Sound absorption in melanin composites

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Melanin is a heterogeneous polymer which exists in both natural and synthetic forms. Most living organisms contain melanin to some degree, and much of the published research deals with its ability to shield from ultraviolet light. We were intrigued by a published report that purely synthetic melanin has a large capacity to absorb sound and found little other mention in the literature of the interaction of melanin with sound. Melanin is known to be present in the ear, and its function is unknown. We decided to test whether natural melanin is capable of absorbing sound. Cuttlefish melanin was dehydrated, grinded, and mixed with polyvinyl alcohol as a binder, and compressed in a hydraulic press into approximately 100 mm x 100 mm blocks, each about 10 mm thick. Six such blocks were assembled into a cube, using a cardboard box as support and a small MP3 player and speaker were inserted in the box. The emitted sounds were measured in decibels. Controls were also tested. It was found that the composite of natural melanin with polyvinyl alcohol showed substantial absorption of sound. The fact that melanin is an abundant, natural, light, biodegradable material makes it suitable for both further experimentation in this area, and possible commercial applications.

B339/P0328
Spatially herding cell migration for optimal wound healing via electrotaxis
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Collective cell migration is critical in many physiologically important processes including cancer invasion, development, and wound healing. Therefore, it is of great interest to develop methods for controlling such collective motions. One promising control method is to use exogenously applied electric fields to leverage electrotaxis, the biophysical mechanism in which cells bias their migration in response to DC electric fields resulting from endogenous ion currents (which occurs during epithelial injury and morphogenesis). In vivo measurements of electric fields show clear spatial patterns of field strength that change over time, yet nearly all electrotaxis studies have focused on instant-ON, global fields that control the motion of an entire tissue, often with consequences for tissue integrity. Here, we explore the more specific and challenging question of whether using localized electric fields to control only part of a tissue can improve electrotaxis and healing performance in engineered tissue models. We present a new electro-bioreactor platform combining custom integrated electronics and 3D printed, electric field shaping inserts to spatially and temporally control patterns of electrical stimulation within engineered skin monolayers made from differentiated primary mouse keratinocytes. We first demonstrate and characterize how local application of electric fields can drive much broader regions of tissue migration response due to collective mechanical coupling. Learning from these dynamics, we then harness this extended range to design unique field patterns that substantially accelerate wound healing of a 2D circular wound (~3.5-fold faster). Specifically, we combine annular, local field patterns with pulsed ON/OFF dynamics to improve cell migration and harmonize with the underlying collective behaviors. Finally, we then used a minimal, biophysics-informed, optimal control model to design a simple double pulse sequence which further accelerates healing. Together, these observations prove that there are more optimal control methods for influencing large crowds and that these methods can be used for important applications like wound healing.

Biophysics, Structural Biology, and Mechanobiology - Cell Size and Scaling

B365/P0354
Cell crowding drives mass density increase through changes in osmotic regulation in epithelial tissue
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Cell size regulation is crucial for proper tissue development and maintenance in all organisms. We have previously shown that MDCK II cell lines undergo a series of volume-reducing divisions (VRD) as cell crowding increases, followed by G1/0 arrest at a minimal volume of ~1,000 μm3. Increasingly, it has become clear that regulation of cell mass density is an important factor contributing to cell size regulation; however, this is currently underexplored. Changes in cell mass density have been previously associated with changing cell size and cell senescence, yet the mechanism by which cells set their mass density is not understood. Additionally, much of the work concerning cell size regulation has been done in single-cell systems, while regulation of cell size in multicellular, tissue contexts is crucial for understanding tissue-scale size organization. Here we propose that cell crowding induces changes in cellular osmotic regulation affecting both mass density and cell volume. We employed model epithelial cell lines, confocal fluorescence and quantitative phase microscopy (QPM) to

measure volume and mass as cell crowding increases leading up to the onset of contact inhibition and cell cycle arrest. Under these conditions, we show that cell density increases by ~1.5-fold while volume decreases by ~2.5-fold. Furthermore, cell crowding induces significant transcriptional changes in osmoregulatory gene transcripts including ATP1A1 (Na+/K+ ATPase), SCNN1A (epithelial sodium channel), and SLC12A2 (Na-K-Cl cotransporter). Based on these results, we propose that cell crowding induces changes in cellular osmotic regulation, and this mechanism may be crucial to both cell size regulation and cellular mass density control in epithelial tissues.

B364/P0353

Determining mechanisms underlying nuclear size control in fission yeast

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The objective of this study was to test a physical model of nuclear size control. Precise regulation of nuclear size relative to cell size is a robust feature of cellular biology, yet the mechanisms governing nuclear size control remain poorly understood. We and others recently proposed a physical model in which the nuclear-to-cell volume ratio (N/C ratio) at steady state is set by a balance between colloid osmotic pressures generated by the numbers of osmotically active macromolecules within the nucleus and cytoplasm, and by nuclear envelope tension. In fission yeast, where envelope tension is minimal, the nucleus can be regarded as an osmometer whose size is primarily determined by intracellular osmotic forces. As a key test of this model, we increased osmotic pressure in a compartment-specific manner by massively expressing exogenous proteins targeted to either the nucleus or cytoplasm. Loading the nucleus with millions of copies of mCherry-GST-NLS proteins caused a striking, dose-dependent expansion of nuclear volume, elevating the N/C ratio up to four-fold. Conversely, expression of mCherry-GST-NES proteins in the cytoplasm reduced the N/C ratio in a dose-dependent fashion. As one control, equivalent high expression of mCherry in both compartments did not affect cell growth or nuclear size. These experimental data, which closely fit model predictions, provide significant quantitative support for this osmotic model for nuclear size and yield fundamental parameters for osmotic theory in living cells. Additionally, these perturbations altered the physical properties of the nucleoplasm. Expression of mCherry-GST-NLS in the nucleus significantly increased diffusion of 40nm-GEMs nanoparticles, consistent with nuclearexpansion and dilution of endogenous nuclear components. Conversely, mCherry-GST-NES expression in the cytoplasm reduced nuclear diffusivity. To test whether changes in macromolecular crowding affect nuclear condensate assembly, we observed consistent effects on Swi6/ HP1 heterochromatin loci and on the nucleolus. Together, this work establishes a tunable experimental strategy to manipulate the N/C ratio and physical properties of the nucleoplasm, providing a powerful tool to study how osmotic forces influence nuclear size control, molecular crowding, and nuclear organization.

B362/P0351

Dimensional Scaling of Mitotic Spindle Assembly

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During eukaryotic cell division, the replicated genome is equally partitioned between daughter cells by the mitotic spindle, a dynamic structure mainly composed of microtubules. Mitotic spindle size scales with cell dimensions during early embryonic cleavages. Several mechanisms including regulation of microtubule length or number have been proposed to explain size adaptation of the mitotic structure, however, it remains unclear how the mitotic spindle can robustly assemble over a large range of cell sizes even within the same organism while satisfying temporal constraints imposed by cell cycle events. Using live imaging of spindle assembly in embryos of different species and of various sizes we identified conserved mechanisms involved in scaling of mitotic spindle size and assembly. We developed a methodology based on labeled-protein injection to visualize and measure microtubule dynamics in different non-model organisms in which genetic tools are limited. We found that the microtubule growth rate scales with cell size across metazoans. In addition, by observing epithelial cell divisions in C. elegans, we provide the first characterization of spindle scaling mechanisms in somatic tissues in intact animals. Our data provide evidence that microtubule growth rate scales with cell volume in somatic cells. Interestingly, our work demonstrates that mitotic spindle size scales with cell size in somatic cells but this size adaptation is driven by a reduction of the width instead of spindle length. This altered mechanism in small somatic cells appears to tend towards a lower limit of spindle length scaling. Together our data provide an exhaustive comprehension of spindle shape, size and assembly across a wide range of size and shed light on the distinct mechanisms that operate to ensure faithful cell division in different tissue or developmental contexts.

B363/P0352
Protein overabundance is driven by growth robustness
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Protein expression levels optimize cell fitness: Too low an expression level of essential proteins will slow growth by compromising essential processes; whereas overexpression slows growth by increasing the metabolic load. This trade-off naively predicts that cells maximize their fitness by sufficiency, expressing just enough of each essential protein for function. We test this prediction in the naturally-competent bacterium Acinetobacter baylyi by characterizing the proliferation dynamics of essential-gene knockouts at a single-cell scale (by imaging) as well as at a genome-wide scale (by TFNseq). In these experiments, cells proliferate for multiple generations as target protein levels are depleted by dilution from wild-type expression levels. This approach facilitates a proteome-scale analysis of protein overabundance. As predicted by the Robustness-Load Trade-Off (RLTO) model, we find that roughly 70% of essential proteins are overabundant and that overabundance increases as the expression level decreases, the signature prediction of the model. These results reveal that robustness plays a fundamental role in determining the expression levels of essential genes and that overabundance is a key mechanism for ensuring robust growth.

Biophysics, Structural Biology, and Mechanobiology - Mechanical Properties of Cells

B327/P0318
Cancer Detection Through Chromatin Biomarkers in a Microfilter-Assisted Plasma Separation Platform
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Detection of cancer remains a major clinical challenge, as current approaches are hindered by tumor heterogeneity, low biomarker abundance, or lack of specificity. Here, we introduce a chromatin-based sensing strategy that leverages the intrinsic responsiveness of cells to tumor-associated signals in human plasma combined with a microfluidic detection platform. We hypothesize that rapid chromatin remodeling in these cells could serve as a functional fingerprint for disease detection. To test this, we treated fibroblast cells, in culture, with human blood plasma from healthy donors or cancer patients. Cells were stained for DNA, and chromatin configuration and intensity-related features were extracted from fluorescence images. These features were then analyzed using a Random Forest Classifier (RFC) to distinguish cancerous versus healthy conditions. Fibroblasts exhibited robust chromatin responses with 74.75% classification accuracy. Finally, to enhance translational feasibility, we developed a microfilter-assisted polydimethylsiloxane (PDMS)-based microfluidic platform that isolates plasma directly from whole blood without extensive processing or specialized equipment, and enables on-chip fibroblast treatment. In conclusion, our findings demonstrate that fibroblast chromatin remodeling offers a sensitive and robust biological readout of cancer-associated signals in blood plasma. Coupled with a scalable microfluidic device, this platform holds promise as a rapid, cost-effective, and biologically integrated approach for cancer detection and could be extended to drug discovery and therapeutic screening in the future.

B326/P0317

Changes in nuclear and actin mechanics from G1 to G2 affect nuclear integrity

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The structural integrity of the nucleus is dependent on nuclear mechanical elements of chromatin and lamins to resist antagonistic actin cytoskeleton forces. Imbalance results in nuclear blebbing, rupture, and cellular dysfunction found in many human diseases. We used Fluorescent Ubiquitin Cell Cycle Indicator (FUCCI) cells to determine how cell cycle changes affect the nucleus and actin force balance. While nuclear blebs are present equally throughout interphase, nuclear blebs form predominantly in G1 and then persist into G2 due to increased actin-based nuclear confinement and focal adhesion density in G1 vs. G2 cells. Use of focal adhesion kinase inhibitor decreased focal attachments, nuclear confinement, and nuclear blebbing which supports this mechanism. Alternatively, G1 nuclei could be mechanically weaker leading to predominant formation of nuclear blebs. Interestingly, upon artificial confinement, G2 nuclei ruptured more than G1 nuclei. Single nucleus micromanipulation force measurements confirmed that G1 nuclei are stronger than G2 nuclei in both the chromatin-based and lamin-based nuclear stiffness regimes. Decreased nuclear stiffness can be explained by loss of peripheral H3K9me3 from G1 to G2, recapitulated by H3K9me3 inhibition via chaetocin. Thus, actin-based nuclear confinement can overcome a stronger nucleus, causing nuclear blebbing and ruptures. Conversely, decreased actin-based confinement in a weaker nucleus does not rescue nuclear blebbing but does decrease the rate of formation. Cell cycle-based changes in nuclear and actin mechanics play a major role in nuclear integrity and shape.

B330/P0321

Changes in nuclear and actin mechanics from G1 to G2 affect nuclear integrity

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B328/P0319

Genome-scale cell surface crowding measurements reveal mechanisms that mediate membrane accessibility

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Living cells process external stimuli and interact with their environments using a wide array of molecules presented on their surfaces. The layer of glycosylated proteins and lipids on the surface, known collectively as the glycocalyx, can sterically block receptor-ligand interactions depending on the crowding level, which can vary with cell type and state. However, it is unclear how cells regulate their surface crowding. To address this, we developed a high-throughput method to quantify the effect of crowding perturbations and combined it with a whole-genome CRISPR interference screen in Jurkat cells to identify protein-coding genes that drive cell surface crowding changes. The screen revealed three key contributors to surface crowding. First, we show that knocking down large and heavily glycosylated cell surface proteins reduces surface crowding. Second, we find that positive regulators of overall gene expression are major determinants of crowding. Consistent with this, we show that pharmacological disruption of transcription or translation reduces surface crowding. Finally and unexpectedly, we found that the actin cytoskeleton can modulate crowding through modification of membrane-cortex attachments that alter effective membrane surface area. These results suggest that cells can regulate their cell surface crowding not only over long timescales by controlling gene expression but also over short timescales by altering membrane-cortex attachment, providing a versatile and dynamic mechanism for controlling membrane accessibility.

B335/P1803
In Vitro Generated Neutrophils Reveal High Deformability is an Emergent Property
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Neutrophils must deform their nuclei up to < 10% their cross-sectional area to migrate in vivo, yet when this ability emerges remains unresolved. Cancer-derived models fail to reproduce the multilobulated nuclear morphology and envelope composition of primary neutrophils. Here, we developed a platform to differentiate human neutrophils from CD34+ stem cells and investigate when high nuclear deformability emerges in neutrophils. We cultured CD34+ stem cells with growth factors and cytokines to differentiate them into functional neutrophils (dCD34-neutrophils) in 14 days with 76.5 ± 6.3-fold expansion. We showed that dCD34-neutrophils expressed canonical human neutrophil markers (99% CD15+, 78% CD11b+), generated reactive oxygen species, and underwent NETosis comparable to primary human neutrophils (41.3%). Using quantitative microscopy, we showed that nuclear circularity, an indicator of multilobulation, decreased from 0.84 to 0.77 as cells transition from early progenitors (D3) to dCD34-neutrophils (D16). Coincidently, nuclear envelope composition changed: Lamin A/C and B1 decreased by 1.6- and 2.7-fold, respectively; Lamin B Receptor (LBR) increased by 7.3-fold, inversely correlating with nuclear circularity in dCD34-neutrophils. To determine if nuclear remodeling correlates with deformability, we measured the migration speed of progenitor and dCD34-neutrophils using microfabricated channels with and without constrictions. We found that migration speed increases from 7.7 ± 3.1 μm/min in early progenitor to 14.1 ± 4.9 μm/min in late progenitor cells. In 2 μm constrictions, progenitors slowed markedly (9.9 ± 3.9 before vs 5.5 ± 2.6 inside), whereas dCD34-neutrophils maintained high speed (11.4 ± 6.2 before vs 14.4 ± 7.6 inside). After constriction, progenitors remained slow (5.9 ± 2.6), while dCD34-neutrophils kept high speed (14.3 ± 6.2). Together, our data show that high nuclear deformability emerges late in human neutrophil differentiation and correlates with LBR upregulation and Lamin A/C and B1 reduction. dCD34-neutrophils provides an accessible platform to dissect how nuclear envelope composition regulates the biophysical mechanisms of human neutrophil functions in health and disease.

B329/P0320
Nuclear stiffness is differentially regulated by lamin isoforms
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The mechanical properties of the nucleus are critical for maintaining nuclear integrity and function, and disruptions in these mechanics are linked to dysfunction. We previously showed that chromatin dominates short-extension mechanics, while lamins provide long-extension strain stiffening. To distinguish the roles of lamin isoforms, micromanipulation nucleus force measurements were performed on isolated nuclei from mouse embryonic fibroblast lines depleted of lamin B (LMNB1-/-), lamin A (LA KD), and lamin A and C (LMNA-/-). Loss of lamin B nuclei exhibit reduced short-extension stiffness while maintaining long-extension mechanics, due to loss of facultative heterochromatin. Loss of lamin A and C did not alter short-extension nuclear stiffness. Nuclei depleted of lamin A display weaker stiffness in long extensions, whereas loss of both lamin A and C nuclei show a dramatic loss of strain stiffening in the long regime. These results support a model in which lamin B has no direct nuclear stiffness role and that lamin A and C are equally responsible for long-regime nuclear strain stiffening. This work illuminates the distinct mechanical roles for lamin isoforms, and establishes a foundation for understanding how nuclear structure is determined in health and disease.

B331/P0322

Temporal Regulation of Epithelial Nuclear and Cellular Mechanics by Lamin A/C

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The cell nucleus plays a central role in regulating essential cellular processes such as polarization, migration, and mechanotransduction. Lamin intermediate filaments are key structural components of the nuclear envelope. They not only maintain nuclear integrity but also engage with Linker of Nucleoskeleton and Cytoskeleton (LINC) complexes to mediate the transmission of mechanical signals from cell cytoskeleton to the genome. Through these interactions, lamins modulate cytoskeletal organization and regulate gene expression in epithelial cells. However, the precise mechanisms and timescales through which lamins influence these processes remain poorly understood. To investigate these roles, we used an engineered colorectal cancer DLD-1 cell line expressing Auxin-Inducible Degron (AID)–tagged lamins, which enables precise temporal control of lamin A/C degradation and restoration (Y. Alabi et al., 2025). This system allowed us to monitor changes in cellular and nuclear mechanics over defined short-term (STS; 16 hours) and long-term (LTS; 96 hours) depletion periods, as well as after lamin rescue (24 hours after auxin washout). We applied our previously established atomic force microscopy approaches (A. Vahabikashi et al., 2022) to quantify the Young's Modulus (E) of the nucleus and cytoplasm in wild-type (WT), STS, LTS, and lamin A/C–rescued cells. We found that the short-term loss of lamin A/C in STS cells (0.28±0.01 kPa) significantly increased average nuclear stiffness (Mean±SE) when compared to the WT (0.16±0.01 kPa), rescued STS (0.19±0.01 kPa), LTS (0.18±0.01 kPa), and rescued LTS (0.14±0.01 kPa) cells ($P<0.0001$), whereas other groups were not statistically different from each other. Additionally, we measured a significantly stiffer cytoplasm in LTS (0.20+0.01 kPa) and rescued STS (0.18+0.01 kPa) cells relative to the WT (0.13+0.01 kPa), STS (0.13+0.01 kPa) and rescued LTS (0.13+0.01 kPa) groups ($P<0.001$). Our findings reveal that changes in lamin A/C expression have distinct time-dependent effects on nuclear and cytoplasmic mechanics. The transient stiffening of the nucleus observed in the STS group, followed by a return to the baseline stiffness in the LTS group, mirrors recently reported observations that link transient chromatin decompaction to reversible changes in nuclear mechanics over time (A. Mitra et al., 2025). We also hypothesize that the sustained increase in cytoplasmic stiffness in the LTS group arises from a compensatory adaptation within the nucleus–cytoskeleton network that aims to restore mechanical homeostasis under prolonged lamin deficiency, which is the focus of or current studies.

B334/P0325

Transcriptional activity generates chromatin motion that drives nuclear blebbing

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Abnormal nuclear blebbing is a hallmark of human diseases, including cancers and age-related disorders. Previous work has outlined that nuclear blebbing is due to an imbalance of nuclear strength resisting actin confinement and contraction. However, our recent work revealed that inhibiting RNA polymerase II suppresses nuclear blebbing independent of altering nuclear spring constant and actin confinement and contraction, but the mechanism remains unknown. Through removing cell culture media serum and then adding it back, we can decrease and then restore transcriptional activity. Decreasing transcriptional activity decreases nuclear bleb formation, stability, and rupture while returning transcriptional activity increases nuclear blebbing. These

modulations of transcriptional activity did not alter nuclear or actin mechanics. Instead, we find that transcription activity regulates chromatin motion measured by tracking the mean square displacement (MSD) of chromatin domains labeled via transfected Cy3-dNTPs. To determine if increasing chromatin motion is a mechanism to increase nuclear blebbing, we used an established RAD51 inhibitor BO2 to increase chromatin motion. We verified BO2 increases chromatin domain motion which resulted in increased nuclear blebbing. We reveal the mechanism by which transcriptional activity drives nuclear blebbing is through chromatin motion. Thus, two hallmarks of human disease are directly linked via transcriptional activity and abnormal nuclear shape.

B332/P0323

Tuning the distribution of tissue structures via their effective temperature

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In vitro organoid models are a compelling system for investigating the basic principles of tissue self-organization and hold promise for high throughput screening of therapeutics. Recent progress towards these goals reveal an impressive amount of heterogeneity and dynamics at the cellular and tissue scales; however, we are lacking in quantitative models which relate outcomes in tissue morphogenesis and its dynamics to the underlying cellular mechanics and fluctuations. We demonstrate how a maximum entropy model of cell sorting allows us to directly consider how both mechanics and noisy cell dynamics combine to determine cell sorting in primary human mammary organoids, and predict the distributions of tissue structures seen in experiment. Further, small molecule inhibitors such as RO3306, a specific inhibitor of cell-cycle regulating protein CDK1, allow us to control the mean and width of this distribution of tissue structures. In particular, the thermodynamic interpretation suggests that we are able to shift the distribution of structures towards greater order by reducing the effective temperature of the tissues via reduction in noisy cell dynamics.

B333/P0324

Understanding centrin-driven ultra-fast contraction in single-cell organisms

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Free-living unicellular organisms must rapidly respond to environmental challenges through ultrafast cellular deformations. Unlike actin-mediated shape changes, these rapid responses occur on much shorter timescales and are mediated by centrin. In unicellular protists, centrin is not only localized at the basal body but also forms highly patterned, cellular-scale networks. While centrin is essential for these deformations, the mechanisms by which its networks generate and transmit forces remain unclear. Using the ciliate Paramecium tetraurelia, we examine how cell-scale shape changes relate to strains within the centrin mesh. Upon calcium stimulation, cells contract non-homogeneously, with most contraction at the posterior, producing a bottle-like morphology. This macroscopic anisotropy in cell shape correlates with the non-homogeneous subcellular organization of the centrin mesh: anterior units are isotropic, middle units elongate perpendicular to the anterior–posterior axis, and posterior units are hexagonal with near-circular aspect ratios. During calcium-mediated cellular contraction, posterior mesh units decrease significantly in area, while anterior units remain unchanged, consistent with the observed global shape changes. Together, these findings reveal how centrin networks exploit geometric organization to encode large-scale rapid deformations, uncovering novel

mechanisms of cytoskeletal polymer function. Beyond their biological relevance, our results establish design principles for engineering deployable, adaptable shape-shifting materials.

Biophysics, Structural Biology, and Mechanobiology - Mechanobiology in Cell Function and Development

B323/P0314
A ratiometric reporter of Mucin-1 SEA domain dissociation
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The SEA domain (Sea urchin sperm protein, Enterokinase, and Agrin) is a ~120 amino-acid protein domain commonly found in mucin-like glycoproteins that are widely expressed in epithelial and endothelial tissues. Structural studies of MUC1, a human cell-surface tethered mucin, have shown that the SEA domain can undergo post-translational autocatalytic proteolysis. While the two resulting MUC1 subunits remain non-covalently associated and traffic to the plasma membrane together, they are thought to be able to dissociate once at the cell surface. However, the physical and biochemical conditions that can drive this dissociation on native cell surfaces remain poorly understood. To directly observe SEA domain dissociation, we engineered an inducible MUC1 construct in which the SEA domain is flanked by two fluorescent proteins, enabling ratiometric monitoring of SEA domain dissociation on cell surface. Together with a wildtype SEA domain, we generated mutant SEA domains with impaired or enhanced cleavage functions. As expected, overexpression of MUC1 on HEK cell surface induced significant morphological changes, including weakened adhesion and tubular structure formation, consistent with prior studies. Interestingly, confocal imaging revealed that wildtype SEA domain dissociation was spatially non-uniform, with enrichment of dissociated SEA domains at adhesion sites. In contrast, cells expressing cleavage-deficient SEA mutants showed minimal morphological change and no apparent SEA domain dissociation. Our work established a versatile reporter for investigating SEA domain dissociation in living cells. Ongoing work is using this system to determine the biochemical and biophysical conditions that induce SEA domain dissociation, including pH, mechanical forces, and charge. By dissecting these mechanisms, we aim to further our understanding of how autocatalytic proteolysis domains sense and respond to cellular microenvironments.

B325/P0316
A two-tether divisome system ensures timely bacterial cell division
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Bacterial cell division requires simultaneous synthesis of new septal peptidoglycan (sPG) and degradation of old cell wall circling the division plane, improper coordination between which leads to osmotic lysis and has been a key target for antibacterial treatments. Despite extensive research, the physical mechanisms of sPG remodeling remain largely unknown. FtsZ, the bacterial homolog of tubulin GTPase, is the central organizer of bacterial cytokinesis and forms treadmilling polymers that coalesce into a composite ring-like structure (termed the “Z-ring”) at the septum. Using E. coli as our model system, we established that the treadmilling Z-ring acts as a Brownian Ratchet to not only recruit the essential sPG enzymes and regulators (termed the “divisome”) to the septum, but to also facilitate complex formation between FtsWIQLB, an sPG synthase complex, and FtsN, its trigger. The Z-ring therefore evenly distributes sPG synthesis activity along the septum

circumference preventing osmotic lysis. Building upon this framework, we further addressed how the Z-ring promotes divisome assembly from the early stage and why sPG synthesis requires two different tethers (i.e., FtsA and ZipA) that relay Z-ring dynamics to FtsWIQLB and FtsN. Combining theoretical modeling, single-molecule tracking, live-cell imaging, and bioinformatics studies, we present evidence that ZipA couples the persistent and directed movements of FtsWIQLB to the shrinking end of treadmilling FtsZ polymers through a Brownian Ratchet mechanism, while FtsA attacts FtsN weakly such that FtsN loosely switches FtsZ filament "tracks". Proper combination of these two distinct motilities, mediated by the two different tethers, underlies coordinated sPG synthase activation and hence, initiation of cell division when the septal concentrations of both FtsWIQLB and FtsN are at their lowest. The physical mechanism established by our model points to a general design principle that is shaped by evolution for efficient cell division that is conserved across gammaproteobacteria.

B284/P0275
Bacteria Distinguish Between Local and Global Envelope Stress
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Sensing of solid surfaces, like epithelia, is critical for bacterial pathogenesis. We discovered that a canonical Escherichia coli envelope stress pathway that is known to respond to various envelope-targeting antibiotics is also triggered by growth on surfaces. However, the transcriptional response of this pathway depends on the type of perturbation. We found that this dependence is mediated by a bistable genetic circuit that is tuned to distinguish between local stress (surface-sensing) and global stress (antibiotics). This result provides a new paradigm for surface-sensing during pathogenesis, and demonstrates how the smallest organisms on earth can specifically respond to local perturbations via a quintessentially prokaryotic mechanism.

B324/P0315
Bulk Actomyosin Partitions Cytoplasm in Large Embryos via Long-Range Cytoplasmic Flows
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Eggs and early embryos of most animals are characterized by their large cell size, varying between 0.1 to 1 mm. With their enormous cytoplasmic volume, which is often 103 to 105-fold larger than somatic cells, comes the challenge to organize cytoplasm over large spatial scales and partition intracellular components during cell division. How cytoplasmic organelles are organized and partitioned at such scale is not understood, and little is known about the function of cytoplasmic F-actin away from the cortex during cell division in general. To study these questions independent of the actin-rich cell cortex, we developed a cell-free system where actin-intact cycling Xenopus egg extract reconstitutes cytoplasmic dynamics over cell cycle progression, and also validated many of our findings in intact frog and fish embryos. We found that cytoplasmic F-actin facilitated robust division of centrosomes and nuclei after mitosis in this cell-free system, as well as robust partition of cytoplasmic organelles. Both F-actin and organelles were depleted from the future cleavage plane, in both actin-intact egg extract and pre-cleavage embryos. By tracking cytoplasmic flow, we found that cytoplasm underwent F-actin-dependent long-range flows into daughter compartments, at velocities corresponding to centrosome separation. Biophysical measurements revealed that bulk actomyosin modulated rheological and

physical properties of both cytoplasm and microtubule asters, making them behave as composite gel crosslinked over large spatial scales. Actomyosin contraction provided driving force that moved astral gels away from the fluid-like future cleavage plane, where the geometry was set by CPC localization and Aurora kinase B activity. With physics modelling and simulation, we showed that spatially heterogeneous stress distribution from bulk actomyosin was sufficient to drive cytoplasmic flows in 2D and 3D space that partition cytoplasm. Overall, our study revealed novel mechanism of cytoplasmic partitioning in large embryos, and highlighted bulk actomyosin's contribution to long-range organization and gel-like behavior of the cytoskeleton-cytoplasm composites.

B314/P0305

Calcium and actomyosin pulses drive Tricellular Zipping to remodel epithelial junctions and facilitate tissue packing

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Epithelial cell-cell junctions and the multicellular vertices that connect them must remodel as cells extend, shrink, gain, or lose cell-cell contacts with their neighbors during developmental morphogenesis. However, the signaling mechanisms and mechanics that regulate vertex remodeling in different contexts remain unclear. Here, using live microscopy in the Xenopus embryonic epithelium, we characterize "tricellular zipping", a vertex remodeling process where a long, thin cellular extension formed following cytokinesis is resolved as two cells zip together via lengthening a new bicellular interface, thus relocating the remodeling tricellular vertex. Tricellular zipping contributes to the transition from irregular cell shapes observed at blastula stage to the more regular hexagonal packing seen in gastrula-stage embryos. Cell-cell junction proteins ZO-1 and E-cadherin remodel as the new bicellular interface elongates, while the tricellular tight junction proteins Tricellulin and Angulin-1 accumulate at the remodeling vertex and exhibit different dynamic properties at the remodeling vertex compared with the stable vertex. Mosaic studies reveal that the receding cell exhibits pulsatile F-actin dynamics near the zipping front, whereas actin from the zipping cells accumulates strongly at the remodeling tricellular vertex. Additionally, Myosin II is enriched in a horseshoe-shaped structure at the zipping front and moves with the remodeling vertex. Inhibiting formin-mediated actin polymerization or expressing dominant negative ROCK perturbs tricellular zipping, suggesting that this is an actomyosin driven process. Notably, pulsatile cytoplasmic calcium flashes occur within the long, thin cellular extension as zipping progresses. Preliminary analysis suggests that calcium flashes are associated with an increase in the rate of new bicellular junction elongation. Similarly, we find that calcium flashes are often associated with actomyosin-driven cell shape changes in the Drosophila epithelium. Together, our results indicate that repeated calcium pulses and actomyosin accumulation facilitates the successful remodeling of higher-order vertices by tricellular zipping as epithelial cells undergo dynamic changes in their packing order during development.

B311/P0302

Cell contractility effects on fibrillar collagen in developing hearts

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Tissue fibrosis is leading cause of deaths in the developed world where collagen fibers overaccumulation as a result of dysregulated remodeling stiffen almost all tissues. Although cell contractility as a therapeutic target

has started to gain interest over canonical therapies focused on extracellular matrix (ECM), key aspects of collagen fiber remodeling in cardiac tissues remain unclear. We employed a recently FDA-approved drug Belumosudil for chronic Graft-versus-host disease (cGVHD) targeting cell-contractility via Rho/ROCK pathway to study effect on heart tissues. We perturbed hearts from chick embryos beating at ~5% strain using the ROCK-inhibitor as well as a pan myosin-II inhibitor Blebbistatin with or without a broad-spectrum matrix metalloproteinase (MMP) inhibitor exposure, and tracked real-time kinetics of fibrillar collagen under perturbations using label-free second harmonic imaging (SHG) imaging. Under ROCK-inhibitor, we find slower contractile beating strain suppression, less severe collagen fiber loss and negligible rescue of collagen-fiber loss under MMP-inhibitor when compared to myosin-II inhibition effects. Such findings are consistent with ROCKs as upstream target of cell contractility unlike myosin-II as a direct target as well as with differential expression of ROCKs across different cell-types (fibroblastic, cardiomyocytes) within embryonic hearts we investigated by scRNA sequencing. Such results motivate further studies on cell-contractility effects on tissue mechanics and on rate/reversibility of collagen loss in mature hearts, and have potential to deepen fundamental understanding of cell-contractile forces on ECM in development, adaptation, and disease for improved therapeutics.

B299/P0290

Chromocenter Decompaction Drives Abnormal Nuclear Morphology and Behavior in Low Cellular Confluence Conditions

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The nucleus is a membrane-bound organelle that contains the genome and regulates cellular function. Abnormal nuclear morphology has long been recognized as a hallmark of disease, serving as a diagnostic marker for cancer, heart disease, muscular dystrophy, and diabetes. Nuclear blebbing, a herniation of the nucleus, induces nuclear rupture resulting in nuclear dysfunction. We investigated the relationship between cell confluence and abnormal nuclear morphology and integrity. We observed that cells in low confluence conditions have significantly increased abnormal nuclear morphology, such as loss of circularity and nuclear blebbing, when compared to cells in medium to high confluence conditions. We also observed significantly increased abnormal mitosis in low confluence conditions. Chromatin and lamins are the components of nuclear mechanical strength that resist actin forces to maintain nuclear shape and function. Measurements of actin confinement and contraction as well as lamin levels did not significantly change based on levels of cell confluence. However, constitutive heterochromatin ratios of H3K9me3/H3K9me2 decrease significantly in low cell confluence resulting in decreased chromocenter compaction and nuclear rigidity. Wound heal assays recapitulate low confluence behaviors including increased cellular motion, upregulation of H3K9me2 leading to chromocenter decompaction, and increased nuclear blebbing and ruptures. Overall, we find that loss of mechanotransducive signals through cellular confluence modulation increases abnormal nuclear morphology and behavior through chromocenter decompaction mediated by H3K9me2. This finding reveals that cellular communication in the two-dimensional space is essential to maintaining nuclear mechanics, morphology, and integrity.

B308/P0299

Collagen-I - Lamin-A axis is mechanoregulated by strain-stabilization across mature, developing, and diseased tissues

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Understanding the rigidity of animal tissues has broad applications, including more realistic designs of organoids, better regeneration of tissues, and explainability of predictions from artificial intelligence (AI). We apply AI to expression from 50 different tissues and more than 1,000 heathy humans to identify possible factors linking nuclear envelope components to extracellular matrix, and we examine the axis within living tissue explants under real-time perturbations. A “mechanoregulated” cluster is discovered that comprises genes encoding Collagen-I fibers and nuclear Lamin-A filaments as well as Piezo-1, Vinculin, Myosin-II (MYH9), and Yap1 but not Lamin-B, or Piezo-2. Since key genes exhibit power-law scaling versus Collagen-I, we next perturbed tissue mechanics via either exogenous-collagenase or acto-myosin contractile-strain inhibition in fresh human heart and other tissue samples. Fibrillar collagen and myosin-based muscle striation level change in ~1 hour exhibited via label-free second harmonic generation (SHG) imaging, and confirmed by quantitative immunostaining of collagen-I, F-actin, and also Lamin-A in intact tissues. Combined with studies of beating embryonic chick hearts and mass-spectrometry (MS) of extracts, we conclude that mechanical forces not only protect tissue collagen from degradation by collagenases including endogenous matrix metalloproteinases (MMPs) but also preserve cytoskeletal organization/striation and nuclear shape and lamin-A. Strain-stabilization is consistent with a tissue-nucleus compliance-matching and can explain mechano-regulation and scaling for adult and developing soft tissues as well as high collagen-I in liver cancer. Tissue viscoelasticity above gelation thus reflects tissue-spanning collagen-fiber networks that mechanoregulate key cellular factors implicated in health, disease and aging.

B320/P0311

Convergent Flow-Mediated Mesenchymal Force Drives Embryonic Foregut Constriction and Splitting

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The transformation of a two-dimensional epithelial sheet into various three-dimensional structures is a critical process in generating the diversity of animal forms. Previous studies of epithelial folding have revealed diverse mechanisms driven by epithelium-intrinsic or -extrinsic forces. Yet little is known about the extreme case where epithelial tube deformation reaches a state where closely apposed folded segments fuse and undergo a topological transition that breaks the continuity of the epithelial tube, which we here define as epithelial splitting. To decipher the biomechanical basis of epithelial splitting, we leverage tracheal-esophageal separation (TES), an essential and highly conserved morphogenetic event in the foregut during tetrapod embryogenesis, as a model system both in vivo and ex vivo. Here, the dorsoventrally patterned foregut tube undergoes extreme medial constriction to form an epithelial junction (septum), which then splits into the esophagus dorsally and the trachea ventrally. Abnormal epithelial splitting in TES leads to life-threatening birth defects impacting 1 in 2,500 humans. Through comparative study of TES in chicken and mouse embryos, we identified an evolutionarily conserved, compressive force exerted by the mesenchyme surrounding the epithelium, as being necessary to drive epithelial constriction and splitting. We established a foregut slice culture system which recapitulates TES ex vivo, allowing direct visualization of tissue dynamics at single-cell resolution through two-photon microscopy as well as pharmacological and biomechanical perturbations. We found that the compressive mesenchymal force is mediated by localized, convergent flow of mesenchymal

cells toward the epithelium, representing a novel mechanism of epithelial folding. We further discovered that Sonic Hedgehog (SHH) secreted by the epithelium functions as an attractive cue for mesenchymal cells, which later give rise to the esophageal smooth muscle and the airway cartilage. Removal of the mesenchyme, inhibition of cell migration, or loss of SHH signaling all lead to defective TES due to a failure in epithelial constriction, which can be rescued by externally applied pressure. These results unveil the biomechanical basis of epithelial splitting and suggest plausible mesenchymal origins of tracheal-esophageal birth defects.

B287/P0278

Cortical microtubule reinforcement enables mechanical resistance of actomyosin-driven contractions in C. elegans and mouse oocytes

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The cell cortex is a network of actin filaments, non-muscle myosin II, and membrane-crosslinking proteins that underlies the plasma membrane. Cortical tension drives changes in cell shape and depends on myosin-generated forces and microfilament architecture. Such mechanical properties of the cortex are emerging as key determinants of oocyte developmental potential. While microtubules are considered separate from the cortex, they may play a greater role during oocyte meiotic cytokinesis. We have shown that loss of the C. elegans microtubule stabilizer CLASP/CLS-2 in oocytes results in excessive membrane ingression throughout the cortex, accompanied by defects in contractile ring assembly or stability and failed polar body extrusion. Because cortical actomyosin dynamics appear normal in cls-2 mutants, we proposed that CLS-2, which localizes both to meiotic spindle microtubules and to patches throughout the cortex, stabilizes cortical microtubules to promote a cortical stiffness that resists actomyosin-driven membrane ingressions throughout the oocyte cortex away from the spindle-associated contractile ring. To directly test this, we used Atomic Force Microscopy (AFM) to measure cortical tension in C. elegans oocytes. Tension was significantly reduced in both cls-2 mutants and nocodazole-treated oocytes, which destabilizes microtubules. Conversely, tension increased when microtubules were stabilized with taxol or by depletion of XMAP215/ZYG-9 or Kinesin-13/KLP-7, consistent with microtubules stiffening the cortex. We extended our work to mouse oocytes, where regulation of actomyosin activity plays a functional role in meiosis. However, microtubule reinforcement of the mouse oocyte cortex remains unexplored. In contrast to C. elegans, nocodazole increased cortical tension, whereas taxol had a conserved effect. We are testing whether nocodazole-induced tension increase reflects non-muscle myosin activation. These findings reveal species-specific roles for microtubules in modulating oocyte cortical mechanics and suggest that microtubules may act as mechanical regulators during these highly asymmetric cell divisions.

B285/P0276

Curved microtubule regions mark sites of lattice compaction in cells and neurons

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Tau is an intrinsically disordered microtubule-associated protein (MAP) best known for its aggregation in Alzheimer’s disease. However, similar tau pathology is also present in a substantial subset of Parkinson’s disease (PD) patients, especially those with LRRK2 mutations, a connection that remains relatively uncharacterized. On microtubules, tau has been observed to form cohesive “envelope”-like assemblies that

preferentially associate with compacted lattices (~41 Å inter-dimer spacing). Here, we demonstrate in cells that lattice expansion - induced by MAP7 or by microtubule-stabilizing agents such as taxol and epothilone D - causes tau to relocalize to regions of high microtubule curvature. At substoichiometric concentrations, tau acts as both a microtubule compactor and curvature sensor, reminiscent of doublecortin. Strikingly, pathogenic LRRK2 mutants (G2019S and R1441C), common in early-onset PD, exhibit microtubule binding patterns similar to tau and compete with it for binding sites. In human iPSC-derived neurons carrying these LRRK2 mutations, we observe elevated levels of pathogenic, hyperphosphorylated tau, consistent with postmortem findings in PD patients, suggesting that specific microtubule lattice conformations influence tau localization and disease-associated modifications. Finally, we show that neurons display micron-sized tau patches on microtubules, whose localization is sensitive to lattice-expanding perturbations. These findings reveal that mechanical and structural changes to the microtubule lattice can modulate tau distribution in neurons and may contribute to cytoskeletal dysregulation in neurodegenerative disease.

B291/P0282

Dissecting the effects of mechanical stretch on rapid changes in Transcription through integrated computational and experimental approaches

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Mechanotransduction is the process by which cells propagate mechanical forces and translate them into transcriptional responses. While cytoplasmic pathways are well-studied, these take several minutes to reach the nucleus, while direct nuclear mechanotransduction acts within seconds. Recent advances in methods to map RNA polymerase II (PolII) progression and nascent transcription (PRO-seq) enabled our lab to achieve a higher temporal resolution of nuclear mechanotransduction and rapid gene activation. Our data show that, upon mechanical strain, paused PolII complexes near mechanosensitive promoters are released, resulting in productive elongation. By combining orthogonal computational and experimental approaches, we dissect the mechanisms regulating PolII dynamics at these rapidly induced genes, thereby bridging the gap on how mechanical forces translate into rapid gene activation. We applied computational approaches to PRO-seq maps of nascent transcription following cyclic biaxial strain to gain insights into aspects of transcriptional regulation. DENR and eNRSA, tools that detect active gene isoforms from PRO-seq data, identified differential transcripts and generated an improved annotation of transcription start sites (TSSs). By using Markov models through groHMM and leveraging the enhanced annotations, we dissected the rates of PolII progression gene bodies following strain. The decrease in TSS-proximal PolII at activated TSSs, as measured relative to the gene body signal through pausing indices, suggests that mechanical stimulation results in a coordinated progression toward elongation. These estimations of PolII wavefronts and pausing indices indicate that mechanical forces rapidly induce transcription through the release of paused PolII or de novo recruitment in a gene-specific manner. Analysis of regulatory elements near the TSSs of induced genes revealed the enrichment of motifs of mechanosensitive factors, including NFκB, MKL1/SRF, and MAP family members. We validated an immunofluorescence-based approach for imaging stretched cells on their silicone membranes. To enable robust detection with fewer cells, we designed an RT-qPCR-based approach assessing the nascent transcription of genes by amplifying pre-mRNA using intronic primers. Our experimental approaches allow us to assess the subcellular localization of candidate mechanosensitive regulators following stretch, as well as quantify their relative effects on rapidly induced transcription through inhibitor studies. By integrating computational and experimental approaches, our findings reveal the gene-specific dynamics of RNA polymerase II during mechanically induced rapid transcription, enabling further experimental assessment of candidate regulators.

B302/P0293
Distinct domains of tensin 3 and cten interact directly with force-bearing keratin filaments
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How epithelial cells respond to mechanical force plays an important role in cell proliferation and their barrier functions. While the tensin family of phosphoproteins is known for its role in cytoskeletal regulation by directly linking actin filaments to integrin receptors, we demonstrate that the tensin family's interaction with the cytoskeletal network extends beyond actin filaments. Tensin 3 and tensin 4 (also known as cten) rapidly accumulate along tensed keratin fibers in epithelial cells, suggesting that keratin fibers, known for its role in the structural integrity of epithelial cells, are also active participants in mechanotransduction pathways. Notably, the highly conserved C-terminal Src Homology 2 (SH2) and phosphotyrosine-binding (PTB) domains are dispensable for the force-sensitivity of tensin 3 and cten. Instead, the force-induced accumulation relies on distinct N-terminal regions: unique alpha-helical structures in tensin 3 and disordered regions in cten. Deletion of these helical sequences within tensin 3 eliminates its ability to bind to tension-bearing keratin fibers. To further investigate whether tensin 3 and cten proteins bind directly or indirectly to the force-bearing keratin filaments, we developed an in vitro force-dependent protein interaction assay using recombinant keratin 8 and keratin 18 and a microneedle to apply strain. Importantly, GFP-tagged force-sensitive regions of tensin 3 and cten proteins selectively and directly bind to the force-bearing keratin filament bundles in vitro. These results collectively demonstrate that distinct domains of tensin 3 and cten interact directly with force-bearing keratin intermediate filaments and that the keratin network may act as a signaling hub for cell's mechanotransduction and mechano-resilience.

B290/P0281
Dynamics of nuclear envelope rupture and cellular recovery
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The nuclear envelope is a composite elastic membrane that encloses the genome and regulates nucleo-cytoplasmic exchange in eukaryotic cells. Subject to intrinsic and extrinsic mechanical stresses, the nuclear envelope can undergo transient rupture, leading to the leakage of chromatin into the cytoplasm. Such rupture has been implicated in DNA damage, particularly in cancer cells. Recovery involves retrieving the leaked chromatin and resealing the nuclear envelope—a process primarily driven by DNA-binding proteins that cross-link chromatin and promote its compaction. Motivated by experiments in which rupture is induced through mechanical compression or laser ablation, we develop a continuum model to study the dynamics of chromatin and cross-linking proteins following nuclear envelope rupture. Chromatin is coarse-grained as a polymeric gel that contracts in response to cross-linking interactions. The nuclear envelope is modeled as a thin elastic shell dynamically coupled to the gel. Cross-linker proteins are transported via advection, diffusion, and reversible binding, allowing them to accumulate at rupture sites and locally compact the chromatin. Our model captures the nonlinear interplay between gel mechanics, protein transport, and membrane elasticity. Numerical simulations reproduce the key dynamical behaviors observed in vivo during chromatin leakage and retrieval. We further show that severe rupture events can trigger global nuclear contraction and wrinkling of the nuclear

envelope, consistent with experimental observations. Our study identifies and characterizes the physical mechanisms that govern chromatin recovery and reorganization following nuclear envelope rupture, highlighting the central role of cross-linking proteins in this process.

B317/P0308

Effects of Local Strain on Epithelial Monolayer Growth and Collective Behavior

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Mechanical cues such as stretch are strong regulators of epithelial growth and organization. This study aims to investigate how spatially localized strain influences proliferation and collective behavior in epithelial monolayers, and our preliminary data demonstrates both local effects on proliferation and surprising, radial waves of cell density. While much work has been done with global strain on cultured tissues, the body routinely experiences hyper local mechanical perturbations, and we wanted to investigate how these local mechanical cues affect global tissue behaviors. To achieve this, we engineered a stretchable silicone culture substrate where only a small, ~500 µm region stretches, while the substrate immediately surrounding this zone stays stationary. We do this by infinitely stiffening the substrate regions around the stretchable zone using bonded glass sheets. The result is that we can apply concentrated, local, nearly uniaxial strains of up to 100% to specific parts of engineered tissues. We tested this device using Madin-Darby Canine Kidney (MDCK-II) cell monolayers expressing fluorescent FUCCI cell-cycle indicators. We engineered 5x5 mm square tissues centered over the stretchable zone, grew the cells to contact inhibition, and then applied 100% strain using 3D-printed fixtures compatible with live confocal imaging. Fluorescent and phase contrast microscopy were captured for up to 24 hours post-stretch, and proliferation was quantified by the proportion and number of cells in the S–G2–M phases of the cell cycle. Strain is known to trigger cell proliferation, and we validated the local strain effects by quantifying a marked increase in cell cycle re-entry within the stretched region. These proliferative responses were accompanied by recovery of tissue density within the strained zone. More surprisingly, we observed large, persistent, and rapid waves of what appears to be collective density fluctuations within the stretched zones. We are currently characterizing how these arise and whether they are related to strain-induced proliferation.

B292/P0283

Elucidating the role of Lamin B Receptor in abnormal nuclear structure in papillary thyroid cancer

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Papillary thyroid cancer (PTC) is diagnosed by nuclear atypia. The nuclei are enlarged, have irregular nuclear contour, and have increased wrinkling. As in most solid and dense tumors, PTC cells experience recurrent mechanical stress which may impact the nucleus, a major mechanosensory organelle. Despite the diagnostic importance of nuclear atypia, the causes and consequences of nuclear atypia in PTC remain unknown. The goals of this study are to 1) identify factors involved in nuclear atypia and 2) determine how PTC nuclei respond to mechanical stress. Our bulk RNAseq data from patient PTC tumors revealed lower expression of lamin B receptor (LBR) and higher expression of lamin A/C compared to the normal adjacent thyroid tissue. To investigate these proteins in vitro and investigate a potential role in PTC atypia, we employed the PCCL3 HRAS cell line, which is derived from rat thyroid follicular cells and have a dox-inducible expression of H-RASv12, an

oncogenic driver of PTC. Agreeing with our bulk RNAseq results, western blot revealed decreased LBR upon HRASV12 expression. Additionally, we found that the nuclei of HRASV12-expressing cells displayed increased size, wrinkles, and shape abnormalities compared to control cells. Interestingly, H-RASv12 expression led to enriched LBR localization to the ER compared to the typical nuclear envelope localization in control cells. In patient tumors, we found a similar striking pattern of LBR enrichment in the ER. LBR is an inner nuclear membrane protein with multiple functions including: 1) linking DNA/lamina to the inner nuclear membrane and 2) as a sterol reductase for lipid biosynthesis. As the ER is continuous with the outer nuclear membrane, we hypothesize that ER-enriched LBR in PTC cells increases the nuclear membrane reservoir by promoting increased lipid synthesis at the ER. This could explain the augmented nuclear size and nuclear envelope wrinkles observed in vitro and in PTC patients. We further hypothesize that this excess of nuclear membrane reservoir buffers mechanical stress on the nuclear envelope. To test this, we employed a microfabricated confiner to apply controlled and precise mechanical confinement. We assessed nuclear envelope rupture by investigating the localization of the cytosolic DNA sensor cGAS (upon nuclear envelope rupture, it forms perinuclear foci, at sites of rupture). We found that PCCL3 cells expressing HRASv12 have decreased frequency of nuclear envelope rupture events at intermediate confinement heights compared to control cells. Using the HRASv12 inducible model to study nuclear atypia, we identified ER-enriched LBR as a strong candidate that might contribute to nuclear atypia and play a role in buffering mechanical stress, a hallmark of dense solid tumors.

B319/P0310

Emulsion Droplets as Deformable Force Sensors Reveal Mechanics of Phagocytosis

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Phagocytosis is a fundamental process in immunity and tissue homeostasis, whereby immune cells ingest and degrade diverse targets ranging from pathogens to apoptotic cells. The objective of this study is to quantitatively link receptor signaling, cytoskeletal remodeling, and mechanical force generation during engulfment. To address some of the limitations of model particles such as polystyrene or gel beads, we developed oil-in-water emulsion droplets as deformable force sensors. These droplets have tunable size, a well-defined surface tension, and can be functionalized with ligands to selectively engage immune receptors. Unlike solid substrates, their liquid–liquid interface allows lateral diffusion of ligands, thereby enabling receptor clustering and more physiological recognition dynamics. Deformation of the droplets directly reflects the normal stresses exerted by cells through Laplace's law, providing a quantitative handle on force generation. Using RAW 264.7 macrophages, we analyzed hundreds of engulfment events. Statistical analysis revealed that droplet deformation correlates with Fcγ receptor engagement and IgG recruitment. Epifluorescence microscopy showed dynamic actin protrusions driving asymmetric deformations at the droplet surface, while confocal microscopy combined with 3D reconstruction algorithms adapted from yielded stress maps across the target surface at distinct engulfment stages. These data link receptor-level recognition to local force exertion patterns, consistent with recent microparticle traction force studies. We conclude that functionalized emulsion droplets constitute a powerful and versatile tool for probing the mechanobiology of phagocytosis. By overcoming the limitations of solid particles, they enable quantitative mapping of cellular forces with molecular specificity. This approach provides new insight into how immune cells regulate force generation during target internalization and establishes a broadly applicable platform for studying receptor-mediated uptake in diverse contexts.

B282/P0273

Epigenetic Remodeling Stores Mechanical Memory and Stabilizes Gene Regulation

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Cells are constantly exposed to physical forces during development, regeneration, and disease, yet how they retain a memory of past mechanical experiences and translate transient inputs into lasting molecular and phenotypic states remains unclear. Here we show that mechanical priming imprints persistent epigenetic and gene-regulatory programs in human mesenchymal stem cells. Cells cultured on physiologically soft or stiff substrates for defined durations retained substrate-dependent morphology and cytoskeletal organization even after removal from the priming environment, establishing a phenotypic signature of mechanical memory. This persistence was accompanied by progressive changes in histone modifications: active marks captured through global acetylation and H3k27ac, while the repressive mark H3K9me3 evolved from transient shifts after short priming to semi-stable patterns with prolonged priming. Super-resolution imaging revealed correlated remodeling of nanoscale chromatin domains and lamina-associated regions, indicating that nuclear architecture encodes the duration of mechanical exposure. Perturbation experiments showed that mechanotransduction pathways are required for memory formation, whereas epigenetic inhibition erases established memory, underscoring complementary roles for cytoskeletal force transmission and chromatin regulation. Transcriptomic profiling further demonstrated reinforcement of gene regulatory networks, with cell–matrix signaling and epigenetic read/write programs becoming progressively stabilized under longer priming. To unify these findings, we developed a mesoscale mechanotransduction–chromatin model that describes a self-reinforcing axis linking mechanical inputs to chromatin remodeling and transcriptional stability. Together, these results establish the epigenome and chromatin architecture as central repositories of mechanical memory and reveal a general principle by which cells record their mechanical history, with broad implications for development, diseases such as cancer and fibrosis, and regenerative medicine.

B316/P0307

Epithelial architecture emerges from physical forces patterned at cell-cell junctions

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Intestinal villi are small, finger-like projections present in our small intestine. Each villus consists of monolayer of epithelial cells called enterocytes that line the intestines and play critical roles in digestion and defense. This monolayer is also the body's fastest self-renewing tissue, whose turnover is driven by balanced rates of cell birth in intestinal crypts, migration along the villi, and death at villi tips. This dynamic equilibrium relies on mechanisms integrating inter and intra-cellular forces that remain elusive. The loss of an epithelial junctional protein Non muscle myosin II (NMIIC) within mouse villi epithelia alters tissue architecture, resulting in distortions in cell shapes and jaggedness in cell edges, suggesting a change in junctional forces mediated by NMIIC. In epithelial cells, NMIIC forms discrete apical junctional puncta [1], that we hypothesize set local packing and tissue geometry. Prior analyses showed that NMIIC knockout (KO) disrupts apical morphology and elicits coarser, compensatory NMIIA puncta at junctions. Here we combine super-resolution imaging with a machine learning-based pipeline for whole-villus apical cartography that registers every epithelial cell to a

canonical villus side surface and quantifies per-cell morphology and puncta organization along the base-tip axis. In wild-type (WT) villi, cells enlarge and relax apical packing from base to tip: cell area rises and perimeter increases while nearest-neighbor puncta spacing widens and perimeter-normalized puncta density drops. Together with the acquisition and analysis of biological data, we develop a bead–spring computational model of cell mechanics [2] that predicts NMIIC puncta act as transient anchoring beads concentrating junctional tension; reduced bead density shifts the system toward less-ordered apical shapes and broken symmetry—consistent with KO phenotypes and the WT axial trend. Here we present the whole-villus cartography framework, quantitative WT maps, KO-informed perturbation results, and model predictions that together support a NMIIC-dependent, physics-first mechanism for sculpting intestinal epithelial morphology. [1] Ebrahim S, Fujita T, Millis BA, Kozin E, Ma X, Kawamoto S, Baird MA, Davidson M, Yonemura S, Hisa Y, Conti MA, Adelstein RS, Sakaguchi H, Kachar B. NMII forms a contractile transcellular sarcomeric network to regulate apical cell junctions and tissue geometry. Current Biology. 2013;23(8):731–736. doi:10.1016/j.cub.2013.03.039. [2] Ray S, Biswas S, Das D. Role of intercellular adhesion in modulating tissue fluidity. eLife—Reviewed Preprint. 2025. doi:10.7554/eLife.106294.

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Flow-stimulated response system in mammalian sperm

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The expression of mechanoreceptors, TRPV, Piezo and TRPC family genes, were detected in human sperm. TRPV1 and TRPC3 were mainly localized acrosome and midpiece of sperm. Piezo1 was also localized in tail. We hypothesized that sperm have the ability to sense physical stimuli such as flow fluid stimulation, which may affect their motility. In this study, we made the microfluidic devices reproduced the flow into female reproductive tract by using PDMS under the microscope and made the condition with various flow rate (0, 3, 5, 10 µl/min), by perfusing the culture medium. The movement trajectories of sperm treated with or without inhibitor of TRPV and Piezo family (Ruthenium red), TRPV1 specific antagonist (SB366791), inhibitor of TRPC family (BTP2), were detected using high-speed CCD camera live analysis. According to increase the flow rate, the amplitude of sperm was significantly increased. The amplitude of sperm was significantly increased in a fluid with a velocity of 3 µl/min. Interestingly, sperm of good quality is more sensitive to the flow velocity. Then, in the absence of flow, the head of motile sperm was oriented in random directions. However, when sperm was received a flow of 3 µl/min, the angles formed by the fluid flow and the motile sperm head became on about 68.6±14.2 degrees against the direction of the flow path. When mechanoreceptors sense the flow-simulation, the inflow of Ca2+ into the cell is occurred to drive the signal transduction. When the flow rate was increased, the fluorescence intensity of Ca2+ in sperm was significantly increased. On the other hand, sperm treated with Ruthenium red or SB366791 decreased sperm amplitude and motility dose dependently and were not activated the motility even when stimulated with a flow of 3 µl/min. In sperm treated with BTP2, when sperm was just swimming in the microfluidic devices, sperm changed their motility pattern to straightly. In this condition, at a flow rate of 0 µl/min, Sperm treated with BTP2 swam straightly with random directions at a flow rate of 0 µl/min. Interestingly, when culture medium was refluxed at a flow rate of 3 µl/min, sperm began to swim straightly forward with their head facing upstream, and even after applying the flow stimulation to sperm, sperm maintained the movement in straight lines and directionality. Similar effects on sperm motility were detected in experiments using TRPV1 KO mice and TRPC3 KO mice. These results suggest that sperm have the ability to sense the speed of flow via mechanoreceptors to activate their motility. Further research to elucidate the relationship between sperm motility and the physical stimulation receptors and signal

transduction mechanisms in sperm will contribute to the drug discovery using potential functional markers and the development of high-quality sperm induction system.

B307/P0298
Harnessing Physical Microenvironments to Modulate Cancer Cell Fate
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Aging is an inevitable biological process and, together with its detrimental effects, is often accompanied by undesirable cell state transitions such as cancer, fibrosis, and senescence. Understanding and controlling these transitions is critical for developing strategies to restore cells to a healthy state. Mechanical cues represent a promising yet underexplored approach to modulating cell fate independently of biochemical factors. Previous work has shown that mechanical interventions alone can induce state transitions in stromal cells and even reverse age-associated phenotypes. However, whether such approaches can reprogram malignant cells and reverse cancer-associated behaviors has remained unclear. Here, we demonstrate that lateral confinement of breast cancer cells on micropatterned substrates is sufficient to induce de-differentiation into a stem-like state, followed by re-differentiation into a more normalized phenotype under extracellular cues. Stem-like induction was validated through alkaline phosphatase activity and high expression of canonical stem cell markers Nanog, Oct4, and ALDH1A1 using fluorescence microscopy and qPCR. In parallel, cancer-associated properties were reduced, evidenced by lower expression of estrogen and progesterone receptors, decreased DNA damage, and reversal of epithelial-to-mesenchymal transition (EMT), marked by altered N-cadherin, vimentin, and cytokeratin-19. We next asked whether chromatin architecture can act as a filter to capture the sequence of transitions during mechanical reprogramming. Using 3D confocal imaging across sequential time points, combined with a computational pipeline, we classified distinct states directly from DAPI-stained nuclei. The method infers lineage trajectories by computing the most plausible paths for a population to transition between time points, assuming cells progress toward similar states. Quantitative profiling revealed state-specific chromatin signatures predictive of outcomes. Notably, only a subset of cells completed reprogramming, highlighting intrinsic heterogeneity and the energetic barriers of epigenetic remodeling. Together, these findings position mechanical reprogramming as a distinct paradigm for controlling cancer cell fate achieved entirely without biochemical factors. By linking nuclear architecture to reprogramming efficiency, our work shows that physical confinement can regulate plasticity. Beyond feasibility, this study introduces a framework for reconstructing the sequence and interdependence of reprogramming events from static nuclear features. This approach deepens understanding of cancer cell plasticity and opens avenues for regenerative interventions guided by physical rather than biochemical principles.

B298/P0289
Investigating Nuclear Mechanotransduction by Mapping Genome-wide Changes in Transcription and Chromatin Accessibility in Response to Mechanical Stretch
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Cells mediate tissue maintenance and growth through mechanotransduction, the translation of mechanical forces into biochemical signals. Whereas several mechanotransduction pathways have been identified at the plasma membrane and cytoplasm, mounting evidence suggests the nucleus also plays a part in

mechanotransduction. It is not yet known, however, if changes in chromosomal organization and concurrent gene activation, initiated by increased binding of transcription factors or polymerases, following mechanical stimuli are a direct consequence of nuclear deformation, or if they are due to upstream cytoplasmic mechanotransduction pathways. To address this question, we developed a custom experimental pipeline to investigate the regulatory mechanisms mediated through nuclear mechanotransduction by probing the immediate (<minutes) genome-wide response to cyclic mechanical stretch. Using precision run-on sequencing (PRO-seq) and Omni-ATAC, we achieved unprecedented sensitivity and resolution in measuring nascent transcription and chromatin accessibility, respectively. Unlike conventional RNA-seq, PRO-seq enriches for newly synthesized RNA and simultaneously maps active RNA polymerases at base-pair resolution. Our data revealed a rapid increase in RNA polymerase II (PolII) activity within the gene bodies of several known mechanoresponsive genes (e.g. EGR1, FOS/B), and stress-associated immediate early genes (e.g. NR4A1, ATF3), as early as 2 minutes after mechanical stretch. PolII processing along the gene body suggests that gene activation was initiated <1min after the stretch, i.e., substantially faster than canonical cytoplasmic mechanotransduction cascades, which typically require several minutes to reach the nucleus. Notably, PRO-seq's sensitivity enabled identification of NFATC2 as a novel mechanoresponsive gene, even in the absence of full-length transcripts. Integration with Omni-ATAC data revealed that promoters of mechanically-induced genes were already accessible prior to stimulation, indicating a poised state. Upstream regulator analysis implicated key pathways such as MAPK, NF-kB, and the FOX family, traditionally associated with cytoplasmic signaling, in rapid nuclear response to mechanical cues. Together, our findings support a model in which nuclear mechanotransduction enables swift transcriptional activation of pre-accessible genomic regions, independent of slower cytoplasmic signaling, offering new insights into the temporal dynamics of gene regulation in response to mechanical stimuli.

B305/P0296

KLF2 Regulates Human Endothelial Cell Size Through Cell-Autonomous Mechanisms

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Atherosclerosis develops preferentially at arterial bifurcations and curves exposed to disturbed flow, where endothelial cells (ECs) suppress Kruppel-like factor 2 (KLF2), adopt cuboidal morphology, and proliferate. In contrast, laminar shear stress induces KLF2, promoting elongation, alignment, and vascular protection. Although EC size influences vessel diameter and perfusion, it has been largely overlooked as a flow-regulated property that may shape focal lesion development. We tested whether KLF2 regulates human EC size and morphology, and whether these effects are cell-autonomous and nitric oxide dependent. Human umbilical vein endothelial cells (HUVECs) were transduced under static conditions with adenoviral murine KLF2 (Ad-mKLF2) or GFP controls. Morphometric analyses quantified area, elongation, and orientation. Mosaic co-cultures assessed cell autonomy, and endothelial nitric oxide synthase (eNOS) dependence was tested with L-NAME. Ad-mKLF2 cells were 22% larger than controls (95% CI: 15–26%) and exhibited significantly greater length-to-width ratios (+0.47 units, 95% CI: 0.36-0.58). Control cultures proliferated over 48 h, whereas Ad-mKLF2 cells hypertrophied without division; nuclear size was unchanged, making senescence unlikely. Aquaporin-1 (AQP1), a candidate KLF2 target, was upregulated, and single-cell analysis showed a moderately positive correlation between GFP intensity and cell area ($r^2 = 0.524$), consistent with a dose-dependent effect. Mosaic experiments demonstrated that hypertrophy is cell-autonomous, whereas elongation and orientation are not: neighboring GFP-only cells aligned and elongated to the same extent as adjacent KLF2-expressing cells. L-NAME abrogated the size increase but not elongation, which remained greater than in controls (+0.51 units, 95% CI: 0.37-0.64). Thus, hypertrophy requires eNOS, while alignment propagates through eNOS-independent, community-level effects. These findings identify KLF2 as a regulator of EC size and morphology, linking eNOS-dependent

hypertrophy with eNOS-independent alignment. Beyond effectors such as AQP1, this work motivates systematic interrogation of the KLF2 transcriptome to define how flow-responsive programs coordinate endothelial growth, shape, and communication. Such pathway-level insights may reveal new principles of vascular homeostasis and targets to stabilize endothelium at atherosclerosis-prone sites.

B281/P0272

Lateral myosin II coordinates cell rearrangement progression in 3D during epithelial morphogenesis

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Cell rearrangements underlie many of the complex structural changes epithelial tissues undergo during development. It is widely thought that cell rearrangements are driven by the apical enrichment and planar polarized localization of cytoskeletal and junctional adhesion proteins. This enrichment of proteins, together with the relative ease of 2D image analysis, has led most studies to focus on the apical side of the tissue, simplifying cell rearrangements as 2D processes. However, by not capturing the full 3D nature of cell rearrangements, rearrangement propagation along the apical–basal axis has been largely overlooked. Recent studies of the Drosophila germband epithelium reveal that rearrangements can initiate at various locations along the apical-basal axis and that myosin II (myosin) is also present at lateral cell-cell contacts, well below the apical surface of the tissue. However, the molecular and mechanical mechanisms that coordinate cell rearrangement progression along the apical-basal axis remain unclear. Here, we use confocal fluorescence and multiview light-sheet microscopy to capture myosin localization patterns and tension inference to study patterns of forces along the apical-basal axis of the Drosophila germband epithelium during convergent extension. We confirm the presence of myosin at lateral positions and show that rearrangement initiation sites correlate with myosin localization at apical and lateral regions. We find that myosin localization at shrinking contacts is associated with narrowing of adjacent cell-cell contact angles prior to rearrangement, consistent with increased mechanical tension at the shrinking contact, suggesting that myosin-driven forces actively propagate these rearrangements along the apical-basal axis. Notably, we also find a subpopulation of rearrangements that lack obvious lateral myosin and exhibit minimal changes in cell-cell contact angles during rearrangement, suggesting that rearrangement propagation along the apical-basal axis may sometimes occur via passive relaxation of active stresses exerted elsewhere in the tissue. These findings indicate that myosin, beyond its established role in the apical domain, contributes to the cell rearrangement process in the lateral domain, providing insight into rearrangement coordination along the apical–basal axis with broad implications in understanding morphogenesis in 3D.

B312/P0303

Lipid Droplets are rigid and mechanically disrupt actomyosin to suppress phagocytosis unless rescued by cell compression or stretching which activates actomyosin

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Lipid droplets (LDs) often accumulate in macrophages and are implicated in various pathologies, but any physical impact on phagocytosis remains understudied. We recently reported that micron-sized LDs remain spherical within cells and behave as rigid bodies, consistent with their high interfacial tension. Here we show that LDs disrupt cytoskeletal structure and impair actomyosin-driven uptake across a range of target sizes.

Importantly, the result is phenocopied by pre-loading microbeads into macrophages prior to a phagocytosis assay, which further supports a biophysical basis for suppressed engulfment. Applying tissue-relevant compressive stress to LD-loaded macrophages activates actomyosin and rescues phagocytosis, and similar effects are observed wih excessive loading of LDs or rigid beads that effectively stretch the cortical network. Consistent with motility defects, LDs and rigid beads hinder macrophage migration through narrow pores, and when LDs press on the nucleus in confined spaces, rapid nuclear envelope rupture ensues independent of actin. This can lead to DNA damage, cell dysfunction, and apoptosis. Mechanical perturbations of the actomyosin network by LDs are thus functionally important and relevant to obesity among other diseases.

B321/P0312

Listeria-infected macrophages promote biomechanical alterations in endothelial cell monolayers for transmigration

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Infectious diseases pose a global health concern, which is further exacerbated by the steady emergence of antibiotic-resistant bacterial strains. A better mechanistic understanding of the processes that drive the onset and progression of infection could inform innovative therapeutic strategies to fight pathogens. Intracellular bacterial pathogens like food-borne Listeria monocytogenes (LM) have developed strategies to manipulate the biomechanics of host cells without disrupting cell integrity,as they rely on it. One way LM can achieve systemic dissemination is by being carried within immune cells like macrophages (MΦ), which are able to transverse the vasculature and reach distant organs such as the brain, further spreading infection and causing devastating fatalities. How do infected MΦ manage to transverse vascular endothelial linings breaching their barrier integrity? Have MΦ developed mechanisms to (in)directly manipulate endothelial cells (EC)? Here, we show that the biomechanical responses of EC to MΦ differ markedly with infection, decisively impacting MΦ transmigration through the EC monolayer. Using long-term videomicroscopy, we find that MΦ exposure, irrespective of their infection status, increases EC polarization and alignment while reducing EC migration speed. However, only EC exposed to uninfected MΦ increase their traction forces, monolayer stresses, and barrier integrity. These EC biomechanical responses are significantly attenuated during exposure to infected MΦ. We show that TNF-α secretion during infection compromises EC barrier integrity and, combined with increased MΦ-EC adhesion, facilitates MΦ transmigration. Using zebrafish, we find that infection increases endothelial permeability, enhancing phagocyte extravasation in vivo. Thus, our findings support a model whereby infection overrides MΦ-induced strengthening of the endothelial barrier, promoting pathogen spread, a remarkable feature that could be harnessed for infection control. By continuing this line of research, we hope to shed critical light into the physical and chemical alterations that allow MΦ carrying bacteria to breach EC barriers like the blood-brainbarrier. This in turn could be very important to understand how to obstruct LM spread and avoid complications associated with LM infection, such as fatal encephalitis and meningitis.

B286/P0277

Long range mutual activation establishes Rho and Rac polarity during cell migration

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In migrating cells, the GTPase Rac organizes a protrusive front, whereas Rho organizes a contractile back. How these GTPases are appropriately positioned at the opposite poles of migrating cells is unknown. Here we leverage optogenetics, manipulation of cell mechanics, and mathematical modeling to reveal a surprising mechanochemical long-range mutual activation of the front and back polarity programs that complements their well-known local mutual inhibition. Rac-based protrusion stimulates Rho activation at the opposite side of the cell via membrane tension-based activation of mTORC2. Conversely, Rho-based contraction induces cortical-flow-based regulation of phosphoinositide signaling to trigger Rac activation at the opposite side of the cell. We develop a minimal unifying mechanochemical model of the cell to explain how this long-range facilitation complements local inhibition to enable robust Rho and Rac partitioning. We show that this long-range mutual activation of Rac and Rho is conserved in epithelial cells and is also essential for efficient polarity and migration of primary human T cells, indicating the generality of this circuit. Our findings demonstrate that the actin cortex and plasma membrane function as an integrated mechanochemical system for long-range partitioning of Rac and Rho during cell migration and likely other cellular contexts.

B280/P0271
Loss of Lamin-A/C Enhances Anti-Tumor Macrophage Function
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Macrophage-based immunotherapies have been found to initiate acquired immunity against solid tumors, but face challenges due to poor infiltration and an immunosuppressive microenvironment. Clinical data has sometimes linked clusters of tumor-associated macrophages (TAM) with improved patient survival, and chimeric antigen receptor macrophages (CAR-Ms) similarly form clusters in vivo. We find macrophage clusters form under maximal phagocytic conditions, where they cooperatively disrupt tumor cell adhesion, and we observe that M1 macrophages increase adhesion receptors, reduce actomyosin factors, and form clusters even without phagocytosis. Decreased cortical tension generally relates to downregulated SRF-target genes and low levels of nuclear lamin-A,C – which we hypothesized facilitates phagocytosis. Using conditionally immortalized macrophages (CIMs), we generated lamin-A,C knockouts that cluster more on low-adhesion substrates, express less vinculin and α-actinin, and phagocytose more cancer cells. Large deformations of the nucleus were also evident, consistent with a softened nucleus. Further combining lamin-A,C and SIRPα deletion in CAR-Ms can further enhance tumor clearance by lowering cortical tension for facile disruption of tumor cell adhesion without the CD47/SIRPα “Don’t Eat Me” signal. Upregulation of adhesion and M1 genes (ITGAL, ICAM1, MHC-II) provide a phenotype well-suited for antigen presentation and tumor immunity. Studies in 3D tumoroid models document infiltration and clustering, while constrained migration assays examine movement through restrictive environments. Targeting nuclear mechanics may thus be broadly leveraged to potentiate immune effector cells for treatment of solid tumors.

B295/P0286
Mapping out the Microtubule Lattice in situ with Cryo-EM: Towards A Statistical Physics of Motor-Mediated Microtubule Interactions
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Collective behavior of microtubules and molecular motors underlies much of sub-cellular self-organization, including the formation of the meiotic and mitotic spindles. While emergent properties of microtubule networks are well-described using continuum theories, how these properties arise from individual microtubules interacting via molecular motors is far from understood due to experimental limitations at the relevant length scales. Recent developments in cryo electron microscopy (cryo-EM) along with our ongoing work in developing novel computational techniques make it possible to resolve the high-resolution details of dynamical steady states of microtubules and molecular motors in situ. Our goal is to measure the positions and conformations of individual motors and microtubule lattice sites in these steady states, thereby informing statistical mechanical models of how interactions between motors give rise to their collective properties, such as traffic jams, which ultimately give rise to microtubule-microtubule interactions.

B289/P0280

Matrix stiffness induces heritable changes in chromosome numbers, aligning with solid tumor heterogeneity

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Extracellular matrix often accumulates in and around solid tumors, and such tumors also evolve diverse mutations that drive cancers and confound therapies. Across cancer types, we observe chromosome number changes associate with collagen-I levels, and our experiments show rare heritable chromosome losses are induced by a stiff 3D matrix around spheroids. Chromosome reporters (ChReporters) reveal losses in as few as ~0.1% of cells, with a mechanism in spheroids based on distortion of mitotic spindles – which increases with myosin-II suppression in 3D stiff matrix. Chromosomes mis-segregate into micronuclei that increase with matrix stiffness despite suppressed cell division. Drugs that increase micronuclei in 2D and that rely on an unperturbed spindle show no effect in 3D where the spindle is perturbed. Tumors in vivo that are surrounded by stiff collagen likewise show more but varied chromosome loss and slower growth than 2D cultures. High variance of ChReporter-negative colonies further illustrate increased heterogeneity with 3D matrix stiffness and heritable mutations per Luria-Delbruck theory. Physical learning models of evolving chromosome numbers in proliferating cells are developed and fit key statistical trends. Overall, stiff 3D matrix fuels mechano-evolution that diversifies solid tumors and complicates treatments.

B300/P0291

Mechanical Aspects of Steatotic Liver Disease: Lipid Droplets as Internal Stressors Driving Nuclear Remodeling and Chromatin Reorganization

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Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) is a growing global health burden, ranging from simple steatosis to steatohepatitis (MASH), fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) and affecting 1/3 of the world population. While matrix stiffening has long been implicated in liver cancer progression, recent clinical observations demonstrate that HCC can arise in non-cirrhotic, relatively soft livers of MASLD patients, suggesting that intracellular mechanical cues may be equally important. We previously identified intracellular lipid droplets (LDs) as potent mechanical stressors that deform hepatocyte nuclei, drive chromatin condensation, and impair hepatocyte function even in soft environments. Here, we combined

super-resolution STORM imaging with transcriptomic profiling to dissect the nuclear and epigenetic consequences of LD-driven nuclear deformation. Primary human hepatocytes (PHHs) were cultured on polyacrylamide substrates of defined stiffness (500 Pa and 10 kPa) and loaded with oleate to induce LD accumulation. STORM imaging revealed reorganization of chromatin nanoclusters and significant alterations in key epigenetic marks associated with heterochromatin stability and transcriptional regulation. Furthermore, bulk RNA-seq analysis of lipid-loaded PHHs showed enrichment of nuclear stress-associated pathways, including transcription-coupled nucleotide excision repair, histone modifying molecular function activity and DNA damage associated pathways, underscoring the impact of lipid driven nuclear deformation on genome maintenance pathways. Collectively, our findings establish LDs as intracellular mechanical effectors that reprogram chromatin architecture, epigenetic state, and gene expression even in soft environments independent of extracellular stiffening. This mechanobiological framework provides new insight into MASLD-associated carcinogenesis and suggests that lipid-nuclear mechanics may represent a critical vulnerability for therapeutic targeting.

B309/P0300
Mechanical signaling upregulates survivin to drive cardiac fibrosis
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Cardiac Fibrosis, characterized by structural and functional remodeling of the myocardium following injury such as myocardial infarction (MI), is a significant risk factor for heart disease. Injury-induced chemical signals from cardiomyocytes trigger the trans-differentiation of quiescent fibroblasts into myofibroblasts, which exhibit increased proliferation and extracellular matrix (ECM) synthesis and deposition. Excessive ECM deposition by activated myofibroblasts reduces myocardial elasticity, increasing stiffness that further promotes fibroblast activation, proliferation, and ECM production, collectively impairing cardiac function. However, the molecular mechanisms by which stiffness drives fibrotic processes in cardiac fibroblasts remain unclear. Survivin (BIRC5), a well-characterized cancer biomarker, has been shown to regulate proliferation and ECM production in vascular smooth muscle cells. Here, we aimed to characterize survivin's role in cardiac fibroblasts to advance our understanding of the molecular mechanisms driving cardiac fibrosis. A publicly available RNAseq dataset comparing cardiac fibroblasts from healthy and post-MI mice was analyzed to identify cellular processes involved in post-injury remodeling. Ingenuity pathway analysis predicted mechanisms by which survivin regulates fibrotic processes, revealing its involvement in cell cycle regulation, differentiation, and ECM synthesis. Furthermore, leveraging the Matrisome database, we performed a matrisome analysis that identified significant changes in matrix components and ECM-associated signaling pathways following MI. To test these predictions in vitro, we used polyacrylamide hydrogels to replicate healthy (soft) and fibrotic (stiff) heart microenvironments. Under stiff conditions, survivin was highly upregulated along with markers of cell cycle progression, differentiation, and ECM synthesis. Pharmacological inhibition (YM155, a survivin inhibitor) or siRNA-mediated knockdown of survivin reduced stiffness-induced cell cycle progression, proliferation, differentiation, as well as ECM synthesis and deposition. These results demonstrate that survivin is upregulated in response to both MI and mechanical stiffness and regulates key fibrotic processes, highlighting its potential as a therapeutic target for cardiac fibrosis.

B294/P0285
Mechanistic model of myosin X procession

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Myosin X is an actin-based molecular motor that localizes at filopodia tips. In dimeric form, myo10 is a processive motor that moves not only toward the barbed end of single actin filaments but also on actin filament bundles. The ability to move between filaments in a bundle is unique to myo10. In literature, the step length of myo10 varies greatly. Most of the variability can be explained by differences in myo10 constructs. However, the step size for full-length myo10 on fascin-bundled actin1 and on filopodia2 were also found to vary. We are proposing a computational mechanistic model to investigate the procession of myo10 on single filaments, bundled filaments, and filaments in filopodia. The model includes the ATP hydrolysis cycle of myo10 and geometry of myo10 on single and bundled filaments. 1 Ropars, V., Yang, Z., Isabet, T., Blanc, F., Zhou, K., Lin, T., ... & Houdusse, A. (2016). The myosin X motor is optimized for movement on actin bundles. Nature communications, 7(1), 12456. 2 Sato, O., Jung, H. S., Komatsu, S., Tsukasaki, Y., Watanabe, T. M., Homma, K., & Ikebe, M. (2017). Activated full-length myosin-X moves processively on filopodia with large steps toward diverse two-dimensional directions. Scientific reports, 7(1), 44237.

B310/P0301

Mechanosensitive myopathy: FHL1 missense variants impair strain sensing response

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Cells are constantly subject to mechanical forces that challenge their ability to function. This is especially true in muscle cells, where high contractile forces propagate within and between cells. The actin cytoskeleton is the dominant mechanical element in all cells, including muscle. Actin filaments are semiflexible polymers, and as such can bend and stretch, but have limits to physical perturbations. Cells, therefore, have developed mechanisms to protect and reinforce actin filaments under load. Previous in vitro and cellular studies identified a subset of LIM (Lin-11, Isl-1, Mec-3) domain proteins that recognize and stabilize strained actin. There are ~70 LIM domain genes in humans, and several have mutations that cause genetic diseases. The molecular mechanisms of these diseases and any contribution from actin strain sensing, however, remain poorly understood. FHL1 (four and a half LIM domains 1) is specifically expressed in muscle. Clinically, FHL1 missense variants are characterized by progressive muscle weakness, joint contractures, cardiac arrhythmias, hypertrophic cardiomyopathy, and rarely, aortic dilation. We hypothesize these diseases derive from impaired FHL1 mechanosensing. To test this, wild-type FHL1 and pathogenic missense variants in the LIM domains (p.W138S, p.C225R, p.C240W, p.C292Y) were tagged with mScarlet and each construct was independently expressed in mouse embryonic fibroblasts stably expressing Zyxin-EGFP as a positive control. Unlike zyxin, which localizes to focal adhesions and stress fibers, FHL1 is predominantly cytoplasmic. Using our established laser photoablation assay, we measured both FHL1 and zyxin recruitment to stress fiber strain sites. Wild-type FHL1, like zyxin, consistently recognized strained actin. All four missense variants, however, demonstrated impaired strain sensing. Our future goals include testing these variants in myocytes and primary smooth muscle cells using both laser photoablation and cyclic stretch. We also plan to test the FHL1 variants' impact on cell contractility by measuring their traction forces. While the role of mechanosensing proteins in maintaining cytoskeletal integrity in muscle remains poorly defined, our findings demonstrate that disease-causing variants reduce FHL1's ability to recognize strained actin.

B322/P0313

Metastatic progression results in upregulation of cholesterol biosynthesis genes resulting in altered membrane fluidity and tension during metastatic invasion

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Metastatic progression is associated with high tumor cell plasticity, enabling malignant cells to evade the immune system, disseminate to distal sites, and resist chemotherapy. One of the most significant adaptations during oncogenesis is in lipid metabolism, which consequently results in abnormal lipid composition within the cell, altering membrane biophysical properties, lipid raft signaling domains and increased storage of energy rich lipid droplets. Here, we sought to determine how genes regulating lipids evolve during metastatic progression, to alter their biophysical properties and promote metastasis. Focusing on cholesterol, as it is known to strongly affect membrane biophysical properties and cellular signaling domains, we utilized public transcriptomic databases of melanoma, breast and glioblastoma tumors, identifying genes controlling cholesterol biosynthesis were frequently upregulated during disease progression. To determine if this results in increased cholesterol in different membrane populations, we exploited fluorescence-lifetime imaging microscopy (FLIM), and phasor plot analysis, paired with laurdan, a membrane dye highly sensitive to lipid order in the blue emission, and cholesterol content in the green emission. Utilizing benign and malignant cell lines representing melanoma, breast cancer and glioblastoma, analysis of laurdan lifetime in the blue emission, revealed malignant cells were significantly more fluid and disordered, largely due to the plasma membrane (PM) lipid composition, compared to benign cells. Analysis of the green emission, an indicator of cholesterol content, showed increased total cholesterol in malignant cancer lines, with the largest concentration present in the PM, and a reduced amount in the IM structures. To determine if these alterations in lipid composition perturbed membrane biophysical properties, we utilized FLIM analysis of the mechanosensitive probe Flipper, determining malignant cells with increased cholesterol also correlated with an increase in local lipid packing and membrane tension. Additionally, utilizing 3d cellular confinement assays to model metastatic dissemination, we find malignant cells with high PM fluidity display increased membrane protrusions during confined migration. Together these data indicate that metastatic progression results in increased cholesterol biosynthesis, resulting in high cholesterol in the PM, altering membrane fluidity, lipid packing and tension and membrane protrusions. Additional work utilizing lipidomic analysis will further elucidate the link between altered membrane lipid composition and metastatic dissemination, potentially elucidating a new avenue to identify and target metastatic cells.

B296/P0287

Micro- and nanoplastics mechanically disrupt actin-dependent mucociliary epithelial development

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Micro- and nanoplastics (MNPs) are pervasive in air and water. Studies have detected MNPs in the placenta, amniotic fluid, and meconium, indicating fetal exposure during gestation. These findings suggest that MNPs may be a risk factor for developmental health, but the extent and mechanisms of their effects remain unclear. Previous work suggests that MNPs can interact with actin; hence tissues whose morphogenesis depends on actin dynamics, such as mucociliary epithelia, provide a relevant model for determining the developmental impacts of MNPs. To study this, we used the embryonic epidermis of Xenopus laevis as a mucociliary model

relevant to the human trachea, as multiciliated cell (MCC) development is conserved. Mucociliary epithelia form by specification of MCC precursors in the inner epithelium, followed by actin-driven radial intercalation and apical emergence into the outer epithelium, and actin-regulated basal-body organization and ciliogenesis. We hypothesized that MNPs disrupt these actin-dependent processes and impair mucociliary epithelial development. Firstly, embryos were exposed to 25 nm polystyrene NPs (PS NPs) during MCC radial intercalation and analyzed after apical emergence and ciliogenesis. Mucociliary function was assessed by an embryo gliding assay. Compared with controls, PS NP-exposed embryos showed a dose-dependent reduction in gliding speed, indicating impaired mucociliary function. To determine how PS NPs produce this functional impairment, we performed confocal imaging of immunostained ciliary axonemes and F-actin. In treated embryos, MCCs still apically emerged and cilia density was comparable to controls, but cilia were shorter and beat more slowly. Notably, the Notch-dependent even spacing between MCCs seen in controls was disrupted after PS NP treatment: MCCs formed clusters whose frequency and size increased in a dose-dependent manner (up to 25 cells/cluster). PS NPs frequently colocalized with cortical actin, suggesting PS NPs directly interact with actin. PS NP-treated embryos also displayed cracks in the outer epithelium, suggesting altered tissue-wide mechanical properties. These data suggest that MNPs interact with actin to mechanically disrupt mucociliary epithelial development. As actin dynamics underlie many morphogenetic processes, these findings raise concern for broader developmental impacts and warrant further study.

B288/P0279

Nanorobot microrheology in vivo: spatiotemporal mapping of the mesenchymal tissue viscoelasticity in development

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The morphology of tissues and organs, whether normal or diseased, is shaped by cell phenotype changes (e.g., epithelial or mesenchymal) that drive tissue mechanics. One central phenotypic change is the epithelial-to-mesenchymal transition (EMT) that not only controls cell behaviours and fate, but also alters tissue mechanical properties. In particular, highly motile mesenchymal cells create fluid-like mesenchymal tissues whose mechanical properties are poorly understood due to their high deformability, limited accessibility of deep regions, and heterogeneity. Here, we propose a nanorobotic approach to measure tissue viscoelasticity in situ and in vivo. Magnetic nanorobots, engineered nanoparticles with precise motion control in programmable fields, are actuated to perform out-of-equilibrium rotational dynamics. Using an in vivo self-assembly strategy, we deployed nanorobots in the presomitic mesoderm (PSM) of the chicken embryo, a region of active mesenchymal cell migration. We combined energy-based theoretical modelling with finite element simulations, providing a comprehensive framework for analysing nanorobot dynamics in diverse tissue environments. We generated a spatiotemporal viscoelasticity map of the tail including the PSM and neuromesodermal progenitor (NMP) region during body axis formation. Gradients of the viscosity and shear modulus along the anterior-posterior and medial-lateral axes were established. The temporal changes in the paraxial mesoderm were tracked as the PSM cells migrate. We are currently investigating the tissue structural basis of the mechanical properties, such as extracellular matrix (ECM) expression patterns (e.g., fibrillin, fibronectin, and hyaluronic acid). We anticipate the nanorobotic approach to be a paradigm for probing spatiotemporal tissue mechanics in vivo and to address broader questions in mechanobiological research.

B304/P0295
Nuclear mechanosensing as a driver of organ fibrosis
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Fibrosis, the pathological stiffening of tissue, is driven by both mechanical and biochemical signaling pathways. We find that SUN2, an inner nuclear membrane component of Linker of Nucleoskeleton and Cytoskeleton (LINC) complexes, is up-regulated in fibrotic conditions in patients and in mouse models of fibrosis, particularly in fibroblasts. SUN2 protein levels also increase in fibroblasts plated on substrates that mimic fibrotic conditions. Genetic ablation of Sun2 leads to protection against bleomycin injury-induced lung and skin fibrotic responses in mice. While loss of Sun2 does not impact tissue injury, activation of the TGFb pathway, or the emergence of myofibroblasts in the lung, it prevents new collagen synthesis in both organs. Based on additional insights from genomics, we suggest that tension on SUN2-containing LINC complexes initiated at cell-matrix adhesions primes fibroblasts to respond to pro-fibrotic biochemical signaling, providing a new node through which to intervene in fibrotic disease progression.

B313/P0304
Small Accelerations Generate Sufficient Nuclear Motion to Modulate Cellular Response Independent of Matrix Strain
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Background: Mechanical signals are essential for cellular adaptation. At the cellular level, mechanosensory complexes convert physical cues into transcriptional responses, altering cytoskeletal and nuclear architecture. While large nuclear deformations have been implicated in sensing the mechanical environment, signals that do not generate large deformations, such as sound and vibration, are ubiquitous. Nuclear positioning is critical for hearing, and LINC complexes are required for sensing dynamic vibrations. This suggests the nucleus enables sensation of dynamic signals through yet-to-be-determined mechanisms. Here, we explore how small motions of the nucleus, through its LINC-mediated connections to the cytoskeleton, enable sensing of dynamic accelerations, independent of substrate deformation. We tested whether small accelerations (<1g) cause nuclear motion relative to the membrane in mesenchymal stem cells (MSCs), as a function of actin connectivity by disabling LINC complexes. We further assessed LIV-induced perinuclear actin remodeling, nuclear mechanics, and associated gene expression changes. Methods: A piezoelectric LIV platform integrated with a Zeiss LSM 900 microscope applied a 0.7g, 90 Hz oscillation. Nuclear and membrane motions were recorded at 1000 fps. LINC connectivity was disrupted via doxycycline-inducible mCherry-KASH. F-actin organization was quantified using deep-learning-based reconstruction. MSCs were exposed to six 20-min LIV bouts; RNA-seq was performed 3 h later (n=3/group). DESeq2 and DAVID identified differentially expressed genes and pathways. Finite element (FE) simulations estimated intra-nuclear stresses under LIV. Results: Nuclei moved ±1.27 µm (17% of displacement) out of phase with the cell body, with a phase lag of 0.2 ± 0.11 radians ($p<0.0001$). Disabling LINC doubled this lag. Phase lag correlated with acceleration magnitude but not frequency. LIV increased apical fibers by 24% and basal by 43%. Apical branching nearly doubled (+88%, $p<0.001$), while fiber cross-section and volume rose by ~20%. Nuclei flattened (−7% height, +7% width). RNA-seq identified 372 genes (218 up, 154 down), enriched in actin assembly, focal adhesion, and cilium organization, with regulators such as Fyn, Pak3, integrin β3, and Zyxin. FE modeling predicted 30% greater F-

actin force for deformation, raising intra-nuclear stress by 18%. Incorporating nuclear stiffening increased predicted stress by 84%. Conclusion: Substrate-independent accelerations induce nuclear motion in a LINC-dependent manner, reorganizing perinuclear F-actin and increasing nuclear stresses. These “inside-out” forces regulate transcriptional machinery, as shown by remodeling and gene expression changes.

B283/P0274

The role of cellular confinement and lipid droplets in hepatocyte dysfunction in metabolic dysfunction-associated steatotic liver disease.

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Metabolic dysfunction-associated steatotic liver disease (MASLD) is defined as hepatic steatosis in the presence of metabolic disorders like diabetes and obesity. The presence of large lipid droplets in hepatocytes (steatosis) is characteristic of this condition. MASLD affects one third of the world’s population and can lead to fibrosis, including so-called chicken-wire fibrosis, where single or small groups of hepatocytes are enclosed by fibrotic tissue. Additionally, MASLD may lead to inflammation, cirrhosis, and hepatocellular carcinoma. Previous work from our group has shown that lipid droplets serve as internal mechanical stressors resulting in nuclear deformation, hepatocyte dedifferentiation, and DNA damage. We hypothesized that external forces, such as from chicken-wire fibrosis, combine with internal forces from lipid droplets to cause increased cellular dysfunction and DNA damage. We lipid load primary hepatocytes and then confine them using a microfluidic confinement device. This system serves to simulate the crowded and confined microenvironment that hepatocytes are exposed to in a MASLD-affected liver. In ongoing work, we are quantitatively examining the nuclear and cell morphology of cells with and without lipid loading under varying degrees of confinement and are assessing disruptions in metabolic and other functions of hepatocytes. These studies will give insight into the cellular stresses and changes that hepatocytes are exposed to that may drive MASLD progression.

B297/P0288

Therapeutic target discovery in liver fibrosis using transcriptomics and organ-on-chip models

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Liver disorders like fibrosis and metabolic-associated steatohepatitis (MASH) continue to be major worldwide health concerns. In addition to the possibility of developing cirrhosis and hepatocellular carcinoma, patients must contend with the absence of effective treatments that can stop or reverse the disease. In order to fill this gap, we integrated pharmacological knowledge bases with publicly accessible transcriptomic datasets from human liver samples at various stages of fibrosis (F0–F4) in order to find potential therapeutic targets. Our investigations uncovered genes and pathways essential to mechanotransduction, cell-death signaling, and cytoskeletal remodeling—processes closely associated with tissue rigidity and the advancement of disease. Drug repositioning is made possible by computational prioritization, which highlights targets with translational potential and druggability, some of which overlap with pathways already modulated by current medications. We established a liver-on-a-chip microfluidic platform that replicates the mechanical and architectural

microenvironment features of liver tissue in order to surpass in-silico predictions. This system reduces the gap between molecular signatures and functional validation in a physiologically relevant context by allowing the controlled co-culture of hepatic and stromal cells under controlled conditions. Our method provides a pipeline that is both clinically significant and scientifically sound by combining organ-on-chip technologies with the power of open omics data. It offers a way to speed up therapeutic discovery, increase the likelihood that current medications can be repurposed, and eventually bring innovative solutions closer to patients with chronic liver disease.

B315/P0306

TMC4/5 regulate epithelial proliferation and gut length through microvillar signaling

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The transmembrane channel-like (TMC) family includes eight conserved membrane proteins, two of which - TMC1 and TMC2 - localize to the tips of actin-based stereocilia and, in complex with calcium- and integrin-binding proteins (CIBs), form the mechanotransduction (MET) channel in auditory hair cells. We recently found that TMC4 and TMC5 similarly localize to the tips of actin-based microvilli in intestinal epithelial cells. Structural modeling predicts that TMC4/5 may function as ion channels or lipid scramblases, potentially interacting with CIB proteins as TMC1/2 do in stereocilia. However, their physiological roles remain unknown. To address this knowledge gap, we generated TMC4 and TMC5 double-knockout (dKO) mice, as single knockouts showed no overt phenotype, likely due to mutual compensation. Strikingly, dKO mice exhibited dramatic elongation of the small intestine, accompanied by deeper crypts and increased Ki67-positive proliferative cells, indicating elevated epithelial turnover. These findings indicate that TMC4 and TMC5 normally act to constrain epithelial growth programs in the small intestine. Consistent with this in vivo phenotype, organoids derived from dKO intestinal crypts exhibited more frequent and complex budding compared to controls, supporting a cell-intrinsic role for TMC4/5 in regulating epithelial proliferation and architecture. These findings identify TMC4 and TMC5 as novel regulators of intestinal growth, opening a new area of investigation into how microvillar TMC complexes integrate signaling and membrane dynamics to regulate epithelial turnover. Given that TMC5 is upregulated in several epithelial cancers and linked to proliferation and therapy resistance, this model sets the stage for mechanistic studies that could illuminate pathways relevant to both normal intestinal physiology and disease.

B293/P0284

TMC5 Complex at the Spermatid Plasma Membrane is Essential for Spermiogenesis

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Objective: Spermiogenesis is the process in which haploid round spermatids differentiate into mature sperm through nuclear condensation, flagellum formation, and cytoplasmic elimination, yet the molecular mechanisms driving these events are poorly understood. Here we identify TMC5, a previously uncharacterized member of the transmembrane channel-like (TMC) family, as essential for spermatid maturation. Methods: We used Tmc5 knockout (KO) and Tmc5-mCherry knock-in (KI) mouse models combined with immunofluorescence, co-immunoprecipitation, mass spectrometry, and single-cell RNA sequencing to define

the expression, localization, and interaction partners of TMC5 in spermatids. Results: Tmc5 KO males were infertile, exhibiting non-obstructive azoospermia due to arrested spermiogenesis but showing no other systemic defects. KO spermatids displayed profound morphological disruptions, including impaired cytoplasmic reduction, disorganized mitochondria, and defective nuclear condensation, leading to failed elongation and spermatid death. In KI mice, TMC5 localized to the plasma membrane of early to late spermatids, suggesting a role in membrane-associated processes. Co-immunoprecipitation and mass spectrometry proteomic analysis identified TMC7, CIB1, and CIB4 as top co-purified proteins, each previously implicated in spermatid maturation, and whose knockout causes the same male infertility phenotype observed in Tmc5 KO mice. Immunofluorescence confirmed that CIB1 co-localized with the plasma membrane in wild-type spermatids, but this localization was lost in Tmc5 KO mice. Single-cell RNA-seq further showed that Tmc7 and Cib4 transcript levels remained unchanged, supporting the model that TMC5, TMC7, CIB1, and CIB4 act as core components of a spermatid membrane complex. Conclusion: These results demonstrate that TMC5 is an essential regulator of spermatid morphogenesis and establish the TMC5 complex as a previously unrecognized molecular hub for spermiogenesis. Although its precise mechanism is not yet fully defined, TMC5 shares structural similarity with calcium-conducting TMC1, and our preliminary data suggest it may function as a calcium-conducting channel responsive to osmotic changes. Importantly, TMC5 is part of the NIH-designated Druggable Proteome, a subset of proteins amenable to small-molecule modulation of which only 5–10% are currently targeted by FDA-approved drugs. Thus, our findings provide new mechanistic insight into sperm development and identify TMC5 as both a fundamental biological regulator and a pharmacologically relevant membrane protein.

B306/P0297

Tricellular Zipping: A vertex remodeling process driven by pulsatile calcium signaling in Xenopus embryos

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Epithelial cells form adhesive connections through cell-cell junctions, maintaining a selective barrier despite significant shape changes during development and tissue homeostasis. This is accomplished through the active remodeling of cell-cell junctions, a process largely driven by actomyosin contractility. Here, using live microscopy of the Xenopus laevis embryonic epithelium, we have characterized “tricellular zipping”, a vertex remodeling process that contributes to the transition from irregular epithelial geometries at blastula stage to the more regular hexagonal packing that emerges at the gastrula stage of development. Tricellular zipping involves the resolution of a long, thin cellular extension that stretches toward a vertex where four or more cells meet. As two cells 'zip' together to lengthen a new bicellular interface, the other cell recedes, thus forming a new tricellular vertex at the end of zipping. Our work reveals that tricellular zipping is accompanied by transient flashes of cytoplasmic calcium within the long cell extension. Each zipping event is accompanied by multiple calcium flashes and an increase in the length of the new bicellular interface between the zipping cells. Preliminary studies show that calcium flashes are associated with increase in the rate of elongation of the new bicellular interface. Both actin and Myosin II accumulate during tricellular zipping. Preliminary data indicate a correlation between calcium and actin pulses in the long cell extension. Ongoing work is focused on further characterizing the cross correlation between calcium and actin pulses and how these pulses may associate with changes in the rate of elongation of the new bicellular interface. We hypothesize that the long cellular extensions are under increased membrane tension, which might activate mechanosensitive calcium channels, leading to the observed pulsatile mechanosensitive calcium signaling. To test this idea, we are using FLIPPER-TR to measure membrane tension at the site of zipping. Together, these findings suggest that calcium-associated actomyosin pulses drive tricellular zipping.

B318/P0309

Tricyclic Antidepressants Disrupt Mechanosignaling and Remodel the Tumor Microenvironment to Suppress Pancreatic Cancer Progression

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Pancreatic ductal adenocarcinoma (PDAC) is a highly fibrotic, stiff, painful, and innervated tumor with a high mortality rate. Unfortunately, antifibrotic treatments have failed clinically, emphasizing the need to identify alternative therapies that target the tumor microenvironment and its mechanobiological drivers. Our prior studies demonstrated that tricyclic antidepressants (TCAs) ameliorate hepatic fibrosis by attenuating acid ceramidase activity (aCMA) in stellate cells. Accordingly, we investigated whether TCAs could modulate PDAC progression, mechanosignaling, and therapy response. Using the ERCHIVES cohort of 754,670 veterans, patient biopsies, and genetically engineered mouse models, we evaluated the effects of TCAs on extracellular matrix (ECM) remodeling, tissue stiffness, mechanosignaling, tumor burden, and metastasis. In ERCHIVES, PDAC patients receiving TCAs had significantly improved overall survival. Patient tumor tissues showed reduced collagen deposition, along with reduced innervation and tumor grade. In KRas/p53-driven GEMMs of PDAC, TCAs suppressed aCMA activity, ECM remodeling, and tissue tension. This was associated with decreased fibrosis, reduced FAK activation, and modulation of immune cell infiltration. Functionally, TCA treatment reduced primary tumor burden, innervation, and liver and lung metastases, resulting in prolonged survival. RNA sequencing revealed enrichment of immune and neural signaling pathways consistent with reduced sensory nerve density. Pharmacologic inhibition of aCMA in GEMMs confirmed its role in ECM mechanoregulation and antidepressant response. Together, these findings demonstrate that TCAs reprogram the PDAC microenvironment by targeting aCMA-driven ECM remodeling and mechanosignaling. By reducing fibrosis, stiffness, fibronectin, and FAK activation, while limiting innervation and enhancing immune surveillance, TCAs could act as mechanotherapeutic agents that suppress PDAC progression and improve outcomes. This research is supported by; 1U01CA300847-01, R35CA242447, and the National Foundation for Cancer Research.

B301/P0292

Using autoencoder-based machine learning to investigate focal adhesion signaling state

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Focal adhesions (FAs) are dynamic macromolecular complexes that link the extracellular matrix to the cytoskeleton. FAs act as mechanical sensors that relay information about the extracellular environment into the cell, initiating intracellular signaling cascades that control processes like migration, proliferation, and gene expression. In addition to outside-in signaling, cytoskeletal tension, myosin activity, and subcellular signals regulate FA morphology and dynamics. Decades of research have identified key biochemical and morphological features that define FAs, such as FA size, composition, and dynamics. However, a comprehensive landscape of adhesions has remained elusive due to their complex and dynamic composition and behavior. Here, we first generated large immunostaining datasets to visualize FA proteins in fibroblasts. In particular, we focused on

paxillin as a key FA protein that marked all stages of FA lifecycle and turnover, along with its phosphorylated form that tunes paxillin activity as a readout of signaling states of FAs. Next, we developed an autoencoder-based machine learning framework to extract latent features that define focal adhesions. This approach enables us to identify interpretable, data-driven representations of FA morphology and signaling activity. Using this framework, we aim to generate a comprehensive latent landscape of focal adhesions that integrates features of protein composition, morphology, and signaling state.

Biophysics, Structural Biology, and Mechanobiology - Membrane Biophysics

B369/P0358
Mapping the Local Membrane Environment at Structures that Sense Plasma Membrane Tension
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The plasma membrane uses a variety of strategies to sense and respond to tension and collective properties of membrane components are hypothesized to play important roles. For example, caveolae protect against tension by flattening to release a reservoir of membrane. Similarly, the curved membrane dome and footprint of the mechanosensitive ion channel Piezo1 are thought to contribute to its gating. In this study, we use Exchange-PAINT microscopy to directly interrogate the local lipid composition of these structures in chemically fixed cells. Exchange-PAINT is a super-resolution microscopy technique that enables the localization of multiple targets in single cells with <10nm localization precision, allowing for direct comparisons in the sorting of membrane components within single cells. We found that an extracellular GPI-linked fluorescent protein was enriched in caveolae, at least in part due to the excess surface area of the 3D membrane. Meanwhile, inner-leaflet lipid probes and transmembrane proteins were excluded, supporting the hypothesis that Cav1 disks displace lipids within these structures. Using the same approach, we found that Piezo1 also displaces lipids, but locally enriches markers of lipid disorder relative to an order-preferring probe in both the resting and Yoda1 stabilized open state. Ongoing work is aimed at further characterizing the lipid environment in caveolae and in the membrane surrounding Piezo1.

B371/P0360
Native-State Structures of Lysosomal Membrane Proteins by Cryo-Electron Tomography
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Lysosomes are central to cellular homeostasis, and their dysfunction is linked to major diseases. While proteomics has mapped their composition, the structures of lysosomal membrane proteins in their native context remain poorly defined. The goal of this study was to purify lysosomes and determine the structures of their membrane proteins in the intact organelle. To achieve this, we developed a cryo-electron tomography workflow to visualize and resolve protein complexes directly in lysosomal membranes. Endolysosomes were isolated by independently targeting two membrane proteins, transient receptor potential mucolipin 1 (TRPML1) and transmembrane protein 192 (TMEM192), yielding organelles that exhibited the expected morphology and proteomic composition of the endolysosomal system. Using subtomogram averaging, we obtained refined structures of V-ATPase, Flotillin, and Clathrin, within native lysosomal membranes. These reconstructions revealed heterogeneous spatial distributions of protein complexes across different

endolysosomal organelles, providing new insights into lysosomal organization at the molecular level. In conclusion, by integrating proteomics with cryo-electron tomography, this workflow establishes a robust platform for studying lysosomal membrane protein architecture and function in health and disease, opening opportunities for new discoveries into lysosomal biology.

B370/P0359

nSMase2 activity modulates membrane fluidity and drives directional migration in neural crest cells

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During embryonic development, a transient group of stem cells known as the neural crest (NC) delaminate from the neural tube and migrate extensively before differentiating into diverse structures that include cranial neurons, glia, and connective tissues. We recently found this process requires NC cells to reprogram their lipid metabolism, which enables them to undergo an epithelial-to-mesenchymal transition (EMT) and thereby adopt their appropriate migratory phenotype. Expression of nSMase2, an enzyme that hydrolyzes plasma membrane sphingomyelin into ceramide, is enriched in NC cells at the onset of EMT and is necessary for this transition and subsequent migration. However, the biophysical effects of these changes in lipid metabolism, and of nSMase2 activity, remain incompletely characterized. Using fluidity-sensitive dyes and live spectral imaging of NC ex vivo, we show that NC cells increase their membrane fluidity when they begin to migrate. Interestingly, while pre-migratory NC cells show similar membrane fluidity with or without functional nSMase2, its knockdown impacted the fluidity increase observed when NC become migratory. This illustrates that nSMase2 function is linked to biophysical regulation of the plasma membrane during developmental cell migration. To better understand the impact of membrane fluidity on migration, we next found that that genetic knockdown or pharmacological inhibition of nSMase2 activity both result in deficits in directional migration but not migratory speed, suggesting that nSMase2 plays a key in role in driving migratory directionality. Our results suggest that ceramide production coordinates migration by changing the biophysical properties of the plasma membrane to enable directionality. These findings provide new insight into how manipulating biophysical properties may offer new opportunities to combat congenital disorders derived from disrupted cell migration.

B368/P0357

Regulation of plasma membrane tension through hydrostatic pressure and actin protrusion forces

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The plasma membrane and its associated proteins form a critical signaling hub, mediating communication between the extracellular environment and the intracellular space. Previous research suggests that both membrane trafficking and signaling activity are influenced by mechanical tension in the plasma membrane. Despite its importance, the mechanisms by which cells regulate membrane tension remain poorly understood. Using the optical tension sensor FliptR and AFM-assisted tether force measurements, we investigate plasma membrane tension regulation in mitotic cells by measuring tension changes following cytoskeletal and cell shape perturbations. Our findings show that in both assays, reported tensions are critically influenced by the cytoskeleton, however, with partially deviating trends highlighting the conceptual differences between bare and apparent membrane tension. By integrating experimental data with theoretical modeling, our results suggest that the actin cytoskeleton regulates bare membrane tension through two distinct mechanisms: (i)

modulation of intracellular hydrostatic pressure and (ii) adjustment of polymerization forces in actin-rich finger-like protrusions.

B367/P0356

Structural Basis of Brain Thyroid Hormone Transport by MCT8 and OATP1C1

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Thyroid hormones, including the precursor thyroxine (T4) and its active metabolite 3,5,3'-triiodothyronine (T3), are essential regulators of development and metabolism in virtually all tissues of the body. Produced by the thyroid gland, these circulating hormones depend on membrane transporters to enter target cells, where they activate thyroid hormone receptors (TRs) to control gene expression. Among these transporters, MCT8 and OATP1C1 are solute carriers (SLCs) of the major facilitator superfamily (MFS) that mediate thyroid hormone transport across the blood-brain barrier (BBB) and into neurons and glial cells. Mutations in MCT8 cause Allan-Herndon-Dudley syndrome (AHDS), an X-linked birth defect characterized by severe neurodevelopmental impairments resulting from cerebral hypothyroidism and elevated serum T3 levels that lead to chronic peripheral thyrotoxicosis. Similarly, OATP1C1 deficiency has been associated with profound brain hypometabolism and progressive neurodegeneration. Despite extensive studies on thyroid hormone transporters, a major gap in the field has been the absence of their structures and the precise molecular mechanisms governing their transport. Moreover, the mechanisms by which these hormones enter peripheral tissues outside the brain are not fully understood. Here, we present cryoelectron microscopy (cryo-EM) structures of human MCT8 and OATP1C1 in complex with T3 and T4 at 2.9 Å and 2.3 Å resolution, respectively. These high-resolution structures, together with functional studies, reveal distinct recognition and transport mechanisms that account for their remarkable substrate specificity for thyroid hormones among all transporters characterized to date. They also provide insight into disease-associated mutations and offer a structural framework for predicting additional thyroid hormone transporters that share similar substrate recognition principles. Such transporters could contribute to peripheral hyperthyroidism observed in AHDS, thereby advancing our understanding of tissue-specific thyroid hormone homeostasis. In addition, we determined cryo-EM structures of OATP1C1 bound to estrone 3-sulfate (E1S) at 2.6 Å resolution and to estrone 3-glucuronide (E1G) at 3.13 Å resolution. These structures unexpectedly reveal an extracellular allosteric regulatory site conserved across the OATP family. We further show that E1G acts as an allosteric ligand that binds exclusively to this site and inhibits OATP1C1 transport activity. Taken together, these findings clarify key aspects of thyroid hormone transport, a process fundamental to development and disease, and open new avenues for designing allosteric modulators targeting OATPs with broader implications for therapeutic modulation of SLC transporters.

B366/P0355

Topology as a Regulatory Code: Antagonistic Membrane Curvature Induced by BCL-2 Family Proteins Regulates Mitochondrial Apoptosis

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Apoptosis, or programmed cell death, is a tightly regulated process that preserves tissue integrity and eliminates damaged cells. Aberrations in apoptotic control underlie numerous human pathologies, including cancer and neurodegeneration. Central to the intrinsic apoptotic pathway is the BCL-2 protein family, which orchestrates mitochondrial outer membrane permeabilization (MOMP). These proteins function as molecular sentinels of cellular stress, integrating signals to govern mitochondrial integrity and the downstream execution of apoptosis. The pro-apoptotic effector BAX translocates to the mitochondrial outer membrane, where it inserts, undergoes major conformational changes, and oligomerizes into putative supramolecular pores—thereby releasing cytochrome c to initiate caspase activation and drive the cell toward apoptosis. The anti-apoptotic protein BCL-XL suppresses apoptosis by inhibiting MOMP and plays a critical role in promoting oncogenic transformation. The regulation of apoptosis the BCL-2 family has conventionally been studied through BH3 domain-mediated protein-protein interactions. However, emerging evidence indicates that BCL-XL can exert anti-apoptotic function independently of direct BH3 engagement, suggesting a complementary mode of regulation. Given the high degree of structural homology between BAX and BCL-XL, a lingering question in the field of cell death is how these homologous proteins manifest strikingly opposing functions and regulate pore formation and repair at the mitochondrial surface. To answer these questions, we developed a novel unsupervised machine learning framework, integrated with structural X-ray scattering data and mean-field theory, to map relationships between the sequence and structure of force-generating motifs and the types of topological curvature and membrane deformation they induce. Using this multiscale framework, we demonstrate that BAX and BCL-XL generate antagonistic Gaussian membrane curvatures, revealing an additional mechanism for regulating transmembrane pore formation and closure. These predictions align with our in vitro observations showing that the inhibition of membrane permeabilization is not exclusive to BCL-2 family proteins, as other curvature-inducing motifs can similarly suppress BAX activity. Together, these findings support a paradigm in which BCL-2 family proteins function not only through direct protein-protein interactions but also via opposing curvature-driven mechanisms. Such membrane-mediated interactions provide a universal, geometry-based layer of regulation that complements sequence-specific binding, underscoring the central role of mitochondrial membrane mechanics in BCL-2 family dynamics.

Biophysics, Structural Biology, and Mechanobiology - Physical Properties of the Cytoplasm

B354/P0343
Beyond Brownian motion: Statistical complexity in cytoplasmic diffusion
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Despite lacking membrane-bound compartments, the cytoplasm of Escherichia coli (E. coli) is not homogeneous. It is shaped by phase separations between chromosomal DNA, actively translated mRNAs, and storage carbohydrates. This spatial heterogeneity is maintained by active processes such as genome-wide transcription and glycogen accumulation, which create cytoplasmic order out of equilibrium. Microrheology is commonly used for studying the physical properties of the cytoplasm and its complexity. The diffusion statistics of tracer particles are confounded by geometric confinement and cytoplasmic flows, but they are also prone to errors associated with the finite sampling of particle positions and their localization accuracy. In our study, we use the μNS particles to quantify cytoplasmic complexity in E. coli. By leveraging the variable particle sizes (~50-150nm), restricting our analysis to long trajectories (>199 positions), examining multiple time scales, and comparing with simulated particle traces, we disentangle the biologically meaningful variability in cytoplasmic diffusion. This variability, which cannot be solely attributed to geometric confinement and methodological

errors, increases during growth in transient environments. Currently we are studying the physiological relevance of this tracer particle diffusion variability, focusing on cellular adaptation and growth.

B358/P0347

Cell wall remodeling maintains cell size and cytoplasmic fluidity during excessive cell growth in S. cerevisiae

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The interior of a cell is a densely crowded environment packed with macromolecules, playing essential roles in processes such as protein synthesis and phase separation. These biophysical properties can drastically change under various perturbations that disrupt intracellular processes. Recent work has shown that cytoplasmic dilution caused by excessive growth leads to cellular dysfunction and potentially alter cell fate. However, the molecular mechanisms regulating biophysical properties during excessive growth remain poorly understood. In this study, we demonstrate that cell wall integrity (CWI) pathway is crucial for maintaining cytoplasmic fluidity during excessive growth in budding yeast Saccharomyces cerevisiae. To probe cytoplasmic fluidity, we employed genetically encoded multimeric nanoparticles (GEMs), which self-assemble into bright, ~40 nm spherical particles. Using an analog-sensitive allele of cyclin-dependent kinase, cdc28-as1, we reversibly arrested the cell cycle in the presence of an ATP analog to induce excessive growth. Consistent with previous reports, increased GEM mobility revealed that cytoplasm becomes diluted in enlarged cells following cell cycle arrest. Strikingly, deletion of MPK1, a mitogen-activated protein (MAP) kinase in CWI pathway, led to even greater cytoplasmic dilution in the cdc28-as1 cells, suggesting that Mpk1 regulates cytoplasmic fluidity in enlarged cells. Moreover, the mpk1Δ cdc28-as1 strain exhibited a higher cell expansion rate than cdc28-as1 cells after arrest. Cell expansion is generally driven by turgor pressure pushing outward against the rigid cell wall. Thus, we hypothesize that the accelerated growth reflects reduced wall stiffness and integrity, which weakens the ability to counteract turgor pressure. Supporting this idea, mpk1Δ cdc28-as1 cells were more susceptible to cell wall stress induced by sonication, resulting in higher levels of cell death. In addition, MPK1 deletion reduced the proliferative potential of cdc28-as1 cells after release from arrest. Similar results were observed in other CWI pathway mutants, including wsc1Δ and bck1Δ in cdc28-as1 background, both of which enhanced GEM mobility and impaired cell proliferation. Collectively, these findings demonstrate that cell wall remodeling via the CWI pathway is crucial for sustaining cell growth, cytoplasmic fluidity, and cell viability during excessive growth. We propose a model that cell wall tension increases with cell size, activating CWI pathway via mechanosensors such as WSC1 to enable cells to adapt to excessive growth.

B347/P0336

Computational Modeling Reveals Active Cytoskeletal Flow-Driven Cytoplasmic Mixing in Giant Amoeba Chaos carolinensis

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Cytoplasmic mixing in extremely large cells such as the giant amoeba Chaos carolinensis cannot be explained by molecular diffusion alone. Fluorescent tracer experiments have shown that rapid intracellular transport relies on coordinated internal flows and dynamic alternation between fluid “sol” and gel-like cortical phases [1]. To investigate these processes, we developed an in-silico model of C. carolinensis that integrates an elastic cortex, a viscous cytoplasmic continuum, and embedded tracer particles using the Immersed Boundary

method. The model was constrained by experimental imaging of cell shape and movement, and incorporated sol–gel phase transitions governed by experimentally measured cytoskeletal dynamics. The simulations recapitulated experimental observations: tracer mixing matched both the time scales and spatial extent measured by a nearest-neighbor mixing index. When active flows were removed in silico, mimicking cytoskeletal disruption, mixing slowed dramatically and shifted from advective transport toward a more diffusive regime. These results underscore the critical role of active cytoskeletal flows and sol–gel transitions in overcoming diffusion limits to ensure efficient intracellular transport in giant cells. More broadly, they demonstrate how computational modeling can connect cytoskeletal dynamics and whole-cell behavior, offering general insights into the physical principles that drive cellular dynamics across scales. The Mid-Cell Cytoplasmic Solution to Gel State Transition Drives Cytoplasmic Mixing of Tracer Beads and Organelles in the Giant Amoebozoa Chaos carolinensis, PhD Thesis, Ulises Diaz, University of California San Francisco (2024).

B346/P0335
Controlled intercellular transport via Tunneling Nanotubes
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Cell-to-cell communication is essential for coordinating cellular responses and occurs between both identical and distinct cell types. Among the known intercellular communication routes, tunneling nanotubes (TNTs) are thin, F-actin–based membranous bridges that mediate the direct transfer of proteins, nucleic acids, and organelles. TNTs have been reported in diverse cell types, including cancer, stem, and immune cells, and are emerging as promising conduits for selective drug delivery. Here, we employed an optogenetically engineered motor system (OptoMotor) to actively drive cargo transport through TNTs. Incorporating CRY2, a light-activated protein, allows regulation of motor activity and precise control of intercellular cargo movement. Using this system, we successfully delivered mRNA via TNTs, demonstrating that nucleic acids can be selectively transferred between cells. Building on this framework, we are developing a photocleavage protein–based strategy for light-triggered cargo release after TNT-mediated transport. This integrated approach of targeted transport and on-demand release positions TNTs as powerful platforms for controllable, selective drug delivery.

B348/P0337
Cytoplasm organization and stress response in a giant syncytial cell of the human placenta
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The human placenta serves the role of many organs in one during fetal development including endocrine, metabolic, immune and barrier roles. Critical to all these functions is the syncytiotrophoblast (STB), a giant multinucleated cell that forms the outer layer of chorionic villi in the human placenta, arising through the fusion of mononucleate cytotrophoblasts (CTBs). Throughout pregnancy, the STB operates within a highly dynamic microenvironment, continuously experiencing varied physiological stresses including hypoxia and variable blood flow rates while buffering maternal and fetal needs. How the cytoplasm of this cell is organized to support both the myriad of functions and fluctuating conditions encountered in gestation remains poorly understood. Moreover, the disruption of the STB function is central to pathological states like preeclampsia (PE) but the cell biological basis of placental disease is mysterious. Biomolecular condensates such as stress granules and P bodies, are highly dynamic in both structure and function, allowing cells to rapidly respond to intracellular stress. We hypothesize that these organelles may fulfill distinct roles in stress response in the

fluctuating conditions experienced by the placenta and shaping the cytoplasmic organization of STBs. Using trophoblast organoids, we show that progenitor CTBs harbor abundant stress granules and P-bodies, whereas surprisingly STBs lack these assemblies and instead display markedly elevated levels of protein translation compared to CTBs. This suggests that CTBs may store RNAs and translational components within condensates, which are mobilized after cell-cell fusion to support the high protein translational demand of STB. Strikingly, STBs adopt a distinct translation regulatory mechanism involving concurrent activation of mTOR signaling and autophagy, potentially enabling them to sustain robust protein synthesis while evading apoptosis under stress. In PE, this balance is disrupted, with disorganized membraneless organelles in both CTBs and STB: P bodies are markedly reduced in CTBs, while stress granules are increased in STB, potentially impairing STB function. These findings highlight fundamental differences in cytoplasm organization between different types of trophoblasts within the placenta, uncover a previously unappreciated mechanism of placental stress response, and provide new insight into the molecular pathology of PE.

B351/P0340

Dynamics of Cytoplasmic Properties in Yeast Cells and Fly Brains

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The cytoplasm forms a crowded, viscoelastic environment whose changing physical state sets the pace of biochemical reactions, guides intracellular transport and phase behavior, and determines mechanical responses. Cytoplasmic properties such as diffusion and density are not constant but may vary between cell types and in various physiological states, including stress or cell division. How do signaling pathways and developmental programs trigger global changes in the physical properties of the cytoplasm? In the budding yeast Saccharomyces Cerevisiae, the pheromone α-factor prepares cells for mating by inducing G1 cell-cycle arrest, altering gene expression, and polarizing the cell. To quantify intracellular rheology during this transition, we expressed cytoplasmic Genetically Encoded Multimeric Nanoparticles (GEMs) and tracked their motion. Remarkably, α-factor elicited a transient cytoplasmic fluidization, with a 40% increase in diffusion that peaked at 60 minutes and then returned to baseline by 120 minutes. Cytoplasmic fluidization occurred before G1 cell cycle arrest and was not evident in G1-arrested cdc28 mutants, indicating that fluidization is independent of arrest. Moreover, mean cellular volume and intracellular density remained constant after α-factor treatment, ruling out osmotic swelling underpinning fluidization. Strikingly, α-factor induced fluidization was blocked by cycloheximide treatment, which inhibits polysome remodeling. This suggests that as with stress responses1 the pheromone signaling pathway may transiently reduce effective cytoplasmic crowding by triggering polysome collapse. We speculate that this transient translational response may allow effective reprogramming of the proteome as cells differentiate for mating. Exploring these principles in a multicellular context, we next probed the cytoplasm of adult Drosophila brains expressing GEMs in diverse cell populations. Explant imaging revealed pronounced 2-fold differences in diffusion between different neuronal and glial cell types. Intriguingly, aging had profound effects on the cytoplasm: aging brain neurons showed a 30% increase in diffusion, while aging cortical glia showed a 50% decrease. In future experiments, we aim to determine how neuronal activation, extracellular signals, and neurodegeneration impact the rheology of neurons and glia. Together, our studies in yeast and flies highlight how dynamic changes in cytoplasmic states governed by extracellular factors, signal transduction pathways, and developmental programs affect global cellular properties across biological scales. Xie, Y., et al. (2024). Mol Cell, 84(14), 2698-2716.e9

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Hag accumulation in the Bacillus subtilis cytoplasm slows Mesoscale Diffusion and limits Z-ring Condensation

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Metabolically active bacterial cells have limits on macromolecule concentrations in the cytoplasm to avoid excessive crowding and impacts on diffusion. However, there is still little direct evidence to support the functional influence of cytoplasm homeostasis on large scale processes such as cell division. Oshiro et al., 2019 (PMID: 31113895) show that accumulation of the flagellin protein (hag) in the cytoplasm of Bacillus subtilis by multiple orders of magnitude results in the misplacement of cell division away from the midcell position, rescued by hag deletion. Here, this system is utilized to show that intracellular accumulation of hag is a major contributor to asymmetric cell division using single-particle tracking of genetically encoded multimeric nanoparticles (GEMs) in live cells. In the hag accumulation mutant (++hag), diffusion of 20 nm GEMs is slowed, with significant rescue of mesoscale diffusion upon hag deletion. Hag was tagged with mNeonGreen (mNG) to show that it does not form localized aggregates in the cytoplasm, indicating that the cell experiences relatively homogeneous crowding throughout the cytoplasm. Furthermore, the crowding effect is shown to be non-specific to hag, where replacement of hag with mNG in ++hag also results in disruption of the precision of division site placement at the midcell position. ++hag shows significantly lower fluorescence of FtsZ localized at the Z-ring using super resolution microscopy. Additionally, speeds of FtsZ treadmilling are significantly lower, suggesting a lack of Z-ring condensation is primarily responsible for inefficiencies in cell division. Current work is exploring the observed positive correlation between cell size and the average GEM diffusion coefficient, which is lost in the hag accumulation mutant. Experiments using GEMs in a cell division incompetent strain which forms long multi-nucleoid filaments are underway. Preliminary results show that GEM diffusion in filaments remains similar to what is seen in divided cells. Since cytoplasm contents remain equivalent in each single cell sized section of the filament, this hints at a correlation between cell cycle and cytoplasm fluidity, where larger cells that tend to be closer to division have more fluid cytoplasms. This work reveals that hyper accumulation of a single protein can slow mesoscale diffusion in bacteria, connecting this to the efficiency of cell division and highlighting the importance of the biophysical properties of the cytoplasm to basic cell function.

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Information-Theoretic and Biophysical Perspectives on Chronological Aging in Saccharomyces cerevisiae

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Aging is a major biological challenge because interventions that slow the aging process have the potential to delay numerous age-associated diseases. Traditionally viewed as a damage-accumulation phenomenon, aging has recently been reframed as a problem of information loss within cells. We explored this perspective using Saccharomyces cerevisiae as a model of chronological aging under nutrient limitation. Yeast cultures were grown in media with varying nitrogen concentrations to modulate lifespan, as nitrogen depletion is known to extend survival. To probe the relationship between cellular state and environment, we quantified the mutual information between the environmental condition and expression of the stress marker Hsp12. Mutual information increased during early aging and declined at later time points, suggesting a transient enhancement

followed by loss of environmental awareness. In parallel, we tracked the motion of genetically encoded multimeric nanoparticles (GEMs) to assess macromolecular crowding. Lower nitrogen concentrations produced higher intracellular diffusivity, consistent with reduced crowding. These findings support a model in which nutrient scarcity promotes longevity by reducing cytoplasmic crowding and temporarily preserving information flow between cells and their environment.

B352/P0341

Muscular dystrophy-associated lamin mutations disrupt cytoplasmic macromolecular crowding via a nucleolar-ribosome axis.

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Understanding how cells control their biophysical properties during development remains a fundamental challenge and its regulation in living animals remains poorly understood. Laminopathies, including Emery-Dreifuss muscular dystrophy (EDMD), arise from mutations in nuclear lamins. Most models of how laminopathies lead to pathologies focus on disruptions of nuclear mechanics and/or gene expression. How lamins regulate the biophysical properties of the cytoplasm is largely unexplored. Here, we reveal a novel pathway linking EDMD-associated lamin mutations to broad cellular disorganization through nucleolar and ribosomal dysfunction. We perform in vivo rheology in C. elegans using our recently developed approach with genetically encoded multimeric nanoparticles (GEMs). We previously used GEMs to discover that C. elegans tissues maintain mesoscale properties that differ from those observed across diverse systems, including bacteria, yeast species, and cultured mammalian cells. We identified two conserved mechanisms controlling particle mobility: ribosome concentration, a known regulator of cytoplasmic crowding, works in concert with a previously unknown function for the giant KASH protein ANC-1 in providing structural constraints through associating with the endoplasmic reticulum. Here, we demonstrated that EDMD-associated mutations significantly disrupt cytoplasmic biophysical properties, reducing mesoscale macromolecular crowding and increasing the diffusivity of cytoplasmic particles in C. elegans models of EDMD. These changes coincide with severe nuclear positioning defects and collapsed endoplasmic reticulum architecture, phenocopying the effects of ribosome depletion. We establish that the C. elegans lamin lmn-1 and ribosomes genetically interact to regulate cellular organization. Crucially, the lmn-1(R64P) EDMD model disrupts nucleolar density and reduces ribosome levels, placing lamin dysfunction upstream of ribosome biogenesis. Together, these findings identify a nucleolar-ribosomal axis through which nuclear lamina defects propagate through biophysical defects of the cytoplasm that in turn disrupt cellular architecture. Thus, we establish a new mechanistic framework for understanding EDMD and other laminopathies, suggesting potential therapeutic approaches targeting ribosome biogenesis or cytoplasmic crowding.

B357/P0346

Nanorheology reveals biphasic cytoplasmic remodeling in aging animals

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Aging involves progressive loss of proteostasis and disabled autophagy, yet the causal relationships between cytoplasmic biophysical properties and cellular aging remain unexplored. We quantified how cytoplasmic biophysical properties evolve during C. elegans aging using genetically-encoded multimeric nanoparticle

(GEM)-based passive nanorheology. We discovered an unexpected biphasic switch in aging animals. In young adult intestinal cells, 40 nm GEMs revealed two distinct cytoplasmic populations, a minority freely diffusing at 1.6 x 10^-2 μm^2/s (albeit 10-fold slower than mammalian cells) and a majority with constrained diffusion at 7.9 x 10^-4 μm^2/s. During early aging (days 1-7 post adulthood), cytoplasmic constraints progressively increased and the freely diffusing population disappeared entirely while constrained diffusion slowed 6-fold to 1.3 x 10^-4 μm^2/s. In contrast, past reproductive age (day 7+), this trend reversed. To probe the molecular basis of cytoplasmic constraints, we examined anc-1 mutants lacking the LINC complex protein orthologous to mammalian nesprins. anc-1 null animals showed dramatically altered cytoplasmic dynamics. Freely diffusing populations increased in young adults, gradually normalized by day 7, but then again exceeded wild-type levels during later aging. These biophysical changes correlated with accelerated organismal decline as assayed by shortened lifespan, impaired locomotion, compromised intestinal barrier function, and defective pharyngal architecture. Our findings establish the aging switch as a fundamental principle of cellular aging where initial cytoplasmic constraint increases (during days 1-7) protect against age-related defects, while subsequent relaxation of constraint (day 7+) drives systemic deterioration. anc-1 mutants accelerate the transition to pathological cytoplasmic states. These findings establish cytoplasmic nanorheology as a quantitative readout of cellular aging and suggests that optimal cytoplasmic organization requires balanced mechanical constraints. Given that nesprin mutations cause human neurodegenerative diseases, our nanorheological approach provides a powerful framework for investigating how cytoplasmic biophysics drives age-related cellular deterioration.

B350/P0339

Nuclear-cytoskeletal coupling through the giant KASH protein ANC-1 maintains neuronal mechanical integrity during aging

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Age-related neurodegeneration affects over 50 million people worldwide, yet current therapies show limited success. Mutations in the giant Klarsicht/ANC-1/SYNE homology (KASH) protein Nesprin-1 giant (Nesprin-1G) of the linker of nucleoskeleton and cytoskeleton (LINC) complex are implicated in amyotrophic lateral sclerosis and ataxia, suggesting that disrupted nuclear-cytoskeletal coupling contributes to pathogenesis. We investigate how the C. elegans Nesprin-1G ortholog ANC-1 affects neuronal mechanical integrity during aging. Using genetically encoded multimeric nanoparticles (GEM)-based nanorheology in C. elegans PVD neurons, we discovered that ANC-1 acts as a critical mechanical scaffold in neuronal cell bodies. Wild-type neurons showed a bimodal distribution of GEM mobility: 43% exhibited confined motion while 57% remained mobile. In anc-1 null mutants, the confined population dramatically decreased to 12% ($p<0.001$), indicating severe mechanical disruption. Notably, GEM mobility in dendrites showed minimal differences between genotypes, demonstrating that ANC-1's mechanical influence is spatially restricted to cell bodies. Domain deletion analysis revealed that ANC-1's N-terminal actin-binding domain as well as C-terminal transmembrane a-helix and KASH peptide play distinct but essential roles in maintaining neuronal mechanics. Confocal microscopy showed that endogenous ANC-1 localizes primarily to nuclear envelopes in the cell body and its expression declines dramatically with aging. Loss of ANC-1 triggered cascading organelle positioning defects: mitochondrial positioning errors increased 3.2-fold, mitochondria detached from the nuclear envelope, and nuclear displacement increased 2.7-fold ($p<0.01$). In aged animals, posterior dendrite mitochondria doubled in anc-1 mutants compared to wild-type. These organelle positioning defects strongly correlated with dendrite deterioration ($r=0.78$) and significantly shortened lifespan ($p<0.001$). Our findings establish mechanical instability as a primary driver of age-related neuronal decline, revealing how LINC complex proteins maintain

cellular architecture through spatially organized mechanical scaffolding. This mechanistic framework offers new therapeutic targets for neurodegenerative diseases.

B349/P0338

Optimization of Genetically Encoded Multimeric Nanoparticles for Probing Intracellular Physical Environments

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The interior of a living cell is a physically unique environment, characterized by high molecular crowding and non-equilibrium dynamics driven by metabolic activity. This physical environment modulates the kinetics of diffusion and biochemical reactions, thereby influencing the behavior of various cellular systems. However, there is a lack of effective methods for non-invasively and quantitatively measuring the physical state of the cytoplasm. This study aims to establish a robust technological platform for these measurements by optimizing Genetically Encoded Multimeric nanoparticles (GEMs), which are fluorescently labeled, self-assembling protein complexes that form uniform nanoparticles (20-50 nm diameter) within cells. Tracking the motion of GEMs in the cytoplasm allows for the evaluation of local viscoelasticity and active diffusion, making them a promising tool for intracellular microrheology. We systematically enhanced GEM performance as intracellular probes through a comprehensive optimization approach. This involved screening various pairs of scaffold and fluorescent proteins to improve particle brightness and assembly efficiency. To enable the clear resolution of individual particles, we also precisely controlled intracellular expression levels by tuning promoter strengths. Furthermore, we developed a strategy to suppress background noise from incompletely assembled subunits, which improved overall imaging quality. Our optimizations led to the efficient intracellular formation of highly bright and monodisperse nanoparticles with an expanded spectral palette for multicolor applications. Precise control over expression levels enabled clear discrimination of individual GEMs and significantly reduced tracking errors, while our strategy to suppress background noise yielded a high signal-to-noise ratio. These combined improvements facilitated stable and high-precision particle tracking in living cells over long durations. In conclusion, these highly optimized GEMs serve as a powerful and robust toolkit for the quantitative analysis of the physical properties of the cytoplasm. This technology is expected to make a significant contribution to understanding the impact of the intracellular physical environment on critical cellular functions, such as signal transduction and phase separation.

B355/P0344

PKA Activity Modulates Cytoplasmic Density and Stress Resistance Through Storage Carbohydrate Production

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Cytoplasmic density and crowding conditions are tightly controlled and narrowly distributed within a given cell type. Deviations from this set point occur in response to various stresses, suggesting that cytoplasmic density is actively adjusted to protect the cell from stress. In budding yeast, different types of stress induce a canonical transcriptional response (environmental stress response, ESR), but how the ESR affects cell density is unclear because different types of stress can have opposite effects on cell density. A central ESR regulator is the cAMP activated kinase (PKA). Using refractive index tomography we show that inhibition of PKA in budding yeast leads to a 70% increase in dry mass density. This increase was not caused by increased protein concentration and depends on the presence of glucose in the growth medium. Mutating the stress-induced transcription

factors Msn2/4, which are normally repressed by PKA, prevents cell density increase upon PKA inhibition. PKA inhibition activates the ESR and induces the production and accumulation of storage carbohydrates glycogen and trehalose, however abrogating their synthesis cannot entirely prevent the density increase. The increased density is accompanied by cytoplasmic restructuring, organelle deformation, increased cell wall thickness and decreased diffusion of genetically encoded nanoparticles. The dry mass density we observe in PKA inhibited cells rivals that of spores, which are highly resistant to starvation and heat stress. PKA inhibited cells exhibit improved resistance to extreme heat stress in an ESR and glucose dependent manner. Similarly, PKA inhibition confers glucose starvation resistance in an ESR and partially glycogen synthesis dependent manner. Curiously, interfering with glycogen degradation also increases starvation resistance, suggesting that glycogen does not only act as energy source but also promotes survival through a different mechanism. We hypothesize that the cytoprotective effect of glycogen and possibly trehalose is conferred by altered cytoplasmic properties. In summary, we show that PKA and the ESR play a central role in regulating cytoplasmic properties and survival during stress - at least in part by accumulating storage carbohydrates. In addition, we developed a reproducible and reversible method of creating extreme crowding conditions in living cells.

B356/P0345

Single-molecule and super-resolution diffusivity quantification elucidates physical properties of the cytoplasm

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The living cell creates a unique internal molecular environment that is challenging to characterize. We discuss our recent efforts to elucidate the physical properties of the cytoplasm via single-molecule and super-resolution diffusivity quantification. With the development of single-molecule displacement/diffusivity mapping (SMdM), a novel super-resolution method, we map molecular diffusivity in living mammalian cells, extracted cytoplasm, and the solution phase with unprecedented spatial resolution and fidelity. We thus resolve nanoscale spatial heterogeneity in intracellular diffusion and elucidate their origins due to macromolecular crowding effects from cytoskeletal and chromatin ultrastructures. Moreover, we identify the protein net charge as an overlooked key determinant of intracellular diffusion, unveiling a striking trend in which the diffusion of positively charged proteins is biasedly impeded and recapitulating this behavior in vitro. Together, these results point to an overwhelmingly negatively charged macromolecular environment in the crowded cell that differentially interacts with molecules carrying different net charges and of different sizes.

B359/P0348

Sol-gel Transition Drives Hyper-fast Mixing in a Giant Cell

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The cytoplasm is a crowded and dynamic fluid within which cellular building blocks such as mRNA, proteins, or organelles, undergo transport and mixing. Although small things like proteins can eventually mix through diffusion, the high viscosity of cytoplasm means that it should be difficult to obtain significant mixing for structures in the size range of organelles. In large amoeboid cells, the cytoplasm undergoes active streaming coupled to cell motility, but this streaming is laminar flow which is not effective for mixing . In this work we used a combination of live cell tracking of injected beads and computational analysis of motion and mixing to discover that the giant amoeba Chaos carolinensis overcomes these limitations using a novel cytoplasmic gel

state capture and release strategy that facilitates the efficient mixing of cytoplasm. While it was previously thought that the amoeba solution to gel state transitions only occurs at the trailing and leading edge of the cell body, our work indicates that these transitions occur frequently throughout the mid-cell region, driving the cytoplasmic mixing of beads and organelles. These results indicate that amoeba reaches a stable mixed state during motility in as little as one cytoplasmic stream/flow cycle, effectively making it a Bernoulli mixing system and thus one of the fastest possible known intracellular mixers.

Biophysics, Structural Biology, and Mechanobiology - Structural Biology of Cellular Machines

B386/P0376
Biochemical characterization of BLOC-2 in melanosome maturation
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Lysosome-Related Organelles (LROs) are a diverse group of cell-type specific, membrane-bound subcellular organelles. Members of this family share some features with lysosomes, but they are functionally and morphologically distinct and contain tissue-specific constituents that confer specific functions that are important for the organism. One example is the melanosome, LRO in which melanin pigments are synthesized and stored in eye pigment cells and in melanocytes of the basal layer of the epidermis and the hair bulb of hair follicles. The melanosome is one of the most common models to study and understand the molecular mechanisms underlying the formation and maturation of LROs. The impairment or absence of melanin underlies oculocutaneous albinism, a feature of the Hermansky-Pudlak syndromes (HPS). HPS is a group of generally rare genetic disorders of intracellular vesicle trafficking that disrupt the function of several LROs including melanosomes. Mutations in any of at least 11 genes underlie the disease; these genes encode subunits of four protein complexes, AP-3 and BLOC-1, -2, and -3. Three of the characterized HPS subtypes result from mutations in subunits of BLOC-2, a protein complex for which the molecular function remains unknown. To better understand the role of BLOC-2 in melanosome maturation we aim to elucidate its three-dimensional structure using cryo-EM. We utilized the baculovirus expression system to express the three subunits of BLOC-2; HPS3, HPS5 and HPS6, in Sf9 cells, and affinity purified the complex using FLAG-tag on the HPS5 subunit. The complex was further purified by glycerol gradient sedimentation with cross-linking, demonstrating that the three subunits were forming the complex. Mass photometry analysis suggests that the purified BLOC-2 is a well-defined particle with a size consistent with either a monomer or dimer of the heterotrimeric complex. Our ongoing efforts are focused on elucidating the three-dimensional structure by cryo-EM and identifying its interacting partners.

B381/P0371
BRD4 inhibition suppresses nuclear blebbing and rupture
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Abnormal nuclear morphology, which includes nuclear blebbing, is used to diagnose various types of cancer. Nuclear blebbing refers to a protrusion on the cell’s nucleus, which leads to nuclear rupture and dysfunction. JQ1 is a molecular inhibitor of the bromodomain-containing protein BRD4, which is important for gene

expression, euchromatin maintenance, and cancer progression. Recent research indicates that JQ1 is an effective BET inhibitor with anti-cancer properties. However, how the BRD4 inhibitor JQ1 impacts nuclear blebbing and rupture, a major phenotype of cancer, remains unknown. Live cell experiments were conducted to test the effects of JQ1 treatment and nuclear blebbing over day long treatments. It was found that there is a reduction in nuclear blebbing and rupture with JQ1 treatment. The suppression of nuclear blebbing by JQ1 can be attributed to changes in transcriptional dynamics or euchromatin levels. We find that overall transcription levels remain unchanged for short day long treatments. However, JQ1 decreases levels of euchromatin and strengthens the nucleus. Overall, we present data that BRD4 inhibition functions through chromatin histone modification state to compact the chromatin and strengthen the nucleus to maintain both shape and integrity. These experiments provide insight into the basic cell biology aiding JQ1's potential as a cancer-treating drug.

B379/P0369

Bridging Scales: A Two-Stage Parametrization for Multi-scale molecular Simulations

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Accurately linking atomistic detail to meso-scale phenomena is a central challenge in computational biophysics. A primary bottleneck is the parametrization of coarse-grained (CG) models, which must capture the essential physics of the underlying high-resolution system. Here, we introduce a powerful bottom-up CG framework built upon a flexible two-stage optimization protocol. While primarily designed to derive parameters directly from all-atom simulations, the approach can also integrate experimental observables for further refinement. The first stage utilizes Bayesian optimization to efficiently navigate the complex parameter landscape and identify a near-optimal parameter region. This globally informed starting point is then passed to a second stage, where a gradient-based relative-entropy minimization is performed to precisely refine the model and maximize its fidelity to the reference atomistic data. We demonstrate the versatility of this method by applying it to two distinct biological systems: the microtubule crosslinker PRC1 and the structural protein collagen, showing the approach can capture both dynamic assembly and material properties. This two-stage strategy enhances the efficiency and accuracy of the bottom-up parametrization workflow, providing a generalizable tool for developing predictive multi-scale models. The framework's capacity to be constrained by experimental data further extends its applicability to a wide range of complex biological processes.

B385/P0375

Channeling the unexpected: A case study in connexin protein modularity

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Gap junctions form the molecular architecture of direct intercellular communication—an essential feature of the cells that make up every tissue and organ system in the human body. Although more than 20 connexin isoforms are expressed across tissues, the isoform-specific features that govern gating, permeability, and interaction specificity remain poorly understood. To investigate how modular domains contribute to these properties, we designed a series of chimeric proteins combining the extracellular loops of Cx34.7 (a homolog of human Cx36) with the transmembrane and intracellular domains of Cx43, a well-characterized, highly permeable isoform. We assessed channel function using a suite of assays: non-injury dye uptake, FETCH (flow cytometry–based evaluation of docking), calcium inhibition, cell death, and imaging-based analyses. Our

results indicate that these chimeras form functional hemichannels with enhanced open probability, even in the absence of injury or other activation cues. To contextualize this phenotype, we benchmarked our constructs against known Cx43 gain-of-function mutants previously shown to promote hemichannel opening. The similarity in behavior supports the conclusion that our chimeras achieve bona fide hemichannel activity. Importantly, while some properties aligned with the expected modular contributions of Cx43 and Cx34.7 domains, others emerged unexpectedly from their recombination. These findings suggest that modular design can be used not only to recapitulate known features, but also to uncover emergent properties—offering a powerful framework to probe isoform-specific differences in basic channel function and to inform future efforts to engineer tailored intercellular communication.

B382/P0372

Characterizing structural determinants of cytoplasmic LINE-1 activity

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Retrotransposable elements (RTEs), genetic sequences capable of “copy-and-paste” self-replication, comprise approximately 40% of the human genome, with Long INterspersed Element 1 (LINE-1/L1) representing the largest and most enzymatically active class of RTEs. RTEs are typically suppressed in healthy tissues by several mechanisms, but cells lose control over their RTEs because of epigenetic dysregulation associated with cellular senescence or cancer development. L1 has directly been shown to cause IFN-I production in senescence and knockdown of L1 has been shown to blunt the senescence associated secretory phenotype (SASP). Consistent with previous studies, we’ve also observed that L1 expressed in cells can produce cytoplasmic DNA:RNA hybrids and L1 derepression results in IFN-I signaling through cGAS/STING. These data strongly indicate that L1 directly stimulates innate immunity in senescent cells by generating cytoplasmic RT products and this is a fundamental process in cellular aging. Canonically, L1 is predominantly primed in the nucleus using endonucleolyticly cleaved genomic DNA (gDNA) during target primed reverse transcription (TPRT), however we have shown that cytoplasmic priming occurs in an endonuclease independent manner. Absent gDNA in the cytosol, the underlying mechanism by which L1 is primed in the cytoplasm is unknown. We show that L1 is capable of priming using RNA and DNA substrates with transient base-pairing interactions and can efficiently prime off self-annealing “snapback” RNAs like those generated by the Alu transcripts. We hypothesize these are a likely source of immune-stimulatory cytoplasmic RT products. Additionally, we demonstrate the structural basis of L1 snapback priming to generate a model for generation of L1 cytoplasmic activity. These findings reveal a targetable mechanism for mediating RT dependent sterile inflammation observed in diseases of aging and cancer immune evasion.

B373/P0362

Constraining sarcomeric protein machinery dynamics through likelihood-based cardiac modelling

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Cardiac contraction relies on the tightly regulated coordination of the sarcomeric protein machinery. However, the dynamics of these molecular interactions remain difficult to quantify, particularly in disease. The objective of this study was to develop and evaluate a computational framework that identifies and constrains

parameters controlling sarcomeric protein kinetics in mutation-induced dilated cardiomyopathy and control. A novel computational model of sarcomeric kinetics and muscle striation was developed and calibrated using fluorescent imaging data. Parameter estimation targeted protein-level mechanisms, with a particular focus on Z-disc alignment. To evaluate estimation strategies, we compared a population-of-models approach, which captures intercellular variability but provides broader parameter ranges, with maximum likelihood estimation (MLE). The latter identifies parameter sets that optimally reproduce experimental recordings of sarcomere shortening and intracellular calcium transients. To assess parameter uncertainty and model robustness, we applied profile likelihood analysis, enabling parameter space reduction while preserving essential model behaviour. This strategy has resulted in improved identifiability and confidence intervals, which in turn enhance interpretation of protein-level dynamics. In conclusion, MLE combined with likelihood profiling constitutes a rigorous framework for characterising the sarcomeric protein machinery and for reducing uncertainty in cardiac models. This protein-centric perspective strengthens the predictive power of computational models and establishes mechanistic links between molecular alterations and whole-cell contractile dysfunction.

B375/P0364

Emerging role of selenoproteins in protein quality control unveiled through Cryo-EM

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Degradation and recycling of aberrant proteins and protein complexes in the endoplasmic reticulum (ER) is key to managing cellular stress. The ATPase p97 (VCP) powers the disassembly and unfolding of dysfunctional proteins through its central pore, using ATP hydrolysis. This mechanism is essential for cell survival and requires p97 enzymatic activity to be regulated by adapter protein partners. Selenoprotein S is a reductase that recruits p97 to the ER membrane, the primary organelle for protein processing. The mechanism of interaction between selenoprotein S and p97, and its ramifications in p97 enzymatic activity, remains unknow and limits our understanding of the cellular stress response. To investigate the interactions of selenoprotein S and p97, we employed cryo-electron microscopy (Cryo-EM) to reconstruct 3D structures of the selenoprotein S/p97 complexes at a resolution of 3 Å. We show that up to six copies of selenoprotein S coordinate with p97, inducing conformational changes in the exit pore. These conformational changes disrupt p97 asymmetry and demonstrate the functionality of p97 asymmetric conformations. We couple structural experiments with biochemical assays and demonstrate that selenoprotein S acts as an activator of p97 ATPase activity, highlighting the relevance of these conformational changes for p97 activity in the ER protein extraction and unfolding machinery.

B374/P0363

In situ intracellular structural mechanism of Epothilone B-induced CNS axon regeneration

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Axonal regeneration in the central nervous system (CNS) remains a significant challenge, with many of the molecular mechanisms involved still poorly understood. To explore the intracellular response to axonal injury,

we established an in situ cryo-electron tomography (cryo-ET) and cryo-electron microscopy (cryo-EM) platform that models axonal damage and uncovers the structural mechanisms underlying Epothilone B (EpoB)-induced regeneration in thalamic axons. We found that stabilized microtubules extend across the injury site, generating membrane tension that drives expansion of the axonal membrane. Cryo-EM reconstruction at 3.19 Å resolution reveals EpoB engaged within the microtubule lattice at the regenerating front. During this process, tubulin clusters are recruited and integrated into growing microtubules at the site of repair. These microtubule projections act as dynamic scaffolds, supporting the transport and localization of vesicles and endoplasmic reticulum (ER), which supply key components for regeneration until membrane tension is balanced. Our findings highlight an unexpected adaptability in neurons, whereby EpoB-induced mechanical strain disrupts cellular homeostasis and activates a regenerative program.

B387/P0377
Membrane-Regulated Oligomerization of SHIP2 Revealed by CryoEM
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Src homology 2 domain-containing inositol-5 phosphatase 2 (SHIP2) is a lipid phosphatase that modulates PI3K/AKT signaling by dephosphorylating PIP3, influencing cell proliferation, adhesion, and survival. Dysregulated SHIP2 activity has been implicated in cancer, type 2 diabetes, and neurodegeneration, yet its regulatory mechanisms at the membrane remain unclear. Using cryogenic electron microscopy, we resolved the structure of the SHIP2 pleckstrin-homology-related (PHR), phosphatase, and C2 domains. The PHR domain engages the phosphatase and C2 domains, aligning lipid-binding sites into a planar configuration that enhances membrane avidity. On PIP3-containing monolayers, SHIP2 undergoes higher-order oligomerization, assembling into arrays stabilized by interdomain contacts between neighboring molecules. This membrane-driven oligomerization, recapitulated in solution by PI(3,4)P2 binding, suggests that lipid composition not only governs SHIP2 recruitment but also its cooperative enzymatic activation. These findings uncover a structural mechanism by which SHIP2 integrates lipid signals to control phosphoinositide metabolism and provide a framework for targeting its activity in disease contexts.

B378/P0368
Molecular Mechanisms of Peroxisomal Fatty-Acid Uptake
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Peroxisomes are highly dynamic subcellular organelles critical for lipid metabolism and cellular detoxification, including roles in fatty acid oxidation and bile acid synthesis. Fatty acyl-CoA substrate translocation across the peroxisomal membrane is mediated through ATP-binding cassette transporters of subfamily D (ABCD1-3). ABCD3 displays broad substrate specificity, facilitating the transport of branched-chain fatty acids, very long-chain fatty acids, bile acid precursors, and dicarboxylic acids into the peroxisomal matrix. Mutations in ABCD3 are linked to metabolic disorders such as congenital bile acid synthesis defects. Using cryo-electron microscopy, we determined high-resolution structures of human full-length ABCD3 in apo and substrate-bound conformations. Our biochemical and biophysical characterization reveals that substrate binding stimulates ATP hydrolysis, demonstrating allosteric communication between substrate-binding and nucleotide-processing sites. Molecular dynamics simulations further provide a detailed insight into substrate recognition and alterations in the binding pocket of the transporter. Comparative structural analysis identifies substrate-

dependent reorganization of nucleotide-binding domains, elucidating key mechanistic features of the transport cycle. These insights advance our understanding role of ABCD3 in peroxisomal metabolite trafficking and associated diseases.

B376/P0365

Preparation and cryo-EM analysis of paired helical filaments in Alzheimer's disease

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Alzheimer's disease (AD) is pathologically defined by the accumulation of misfolded amyloid-β into extracellular plaques and hyperphosphorylated tau into intracellular paired helical filaments (PHFs) forming neurofibrillary tangles. This project focuses on (i) developing expression and purification protocols for PAD12, a full-length recombinant tau construct with twelve phosphomimetic mutations which drive AD-relevant PHF assembly and (ii) establishing a high-resolution cryogenic-electron microscopy (cryo-EM) data processing pipeline for PHF structural reconstruction. PAD12 plasmids encoding 0N4R and 0N3R tau isoforms were generated through site-directed mutagenesis. Plasmids were verified through restriction enzyme digestion and Sanger sequencing. PAD12 constructs were transformed into BL21-CodonPlus (DE3)-RIPL competent cells, overexpressed, and purified through cation exchange and size exclusion chromatography. Expression level and purity were evaluated by SDS-PAGE. Concurrently, PHFs extracted from an AD subtype brain were screened by cryo-EM, and micrographs were uploaded to RELION for structural determination. The next phase of this work will focus on PAD12 purification optimization and assembly into PHFs in vitro, which will be validated with the developed cryo-EM pipeline. Subsequent in vivo seeding studies in Aβ-expressing mouse models will assess the propagation capacity of PAD12 PHFs. Site-directed mutagenesis successfully generated sequence-verified 0N4R and 0N3R PAD12 tau variants, and overexpression was validated by SDS-PAGE at 40 kDa. Additionally, RELION-based cryo-EM reconstruction and refinement resolved the PHF structure at 2.8 Å with a 750 Å crossover distance, thereby establishing a computational pipeline for high-resolution PHF reconstruction. These results demonstrate a reliable workflow for producing and characterizing disease-relevant recombinant tau constructs and yield structural insights into AD subtype-derived PHFs.

B384/P0374

Reversible lipid-mediated gating of gap junction ion channels revealed by cryo-EM

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Gap junctions, formed by connexin proteins, establish direct electrical and metabolic coupling between cells, enabling coordinated tissue responses. These channels universally respond to intracellular pH changes, closing under acidic conditions to limit the spread of cytotoxic signals during cellular stress, such as ischemia. Using cryo-electron microscopy, we uncover insights into the structural mechanism of pH-gating in native lens connexin-46/50 gap junctions in lipid nanodiscs. Mild acidification drives lipid infiltration into the channel pore, displacing the N-terminal (NT) domain and stabilizing pore closure. We show that lipid involvement is both essential and fully reversible. Furthermore, structural transitions involve an ensemble of open and gated-states formed through non-cooperative NT domain movement of individual subunits across the dodecameric channel. These findings provide molecular insights into pH-gating dynamics, illustrating how structural changes may regulate gap junction function under cellular stress and linking connexin-46/50 dysregulation to age-related cataract formation.

B383/P0373
Seeing is Believing - A snapshot into how structural biology is enabling drug discovery at Bristol Myers Squibb
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Structural biology has long been at the forefront of drug discovery, driving the development of new therapeutics across diverse modalities and deepening our understanding of drug mechanisms of action (MOA). The use of X-ray crystallography and, more recently, cryo-electron microscopy (cryoEM) has illuminated drug-target interactions at the molecular level and advanced our knowledge of molecular biology. At Bristol Myers Squibb (BMS), both X-ray crystallography and cryoEM are integral to enabling programs throughout our pipeline. These efforts have contributed to the discovery and development of over eight clinically used medicines, with several more awaiting approval. Successes in this area have led to significant investment in structural biology at BMS, particularly in cryoEM, with the recent installation of an in-house facility at our Lawrenceville, NJ site and plans for a second in San Diego, CA. In this presentation, we highlight the innovative strategies and workflows of the BMS structural biology teams that have enabled drug development and have revealed novel MOAs across a range of drug modalities. This includes small-molecules, millamolecules (cyclic peptides), antibody-derived biologics, and cereblon degraders. For example, we show how X-ray crystallography was pivotal in the discovery and development of Sotyktu®, a Tyk2 pseudokinase-domain inhibitor, and share recent findings on never-before-seen MOAs of small-molecule inhibitors targeting regulators of cell-surface glycosylation in tumors. We next describe how cryoEM is being harnessed for structure-based drug discovery for a range of targets. In particular, we show its use in high-resolution epitope mapping of antibody-based biologics that inhibit regulators of neutrophil extracellular traps during chronic inflammation. Additionally, we demonstrate how cryoEM has enabled the determination of structure-activity relationships (SARs) for small-molecule cereblon degraders and millamolecules, advancing their potential in cancer treatment. In summary, these results underscore the transformative impact of structural biology within the BMS pipeline. With our recent investment in cryoEM and rapid technological advancements, we are poised to study more complex biological systems and enhance our ability to discover novel therapies that will shape the future of medicine.

B388/P0378
Structural insights into mitotic-centrosome assembly
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Centrosomes are essential organelles that orchestrate cell division in most eukaryotic cells. A key event in centrosome maturation is the expansion of the pericentriolar material (PCM), which transforms the centrosome into a micron-scale structure capable of organizing microtubules. This process requires the precisely regulated recruitment and interaction of numerous proteins, among which Centrosomin (Cnn) plays a central role. Cnn, a core PCM protein in Drosophila melanogaster, has proven to be a valuable model for understanding centrosome maturation, as its structure and function are evolutionarily conserved, including in humans. Mutations in the human homolog, CDK5RAP2, have been associated with developmental disorders such as microcephaly and dwarfism, as well as with cancer, emphasizing the critical role of this protein in cellular homeostasis. In this study, we demonstrate how Cnn self-assembles into an extensive scaffold network and identify the specific protein regions that regulate this assembly. We also pinpoint key residues phosphorylated by Polo kinase that are essential for scaffold formation in vivo. Finally, we identify conserved

functional domains within human CDK5RAP2, revealing mechanistic parallels and evolutionary conservation in centrosome maturation across species.

B377/P0367
Structural Modeling of PARP1 Isoforms and Breast Cancer Mutations: Exploring Nucleolin Interactions and Functional Outcomes
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In our previous work, we identified the target genes of six microRNAs (miR-15, miR-16, miR-21, miR-103, miR-221, and miR-222) regulated by nucleolin (NCL), an RNA-binding protein involved in transcription, translation, and DNA repair, all of which have established roles in breast cancer. In a complementary study, we also identified common interactors of NCL and BRCA1 to uncover their interconnected roles in breast cancer. Based on the results of these studies, we selected Poly(ADP-ribose) polymerase 1 (PARP1) as a promising focal point in dysregulated DNA repair in breast cancer with NCL and BRCA1 playing interconnected roles. PARP1 is a BRCA1 C Terminus (BRCT) domain containing protein, a key player in DNA repair and transcriptional regulation, and a known interactor of NCL. Expression levels of PARP1 and its different isoforms are variable across different breast cancer cell lines as well as across normal and tumor breast tissue samples. To investigate their roles and their interactions with NCL, we modeled the full-length structures of all PARP1 isoforms and performed docking analyses with a previously generated full-length NCL model using ClusPro and HDOCK. Based on these analyses, we show the predicted PARP1-NCL interaction interfaces for the different PARP1 isoforms. We further identified breast cancer–associated mutations in key PARP1 domains using ENSEMBL, COSMIC, and The Cancer Genome Atlas (TCGA), modeled these variants, and docked them with NCL. Comparative analyses of mutant and wild-type isoforms revealed mutation-driven alterations in the interaction interfaces. Overall, this study provides new structural and functional insights into PARP1 isoforms, their interactions with NCL, and the potential implications of these molecular interactions as potential therapeutic targets for breast cancer progression.

Cancer Cell Biology - Cancer cell metabolism

B492/P1616
APE1-Mediated Regulation of Mitochondrial Bioenergetics Following Alkylation DNA Damage in HepG2 Cells
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Liver cancer, particularly hepatocellular carcinoma (HCC), has a poor prognosis due to early detection. Its incidence has more than tripled since the 1980s, underscoring the need for research to identify therapeutic targets. Evidence indicates that a reduction of mitochondrial components and function, along with errant mitochondrial DNA (mtDNA) repair mechanisms, may contribute to several hallmarks associated with carcinogenesis. In eukaryotes, the main route of mtDNA repair is base excision repair (BER), facilitated by Apurinic/Apyrimidinic Endonuclease 1 (APE1). APE1 is a multifunctional protein and contains a redox domain, responsible for modulating the activity of several transcription factors. Although the role of APE1 in DNA repair is well understood, the contributions of its redox function to mitochondrial bioenergetics following a genotoxic insult are limited. Our hypothesis is that APE1 redox function is central for maintaining mitochondrial

bioenergetics in response to mtDNA damage. To test this hypothesis, the human hepatoma HepG2 cell line was exposed to methyl methane sulfonate (MMS), a DNA alkylating agent, along with E3330, an inhibitor for APE1 redox function. Crystal violet viability assays were performed to determine the IC-50 of MMS and E3330. Cells were exposed to increasing concentrations of MMS for 30 minutes or EE3330 for 1 hour, washed, and then allowed to grow for 6 days. Cells were then stained with Crystal Violet, and total absorbance was determined at 575nM. The IC-50 of MMS was found to be 18.5 µM. Preliminary results show the IC-50 of E3330 in the range of 13.58 to 34.55 µM. To measure changes in mitochondrial bioenergetics, cells were treated with MMS for 30 minutes and analyzed using an Agilent Seahorse XF instrument, performing a Mito Stress Test immediately after treatment. Maximal respiration decreased by 16% ($p<0.05$) and the spare respiratory capacity decreased by 25% ($p<0.05$) after 6mM MMS treatment, as compared to the untreated control group. Preliminary experiments combining 6mM of MMS and 25µM of E3330 resulted in a 26% decrease in maximal respiration ($p<0.05$) and a 19% decrease in the spare respiratory capacity ($p<0.05$) as compared to untreated controls. We conclude that the APE1 redox function contributes to the maintenance of mitochondrial bioenergetics following alkylation DNA damage. This study underscores the importance of mtDNA repair in the maintenance of mitochondrial bioenergetics in the context of HCC.

B491/P1615

CLUH Drives Metabolic Plasticity and Radioresistance in Glioblastoma Stem-like Cells via ß-Catenin Signaling

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Glioblastoma multiforme (GBM), the most aggressive primary brain tumor, has a poor prognosis and frequent recurrence. Recurrence is largely driven by glioblastoma stem-like cells (GSCs), which possess self-renewal capacity and intrinsic resistance to ionizing radiation. Mitochondrial metabolism is essential for GSC survival and adaptability, yet the molecular regulators of this process are not fully understood. In this study, we identify the cytosolic RNA-binding protein CLUH (Clustered Mitochondria Homolog), which binds nuclear-encoded mitochondrial mRNAs, as a key regulator of GSC metabolism and maintenance. CLUH was enriched in $CD133^+$ cells and consistently higher in GSCs compared with differentiated counterparts. Knockdown of CLUH reduced neurosphere formation, impaired self-renewal, and increased radiosensitivity, whereas CLUH overexpression reinforced stem-like properties and promoted radioresistance. Mechanistically, CLUH supported the energy and biosynthetic needs of stemness by enhancing oxidative phosphorylation and anabolic metabolism.We further demonstrate that CLUH is transcriptionally regulated by Wnt/β-catenin signaling. Pharmacological inhibition or genetic suppression of β-catenin lowered CLUH expression and mirrored the effects of CLUH depletion. Collectively, these findings establish CLUH as a central mediator linking mitochondrial metabolism to GSC maintenance and therapeutic resistance, highlighting the β-catenin–CLUH axis as a promising metabolic vulnerability in GBM.

B493/P1617

Heat shock factor 1 regulates copper-induced metabolic stress and autophagy in pancreatic cancer

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Pancreatic ductal adenocarcinoma (PDAC) is the most prevalent pancreatic cancer type, with a low 5-year survival rate of 13%. This poor prognosis arises in part from the capacity of PDAC cells to adapt to diverse cellular stresses and preserve mitochondrial function under chronic metabolic and proteotoxic stresses. Copper is an essential cofactor that supports key mitochondrial enzymes; however, elevated levels of copper disrupt mitochondrial integrity, leading to the aggregation of the mitochondrial enzyme dihydrolipoamide S-acetyltransferase, which converts glucose-derived pyruvate into a substrate for the mitochondrial energy cycle, and simultaneously triggers the activation of a cytoprotective proteotoxic stress response. PDAC tumors accumulate elevated copper yet remain metabolically active, suggesting the existence of an adaptive program that protects mitochondrial function from copper-induced damage. Our study demonstrated that copper exposure in human PDAC cells, mimicking elevated copper stress, induces the activation of the stress-responsive transcription factor heat shock factor 1 (HSF1), a key regulator of protein folding and proteome homeostasis. Results from Western blotting, ELISA, and fluorescence imaging show that the elevated copper exposure induces global protein polyubiquitination, protein aggregation, and intracellular copper accumulation. Overexpression of HSF1 rescues these defects. Conversely, loss of function of HSF1 exacerbates copper-induced amyloid fibril formation. HSF1 prevents excessive oligomerization and accumulation of the insoluble dihydrolipoamide S-acetyltransferase aggregates under copper stress. Additionally, pharmacological inhibition of HSF1 further decreases PDAC cell survival in response to copper toxicity. In addition to maintaining proteostasis, HSF1 regulates mitochondrial quality control through autophagy, including mitophagy. Overall, our findings reveal a novel function of HSF1 in coordinating copper-induced mitochondrial stress responses and its regulation of autophagy. Targeting HSF1-mediated proteostasis and copper homeostasis may offer a new therapeutic strategy for treating pancreatic cancer.

B489/P1613

Heme biosynthetic pathway functions as a novel negative regulator of cancer cell proliferation and malignancy

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Most aerobic organisms generate energy through the electron transport chain, in which heme, a central component of the respiratory chain, plays an indispensable role. Heme is synthesized exclusively via a single biosynthetic pathway, consisting of seven enzymatic reactions starting from 5-aminolevulinic acid (5-ALA). Because heme is essential, its biosynthetic pathway also has long been considered indispensable as well. However, most cancer cells maintain high proliferative activity despite their markedly reduced heme biosynthetic capacity. We hypothesized that cancer cells may actively suppress heme biosynthesis in order to sustain their proliferative potential. This study aimed to elucidate the physiological significance of the heme biosynthetic pathway in cancer cells. The inhibition of ALA dehydratase (ALAD), the enzyme catalyzing the step immediately after 5-ALA, by succinyl acetone (SA) suppressed proliferation in normal cells but not in cancer cells. Using CRISPR/Cas9, we successfully generated ALAD-knockout cancer cell lines. Measurement of protoporphyrin IX (PpIX), the terminal intermediate of the pathway, revealed a marked decrease in PpIX levels even after 5-ALA supplementation in the ALAD-knockout cells. These findings suggest that downstream steps from ALAD to PpIX synthesis are dispensable in cancer cells. Notably, loss of ALAD induced epithelial–mesenchymal transition (EMT) and enhanced malignant phenotypes. Analysis of TCGA datasets further demonstrated a strong correlation between low ALAD expression and poor prognosis, indicating that loss of heme biosynthesis downstream of ALAD confers a survival advantage to cancer cells. Conversely, activation of the heme synthetic pathway by 5-ALA administration specifically induced DNA double-strand breaks and cell-cycle delay in cancer cells, but not in normal cells. Taken together, our results suggest that certain genes or metabolites within the heme biosynthetic pathway act as negative regulators of specific cancer cell

proliferation. Cancer cells appear to suppress this pathway to evade such regulation. We are currently investigating the identity of these regulatory factors and will present our findings for discussion.

B488/P1612

Non-enzymatic Glycoxidation Coordinatively Activates De Novo Lipogenesis and Oxidative Phosphorylation in Prostate Cancer

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Background: Advanced glycation end-products (AGEs) are bioactive metabolites formed through non-enzymatic glycoxidation, a process accelerated by aging, poor diet, and high-temperature food processing. AGE buildup alters mitochondrial function and redox balance by disrupting metabolic homeostasis. Prostate cancer (PCa), a lipid-dependent malignancy, relies on de novo lipogenesis to sustain growth and survival. This study investigated whether dietary AGE accumulation promotes PCa progression by reprogramming mitochondrial metabolism to support acetyl-CoA–driven fatty acid synthesis and augment tumor lipogenic capacity. Methods: The impact of dietary AGEs on de novo lipogenesis in PCa was evaluated using integrated in vitro and in vivo models. MYC-CaP prostate tumor epithelial cells were co-cultured with WPMY1 human prostate fibroblasts in Transwell systems and treated with BSA-derived AGEs (25 μg/mL, 24 h). Tumor epithelial cell lysates were analyzed by Western blot for lipogenic enzymes including phosphorylated ATP citrate lyase (p-ACLY), total ACLY, acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FASN) and acyl-CoA synthetase long-chain family member 1 (ACSL1). Mitochondrial function in AGE-treated LNCaP cells was measured using Seahorse XF Analyzer. The metabolic changes were recapitulated in vivo, male C3-TAg transgenic mice were fed either a regular diet or a high-AGE diet for 8 weeks. Tumors from MYC-CaP allografts were subjected to targeted metabolomics, lipidomics, and RNA sequencing. Results: AGE-treated MYC-CaP tumors showed marked induction of fatty acid biosynthesis enzymes, including p-ACLY, ACC1, FASN, and ACSL1, indicating acetyl-CoA–driven lipogenesis. In LNCaP cells, AGE exposure elevated basal and maximal oxygen consumption rates and proton leak ($p = 0.05$), reflecting enhanced oxidative phosphorylation and mitochondrial stress. Targeted metabolomics revealed significantly increased levels of phosphorylethanolamine, ATP, L-aspartate, and succinyl-CoA (fold change ≥ 2, $p < 0.05$). Lipidomic profiling identified upregulation of phosphatidylethanolamine PE(P-18:0_22:4), in AGE-fed mice. Transcriptomic analysis revealed enrichment of mitochondrial ATP synthesis (FDR = 1.40×10^{-4}) and oxidative phosphorylation pathways (FDR = 1.00×10^{-24}), supporting a coordinated metabolic shift toward mitochondrial energy production and anabolic lipid metabolism in AGE-exposed tumors. Conclusion: Dietary AGE exposure coordinately activates mitochondrial respiration and acetyl-CoA–driven lipogenesis in prostate tumors, creating a metabolically permissive environment for growth. These findings highlight diet-induced metabolic vulnerabilities as potential targets for intercepting PCa progression.

B494/P1618

O-GlcNAcylation-Dependent Regulation of p90-RSK1 Reveals a Therapeutic Vulnerability in Breast-Cancer Brain-Metastases

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Triple-negative breast cancer is one of the most aggressive forms of breast cancer, as it has the highest occurrence of brain-metastasis (BCBM). Unfortunately, over 80% of patients diagnosed with BCBM die within one year, highlighting an urgent need to identify novel therapeutic targets. BCBM cells exhibit elevated levels of O-GlcNAcylation, a nutrient sensitive post-translational modification catalyzed by O-GlcNAc transferase (OGT) that adds O-GlcNAc moieties to nuclear and cytoplasmic proteins. Importantly, BCBM cells depend on OGT for growth. Using proteomic analysis, we identified the ribosomal protein S6 kinase alpha-1 (p90-RSK1), a key MAPK/ERK-regulated signaling protein, as highly O-GlcNAcylated in BCBM cells compared to parental breast-cancer cells. We found that p90-RSK1 protein levels correlated with O-GlcNAcylation status and that genetic knockdown of p90-RSK1 significantly impaired BCBM growth in vivo. Furthermore, pharmacological inhibition of p90-RSK using BRD-7389 induced BCBM cell death and reduced proliferation in vitro, while increasing doses of the inhibitor blocked BCBM growth ex vivo. Together, these findings identify p90-RSK1 as a novel O-GlcNAc-regulated protein and represent a promising therapeutic target for treating breast-cancer brain-metastases.

B495/P1619

Post-Translational Modifications of MYC Regulate Metabolic Reprogramming in Breast Cancer

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The objective of this study is to investigate how post-translational modifications (PTMs) of MYC regulate metabolic gene expression and energy pathways in breast epithelial cells, and to evaluate the broader significance of these changes in breast cancer models. MCF10A breast epithelial cell lines carrying mutations at specific MYC PTM sites were generated, rendering those modifications inoperable. RNA sequencing was performed to compare global transcriptional changes in PTM mutants relative to MYC-overexpressing and wild-type cells. Candidate metabolic enzymes were validated at the transcriptional level by qPCR and confirmed at the protein level by western blotting. To assess clinical relevance, the TCGA-BRCA cohort was analyzed to evaluate expression of candidate enzymes in breast tumors, and enzyme expression was further examined in a panel of breast cancer cell lines representing distinct molecular subtypes. RNA sequencing identified several metabolic enzymes that were upregulated under MYC overexpression but downregulated in PTM mutant lines. Analysis of mRNA and protein levels confirmed altered expression of these enzymes across mutant and control lines. In the TCGA-BRCA cohort, enzymes displayed subtype-specific expression patterns and were significantly elevated in tumor samples, underscoring their potential clinical relevance. Pearson correlation analysis further revealed that key metabolic enzymes exhibit modest but highly significant correlations with MYC expression. Comparison across breast cancer cell lines revealed consistent subtype-dependent regulation. These findings support a model in which MYC PTMs act as molecular switches that enable metabolic reprogramming through regulation of key enzymes. By combining in vitro modeling, protein-level validation, patient cohort analysis, and cell line comparisons, this work highlights how MYC PTMs integrate oncogenic signaling with metabolic adaptation. These insights reveal new therapeutic opportunities through enzymes that regulate or depend on MYC PTM activity.

B490/P1614

The macropinosome: uncovering the molecular anatomy of an oncogene-driven organelle

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Pancreatic ductal adenocarcinoma (PDAC) cells rely on macropinocytosis, a form of bulk endocytosis that enables the internalization and lysosomal degradation of extracellular protein. By supplying protein-derived amino acids, macropinocytosis allows PDAC cells to circumvent fluctuations in nutrient availability. Glutamine is a vital nutrient to tumors supporting the metabolic reactions necessary to sustain tumor growth; however, elevated consumption by the tumor often leads to a glutamine-depleted tumor ecosystem, prompting the need for alternative glutamine sources. In PDAC, glutamine is the most depleted amino acid relative to normal adjacent pancreas tissue, yet elevated macropinocytosis enables tumor cells to sustain proliferation despite the nutrient-scarce conditions of the tumor microenvironment. While the tenets of the signal transduction events that drive macropinocytosis in cancer have emerged, a detailed picture of the macropinosome itself is not yet available. To address this critical gap, we developed a nanotechnology-based platform to isolate intact macropinosomes from PDAC cells. Proteomic analysis of these purified macropinosomes revealed that the zinc transporter SLC30A1 is highly enriched in macropinosome fractions. We further demonstrated that SLC30A1 localizes to nascent macropinosomes and that its expression is upregulated following glutamine depletion. Using a genetically encoded Zinc FRET-biosensor, we observed a decrease in cytoplasmic zinc levels after SLC30A1 induction under glutamine-depleted conditions. Knockdown of SLC30A1 or supplementation with zinc impaired macropinocytic uptake without affecting plasma membrane ruffling, suggesting a specific role for SLC30A1 in macropinosome closure. Based on these findings, our central hypothesis is that SLC30A1-driven zinc efflux is a key metabolic adaptation that enables macropinocytosis and supports PDAC growth under conditions of nutrient stress. By elucidating the molecular anatomy of KRAS-driven macropinosomes, we will gain further insight into the regulation and function of macropinocytosis in cancer. Moreover, having a clearer picture of the macropinosome will make us better positioned to exploit this pathway therapeutically and deliver breakthroughs to patients.

Cancer Cell Biology - Cancer Cell Plasticity, Invasion, and Metastasis

B520/P1644
A Novel p300 Inhibitor Bisamidoxime, JJMB9, Alone and in Combination with Cisplatin, Significantly Inhibited Breast Cancer Tumor and Lung Metastases Growth
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Chemoresistance remains a major obstacle to the successful treatment of estrogen receptor–positive (ER+) and triple-negative breast cancer (TNBC). We investigated the antiproliferative activity in vitro and the antitumor efficacy in vivo of JJMB9, a novel p300 histone acetyltransferase (HAT) inhibitor, as a single agent and in combination with cisplatin. In vitro, JJMB9 and cisplatin exhibited synergistic growth inhibition in MCF-7 and 4T1 cells. This synergistic effect could be explained by JJMB9 inhibition of p300, which, based on our RNA-seq and RT-qPCR, superseded the expression of replication factors, needed also for DNA-repair. In the ER+ MCF-7 model, female nude mice were orthotopically implanted with tumor cells following estradiol pellet (0.36 mg) insertion and treated with vehicle, JJMB9 (25 mg/kg, 5 days a week for 3 weeks), cisplatin (2 mg/kg, twice daily for 2 weeks), or the combination. JJMB9 inhibited tumor growth ($P \leq 0.05$), while cisplatin alone or in combination produced more potent effects ($P \leq 0.001$). Final tumor weights were reduced from 0.47 ± 0.04 g (control) to 0.34 ± 0.05 g with JJMB9, 0.10 g with cisplatin, and 0.14 ± 0.02 g with the combination (all $P \leq 0.001$). Cisplatin alone induced severe weight loss and mortality in 9/13 mice, whereas the combination initially caused weight loss that later recovered, ultimately rescuing 12/13 mice. In the 4T1 TNBC model,

BALB/c mice received vehicle, JJMB9 (25 mg/kg), cisplatin (3 mg/kg), or the combination, 5 days a week for 3 weeks. Tumor volumes were significantly reduced in all treatment groups compared with controls ($P \leq 0.001$). Final tumor weights were reduced from 0.63 ± 0.06 g (control) to 0.42 ± 0.04 g with JJMB9 ($P \leq 0.05$), 0.24 ± 0.04 g with cisplatin ($P \leq 0.001$), and 0.36 ± 0.06 g ($P \leq 0.05$) with the combination. Lung metastases occurred in 3/13 control mice with a mean metastatic area of 0.171%, JJMB9 reduced metastasis to 0.026% ($P \leq 0.01$), and no metastases were observed with the combination. Body weights remained stable with JJMB9 or cisplatin monotherapy but decreased modestly with the combination ($P \leq 0.05$). Together, these results demonstrate that JJMB9 suppresses tumor growth and metastasis, and its enhanced efficacy with cisplatin supports further development of JJMB9 as a chemotherapeutic agent, either alone or in combination with DNA-damaging drugs.

B499/P1623

A novel ROR1 inhibitor potently and selectively inhibits triple-negative breast cancer cells

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Breast cancer is the second most common cancer in women in the United States, with triple-negative breast cancer (TNBC) accounting for 15-20% of new cases. TNBC cells lack hormone receptors and human epidermal growth factor receptor 2 (HER2), making them resistant to standard hormone therapies. Current treatments, including chemotherapy and radiation, often damage both cancerous and healthy cells, highlighting the need for targeted therapies. The receptor tyrosine kinase-like orphan receptor 1 (ROR1) is an oncoprotein overexpressed in several cancers, including breast cancer, but minimally present in normal tissues. Inhibiting ROR1 signaling has been shown to induce apoptosis and reduce proliferation of TNBC cells. A novel compound, CPD4, was identified through in-silico docking for its ability to inhibit ROR1's pseudo-kinase domain. In-vitro studies demonstrated that CPD4 suppresses cell viability and induces apoptosis at 2-10 μM concentrations in different TNBC cell lines, while sparing normal breast cells. CPD4 also inhibits migration, invasion and induces G2/M-phase arrest in TNBC cells. Its mechanism of action involves reducing the downstream markers of ROR1 signaling, specifically the phosphorylation of the pro-survival AKT/GSK3β pathway. In 3D spheroid culture, CPD4 reduces TNBC spheroid size and stemness development, indicated by the ratio of CD44/CD24 expression. These findings suggest that CPD4 is a promising therapeutic option for TNBC and may have potential for other cancers that overexpress ROR1.

B503/P1627

Activation of WTp53 Following Prolonged Stress Drives Melanoma Progression

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Metastatic melanoma is highly aggressive and resistant to multiple types of stress. Stress induced changes in tumor cells and the surrounding environment alter progression by changing the ability of immune cells to infiltrate the tumor, the extracellular matrix, tumor cell proliferation, and response to treatment. Multiple

types of stress (DNA damage, targeted therapy, aging) increase WTp53, which promotes a slow-cycling metastatic phenotype. Inhibiting WTp53 in an aged microenvironment promotes sensitivity to targeted therapy. Age induced changes in fibroblasts and tumor cells promote a slower growing, but highly invasive phenotype. In metastatic melanoma, RNA seq analysis of pathways downstream of WTp53 revealed a connection between WTp53 and markers of poor patient prognosis. Many of these markers are also markers of a cancer associated fibroblast phenotype including FAP, NTNG1, LRRC15, and FN1. Fibronectin (FN1) has been linked with invasion and therapy resistance in melanoma. Melanoma differs from other solid tumors, in that invasion and proliferation are largely uncoupled and often antagonistic such that cells that are more proliferative tend to be less invasive. Inducing a phenotype switch by adding recombinant Wnt5A to proliferative melanoma cells, increases invasion but is not sufficient to increase WTp53 expression. Long term stress such as treatment with targeted therapy, to generate resistant cell lines, increases WTp53 expression. Knocking down WTp53 decreases invasion and promotes sensitivity to treatment. While WTp53 is classically regarded as a tumor suppressor, our findings suggest a context-dependent role in promoting melanoma survival and progression following stress.

B515/P1639

AMPK Regulation of Syntaphilin Controls Cancer Cell Proliferation

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Metastasis is a multistep process by which primary tumors acquire malignant and invasive properties and successfully adapt to the changing environment as they disseminate and colonize distal sites. Cancer cells are continually reprogramming their metabolism to survive in these new environments. In this context, mitochondria have been shown to facilitate tumor progression via their pleiotropic functions. However, we lack a comprehensive understanding of how mitochondria support metastasis, particularly in the context of nutrient deprivation, which is a common feature of the tumor microenvironment. The serine/threonine AMP-activated kinase (AMPK) is responsible for maintaining energy homeostasis and becomes activated during nutrient deprivation. In cancer, AMPK has been shown to act as a tumor suppressor and our lab previously demonstrated that expression of a constitutively active (CA)-AMPK in tumor cells reduces metastatic burden in mice. While AMPK is known to regulate mitochondrial function, only a few mitochondrial proteins are direct AMPK substrates. Here we identified the outer mitochondrial membrane protein Syntaphilin (SNPH) as a novel substrate of AMPK. SNPH has pleiotropic roles in mitochondrial bioenergetics and mitochondrial trafficking and modulates cell plasticity (sustains cell proliferation at the expense of cell motility) in the context of metastasis. Using glioblastoma cells as a model, we show that SNPH knockdown led to impaired cell proliferation, without affecting viability. To learn the role of AMPK-mediated phosphorylation of SNPH in cell proliferation, we expressed wildtype (WT) SNPH, non-phosphorylatable and phospho-mimetic SNPH mutants. We also characterized the interactome associated with SNPH phosphorylation and found that cell cycle and signal transduction were amongst the top pathways regulated by SNPH phosphorylation. Overall, we propose a novel signaling axis where AMPK phosphorylates SNPH to control cancer cell proliferation.

B501/P1625

An Approach to Inhibit Cancer Cell Migration and Growth by Targeting Non muscle Myosin II And Topoisomerase I

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Conventional combination cancer therapies largely inhibit different signalling pathways within the same cellular process. However, limited studies have simultaneously targeted two distinct cellular processes like growth and migration—to combat cancer metastasis. Cancer metastasis relies on the ability of cancer cells to adapt their migration modes to navigate complex tissue environments, while proliferation is driven by growth and DNA repair mechanisms. It has been hypothesized that inhibiting mechanoresponsive and growth pathways could provide an effective strategy in treating cancer. Here, we report a polymersome-based delivery approach using PEG-PDS-PEG (Poly) to encapsulate (-) Blebbistatin (B), the selective inhibitors of Non muscle Myosin II, an actin binding motor protein and Camptothecin (C), inhibitor of Topoisomerase I, a DNA binding protein involved in replication and repair. This PolyB+C system enables controlled and concurrent release of both inhibitors in mouse 4T1 and human MDA-MB-231 breast cancer cells, leading to reduced cell growth and migration as demonstrated by MTT, soft agar colony formation, transwell, wound healing, and live-cell imaging assays. Importantly, tail vein administration of PolyB+C in tumor-bearing mice significantly suppressed tumor growth and metastasis. Our findings establish a therapeutic paradigm that efficiently prevent cancer progression by inhibiting cancer cell migration and proliferation.

B521/P1645

ANP32B Upregulation by the TGF-ß1/PI3K/AKT Cascade Promotes Oral Cancer Progression through THBS1 Secretion

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Oral cancer ranks sixth among cancer-related deaths and causes over 3,100 deaths annually in Taiwan. More than 90% of oral cancers are oral cavity squamous cell carcinomas (OSCCs). OSCC cells frequently metastasize through the lymphatic system, leading to treatment challenges and poor patient outcomes. This study aimed to identify drivers of OSCC metastasis and elucidate the molecular mechanisms by which drivers promotes OSCC progression. Using iTRAQ-based tissue proteome analyses, we identified acidic nuclear phosphoprotein 32 family member B (ANP32B) as a candidate driver of OSCC progression. ANP32B gene expression was increased in OSCC tissues, and its higher level was associated with poorer survival of OSCC patients, suggesting its critical role in OSCC progression. Subsequently, functional assays demonstrated that ANP32B enhanced the migration and invasion abilities but did not affect proliferation of OSCC cells. Moreover, ANP32B was upregulated by the TGF-β1/PI3K/AKT cascade. To further investigate the underlying mechanism of ANP32B-mediated migration promotion, we conducted TMT-based mass spectrometry to detect proteins differentially expressed in ANP32B-knockdown OSCC cells. We found that thrombospondin 1 (THBS1) is a downstream protein of ANP32B. Knockdown of ANP32B reduced the expression and secretion of THBS1 in OSCC cells, and higher THBS1 gene expression was correlated with lymph node metastasis in OSCC. In summary, ANP32B is upregulated by the TGF-β1-mediated activation of PI3K/AKT pathway and promotes OSCC cell progression by increasing THBS1 expression. These findings may offer valuable insights for the development of novel therapeutic strategies for OSCC.

B512/P1636

Chronic glyphosate exposure at low doses drives acquisition of stemness in breast cancer cells

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The objective of this study was to determine whether chronic exposure to glyphosate, the most widely used herbicide worldwide and classified as a probable human carcinogen, promotes stem-like and aggressive properties in breast cancer cells. Glyphosate is a broad-spectrum organophosphate herbicide extensively applied in agriculture, and human exposure occurs through food residues, water contamination, and occupational handling. Although its carcinogenic potential remains debated, growing evidence indicates that glyphosate can act as an endocrine disruptor and modulate signaling pathways implicated in tumor progression. To address this question, human breast cancer cell lines MCF7 and MDA-MB-231 were continuously exposed to low, non-cytotoxic concentrations of glyphosate for 8 weeks. Functional assays included mammosphere formation efficiency, soft agar colony formation, cell proliferation, transwell migration and invasion, and cytotoxicity assays with doxorubicin. Molecular analyses of stemness-associated transcription factors were performed by qPCR and Western blotting. The results revealed that chronic glyphosate exposure significantly enhanced mammosphere size and self-renewal efficiency, indicating enrichment of stem-like features. Treated cells exhibited increased proliferation, anchorage-independent growth, and marked stimulation of migratory and invasive behaviors compared with controls. Importantly, glyphosate exposure conferred a survival advantage under doxorubicin treatment, demonstrating the acquisition of chemoresistant traits. At the molecular level, prolonged exposure induced strong upregulation of OCT4 and SOX2, while NANOG expression was diminished, suggesting a selective reprogramming of transcriptional networks governing pluripotency. In conclusion, chronic low-dose glyphosate exposure promotes the acquisition of stem-like properties and enhances aggressive behavior in breast cancer cells. These findings provide novel evidence that an environmental contaminant, at concentrations relevant to human exposure, may contribute to breast cancer progression and therapy resistance by reshaping the molecular circuitry of stemness.

B517/P1641

Deficiency of vasodilator-stimulated phosphoprotein exacerbates the cell mobility of oral squamous cell carcinoma by modulating focal adhesion adapter proteins

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Focal adhesions (FAs), which anchor cells to the extracellular matrix (ECM) and connect to actin-based structures such as lamellipodia, microspikes, and filopodia, are essential for mediating cell movement. FAs are large, dynamic, and complex structures composed of integrin clusters and multiple FA-associated proteins. Vasodilator-stimulated phosphoprotein (VASP), a member of the Ena/VASP family, plays a critical role in regulating the actin cytoskeleton, thereby influencing processes such as cell shape, cell–cell interactions, and migration. However, the specific role and underlying molecular mechanisms of VASP in oral squamous cell carcinoma (OSCC) motility remain poorly understood. In this study, we investigated VASP expression in metastatic and non-metastatic OSCC tissues using immunohistochemistry (IHC). We further examined the co-localization of VASP with FA adaptor proteins (e.g., paxillin, zyxin) by immunofluorescence (IF) and assessed the functional role of VASP in OSCC cell motility through transwell assays following VASP knockdown with small interfering RNA (siRNA) both in vitro and in vivo. Our results demonstrated that VASP was overexpressed in metastatic OSCC tissues compared with non-metastatic tissues. Loss of VASP disrupted its co-localization with FA proteins and impaired the formation of filopodia and actin cytoskeleton remodeling at the leading edge of cells. Functionally, VASP inhibition significantly reduced the migration and invasion capacity of OSCC cells in vitro. In vivo, VASP knockdown not only suppressed tumor growth in the lungs of nude mice but also reduced

the expression of the metastatic marker hAlu. Taken together, our findings indicate that VASP is a key regulator of OSCC metastasis, acting through interactions with FA adaptor proteins and the actin cytoskeleton. These results suggest that VASP-mediated cytoskeletal remodeling plays a major role in OSCC progression and may represent a promising therapeutic target for controlling metastasis.

B508/P1632

Defining How Rnd3, a Novel Regulator of Breast Cancer Invasion, Affects the Metastatic Potential of Triple-Negative Breast Cancer

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The purpose of this study is to investigate whether the atypical Rho GTPase, Rnd3, affects triple-negative breast cancer (TNBC) cells metastatic potential (invasion and migration rates). One of the most dangerous attributes of cancer is its ability to metastasize and spread. The 5-year survival rate for localized breast cancer is 90%. However, this drops to 29% when breast cancer metastasizes. Identifying key regulatory proteins and signaling pathways involved in cell migration and invasion, two hallmarks of metastasis, will pave the way to potential therapeutic targets for reducing the metastatic potential of breast cancer. Rho GTPases regulate cellular processes that are commonly dysregulated in cancer with many studies focusing on what roles these proteins play in tumorigenesis. The expression level of the atypical Rho GTPase, Rnd3, rather than Rnd3 mutations, has been linked to cancer. High expression of Rnd3 increases cellular migration and invasion in several cancers, while depletion of Rnd3 decreases cellular proliferation and migration. How Rnd3 expression affects breast cancer progression is unknown. Here we assayed a panel of TNBC cell lines and assessed their migration and invasion potential, we observed a correlation of high Rnd3 expression and high cell migration and invasion rates. After knocking down Rnd3 expression in these cell lines we observed a significant reduction in cell invasion in MB-231, HCC1937, and MCF7 cells, and decreased cell migration in MB-231 cells. Rnd3 has been shown to regulate cell migration via the RhoA-ROCK1 pathway in melanoma. We were unable to rescue invasion rates in Rnd3 knockdown MB-231 cells following either chemical inhibition or knockdown of ROCK1, indicating Rnd3 is regulation cell invasion via a novel pathway in TNBC cells. Our findings indicate that Rnd3 regulates cell invasion and migration, two hallmarks of metastasis, in TNBC cells.

B522/P1646

FILIP1L regulates breast cancer invasion in a cell cycle- and EMT-dependent manner by controlling invadopodia function

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The epithelial-to-mesenchymal transition (EMT) is a central driver of breast cancer invasion and metastasis, yet the contribution of intermediate EMT states remains unclear. The objective of this study was to determine how different EMT states regulate the cell cycle phase during which Triple Negative Breast Cancer (TNBC) cells degrade the extracellular matrix (ECM) and invade. We used the hybrid epithelial/mesenchymal (E/M) TNBC breast cancer cell line 4T1 and induced EMT progression by either transforming growth factor β1 (TGFβ1) treatment to achieve a late E/M state and Slug overexpression to generate a fully mesenchymal (M) phenotype. Cell cycle phases were monitored using FUCCI reporter, and invasion was assessed with invadopodia degradation assay and 3D spheroid invasion assays. Bulk mRNA sequencing was performed on

cells sorted by EMT state and cell cycle phase to identify molecular regulator of invadopodia-dependent invasion. We found that early E/M cells primarily degraded ECM and invaded during the G2 phase, whereas late E/M and M cells shifted invasion to the G1 phase. Transcriptomic analysis identified FILIP1L as a top candidate linking EMT and cell cycle control of invadopodia. FILIP1L expression was enriched in G2-phase early E/M cells and G1-phase late E/M cells, mirroring the invasion window of each population. FILIP1L colocalized with invadopodia markers Tks5 and cortactin, confirming it as a novel invadopodia component. Functional studies revealed that loss of FILIP1L increased ECM degradation but reduced motility, leading to impaired 3D invasion. Kaplan–Meier survival analysis further showed that high FILIP1L expression correlated with poor breast cancer prognosis. In conclusion, EMT progression determines the cell cycle phase of invadopodia-mediated invasion, with early E/M cells invading in G2 and mesenchymal cells invading in G1. FILIP1L acts as a novel invadopodia component that links EMT and the cell cycle to invasion dynamics and may serve as a prognostic marker in TNBC.

B510/P1634

Identifying Drivers of Cell Invasion and Metastatic Progression in Triple Negative Breast Cancer

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The 5-year survival rate for localized breast cancer is 90%, this drops to 10% when metastasis occurs. Triple negative breast cancer (TNBC) exhibits a worse prognosis compared to other types of breast cancer due to its highly metastatic phenotype. The prevalence and lethality of TNBC makes identifying drivers of metastatic progression vital. The first crucial step of metastasis is invasion through the basement membrane. Cell invasion is considered a hallmark of metastasis and can be used as a predictor of metastatic potential in vitro, yet the signaling pathways that regulate cell invasion are not fully defined. Here we focus on cell invasion as a hallmark of metastasis, we generated highly invasive TNBC cell lines and performed proteomic analysis with the goal to identify novel regulators and signaling pathways regulating cell invasion and metastatic potential. We generated a panel of increasingly invasive TNBC (MDA-MB-231, HCC1937) cell-lines by seeding parental cells onto Matrigel coated transwells, propagating only invading cells for subsequent rounds of selection, generating invasive cell-lines (i8) cells after 8 rounds of selection. Multiple i8 cell lines were generated, and we observed a significant increase in invasion in several of our generated lines, which were taken forward for further proteomic analysis. Phenotypic analysis of other metastatic hallmarks was performed on our i8 cells and compared to parentals: Cell migration was analyzed with a wound healing assay and no significant changes in cell migration were observed. A significant increase in cell viability (CCK8 assay) was observed for our MB-231 i8A cell line only, whilst no change was observed in cell cycle progression (propidium iodine, Flow Cytometer) in either the MB-231 i8A or any other i8 cell line compared to their parental cells. We seemed to have selected specifically for increased invasiveness and not just increased mobility or proliferation. We sent 4 samples from each of our i8 lines and their parental controls cells (MB-231 parental, i8A, i8B and HCC1937 parental, i8C) for data independent acquisition (DIA) mass spectrometry to obtain a global protein expression profile comparing i8 protein expression to parental. Analysis of differentially expressed proteins revealed changes in pathways known to affect cell invasion including Hedgehog signaling, cell adhesion, and the Rho GTPase cycle, to name a few. We also observed changes in pathways not known to affect invasion for example: response to osmotic stress, ion homeostasis, and immune system interaction. Identifying how these pathways converge to regulate cell invasion will aid in understanding how cell invasion is regulated. The current findings from this project provide a promising framework for future discovery and drug development.

B496/P1620
Mitochondria Anchor Local Translation and Drive Metastatic Migration
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Background & Aim: Metastasis remains the principal cause of mortality in breast cancer. While cell migration is essential for metastasis, how spatio-temporal mitochondrial dynamics at the leading edge drive this process remains elusive. We hypothesised that spatially polarised mitochondrial potential and VDAC1 activity at the leading edge fuel persistent and high-speed migration (needed for metastasis) through control of local translation. Methods: We combined high-resolution live-cell microscopy, super-resolution immunofluorescence imaging, with optogenetic tools, micropatterning, and various high-dimensional microscopy techniques on breast cancer cell lines with distinct metastatic potentials (MDA-MB-231, MDA-MB-468, MCF7), pharmacological perturbation of mitochondrial potential and VDAC1 oligomerisation, and multi-omics analyses (TCGA, CCLE, proteomics) along with molecular biology to dissect mitochondria–cytoskeleton–ribosome crosstalk. Results: Highly metastatic MDA-MB-231 cells displayed fragmented mitochondria spatially enriched at protrusions, with elevated mitochondrial membrane potential (ΔΨm) and dynamic ATP flux compared to perinuclear, mostly elongated mitochondria. VDAC1 expression was significantly upregulated in breast cancer patients (TCGA, n>1,000) and >55 BRCA cell lines, correlating with poor survival. VDAC1s also oligomerize and are enriched in protrusions, and conductance via oligomeric VDAC1s is hindered by blocking the pores with microtubule interaction. Fragmented mitochondria transport ribosomes to the leading edge, enabling local translation of focal adhesion and mechanosignaling proteins that sustain high-speed migration. Perturbation of ΔΨm (with FCCP or photoactivable uncoupler) or VDAC1 oligomerisation (with VBIT-12) activity reduces the migration speed and directionality. It closely mimics the photo-activable translational inhibition. These perturbations collapsed mitochondrial polarity, impaired cytoskeletal organisation, and reduced membrane ruffling. Mechanistically, due to impaired ribosome hitchhiking and local translation (reduced expression of EIF1B, EEF1A2), migration speed and persistence were altered. Conclusion: We identify a heretofore unknown mechanism of leading-edge mitochondrial potential dynamics, maintained by VDAC1, as a mechanometabolic driver of metastatic migration. Targeting this axis disrupts local translation and cytoskeletal organisation, offering a new therapeutic avenue to hinder metastasis.

B519/P1643
Mutation at loop 2 of Nonmuscle Myosin II-C Causes Cellular Plasticity in Cancer Cells
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Nonmuscle myosin II (NMIIs) are cytoskeletal motor proteins that regulate mechano-adaptation in cancer progression. Although mutations in one of the paralogs, NMIIC, have been detected in cancer, their roles in cancer progression remain elusive. Here, we characterize six point mutations in the NMIIC identified in gingiva-buccal oral squamous cell carcinoma, which were distributed across the N-terminal motor (head) domain (Glu291Gln, Asp597Tyr, Ser655Arg and Gln890Lys) and the C-terminal coiled-coil (tail) domain (Glu1733Gln, Glu1797Asp). Out of these six mutations, Ser655Arg, located at loop 2 of the motor domain, enhanced actin-dependent ATPase activity. Confocal microscopy and FRAP analyses revealed that Ser655Arg displayed slow fluorescence recovery and markedly reduced mobile fractions at the lamellipodia. Notably, unlike other variants, Ser655Arg did not form hetero-filaments with NMIIA or NMIIB. Interestingly, all the motor domain mutants disrupted lamellipodial localization and induced blebbing phenotype in human breast cancer cells,

MDA-MB-231, but this effect was statistically significant with Ser655Arg. Altogether, these data suggest that point mutation, Ser655Arg at loop 2 of motor domain displayed different biochemical properties compared with wild type, that can facilitate cancer progression by enabling cellular plasticity.

B497/P1621

Nuclear envelope rupture of breast cancer cells drives epigenetic and inflammatory reprogramming

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Rapid growth of in situ solid tumors causes the accumulation of compressive stress at the tumor periphery, resulting in cancer cell confinement and nuclear deformation. This can lead to nuclear envelope rupture (NER), which exposes nuclear DNA to the cytosol where nucleases generate extensive DNA damage. These NER events are found at the invasive margins of many solid tumors, including human ductal carcinoma in situ (DCIS), and are thought to promote the progression from pre-invasive to invasive disease. To study these events in vitro, we use 2D microfabricated devices to confine cells to precise heights. Following NER, malignant cells recover and acquire a partial-EMT phenotype characterized by Snail1 upregulation and increased collagenolytic activity. As the nuclear envelope and lamina are known to regulate chromatin accessibility, loss of nuclear envelope integrity due to mechanical stress may trigger extensive chromatin reorganization. Indeed, epigenetic reprogramming has been documented as an adaptive response to mechanical stress in many cell types, as it directly modulates the physical properties of the nuclear envelope and, in addition, supports cell plasticity. However, we do not yet appreciate the extent of the reprogramming, nor its longevity, following NER. Given this, we hypothesized that confinement-induced NER alters chromatin organization, and that this epigenetic reorganization maintains NER-induced behavioral changes. To test this, we performed bulk RNA-sequencing on confined and non-confined DCIS cells, which revealed significant epigenetic remodeling. We observed an increase in histone demethylases and acetyltransferases following confinement-induced NER, confirmed by western blots and immunofluorescence. These results are consistent with an open, active chromatin state. Additionally, we found that DCIS cells maintain this euchromatic, invasive state up to 3 days post-confinement, indicating that NER-induced reprogramming is durable. Interestingly, our RNA-seq also revealed an NER-induced inflammatory response driven by NF-κB. Because myeloid cells represent the largest immune cell population in solid tumors, we focused on DCIS cell regulation of monocyte differentiation and activation. By culturing primary human monocytes in supernatant from DCIS cells previously subjected to confinement-induced NER, we found that NER shapes paracrine signaling of DCIS cells, which directs monocytes towards a phagocytic and anti-inflammatory macrophage state. This work suggests that NER events drive durable signaling changes that promote both invasiveness and immune evasion.

B506/P1630

Padina arborescens extract inhibits oral squamous cell carcinoma via double-strand DNA break-mediated cell death

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Oral squamous cell carcinoma (OSCC) is an aggressive malignancy characterized by high recurrence, poor prognosis, and limited treatment options, highlighting the urgent need for novel therapeutic strategies. This study aimed to investigate the anti-cancer potential of Padina arborescens (P. arborescens) extract in OSCC.

Bioactive compounds were obtained through enzymatic hydrolysis using Viscozyme. To evaluate anti-cancer efficacy, EdU and TUNEL assays were performed, and Western blotting was used to analyze underlying mechanisms. Our results showed that P. arborescens significantly reduced OSCC cell viability and proliferation, accompanied by the induction of double-strand DNA breaks and suppression of DNA repair. Moreover, apoptosis was promoted, as evidenced by increased cleaved caspase-3 expression and a higher proportion of TUNEL-positive cells. In vivo, xenograft experiments demonstrated that P. arborescens effectively reduced tumor size and weight, while in a metastasis model, bioluminescence analysis revealed decreased lung metastasis. Collectively, these findings indicate that P. arborescens exerts anti-cancer effects against OSCC by promoting DNA damage–mediated apoptosis and inhibiting tumor progression, supporting its potential as a candidate for further development in OSCC therapy.

B498/P1622

Perturbations in thyroid hormones promote invasiveness in breast cancer

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For decades, thyroid hormone (TH) signaling has been linked to breast cancer. Breast cancer patients have a higher incidence of thyroid cancer, and thyroid cancer patients have a significantly higher incidence of breast cancer, supporting a link between TH signaling and breast malignancy. However, despite numerous correlative studies, the role THs play in mammary tumorigenesis remains unclear. In a small pilot study utilizing the City of Hope Cancer registry, we examined outcomes from 984 breast cancer patients who had undergone comprehensive TH analysis. Interestingly, we found that thyroid stimulating hormone (TSH) was elevated in breast cancer patients with invasive disease compared to their in situ counterparts. Additionally, higher levels of TSH and total T4 correlated to later metastasis. Together, these data implicate heightened THs in worsened disease outcomes for breast cancer patients. To more acutely investigate the impact of THs in breast malignancies, our lab utilized the MCF10a human mammary epithelial cell line. We found that these cells display sensitivity to both T4 and TSH added to culture medium. Additional in vitro work shows that breast cancer lines (MDA-MB-231, MCF7, T47D) treated with THs experience increased capability for anchorage-independent growth as well as mammosphere formation when compared to vehicle-treated controls. Mammospheres also display cell-line dependent shifts in average size and circularity. Overall, this indicates that THs may promote a more invasive or stemlike phenotype. To interrogate the effect of THs in vivo, we utilized the MMTV-PyMT mouse model of breast cancer. Mice were treated with LT4 or the anti-thyroid drug PTU (to lower T4 levels, thereby increasing TSH). Both cohorts of mice developed significantly larger and more invasive and necrotic tumors than untreated controls. Future work in the lab will focus on utilizing RNA-sequencing to assess changes in gene sets related to stemness or invasion, as well as evaluate shifts in breast cancer subtype that may result from TH treatment. From these data, we concluded that even minimal perturbations in normal TH levels can stimulate mammary cells, thereby increasing the risk of breast cancer development as well as progression to aggressive disease. Our data highlight a strong need to further investigate the role of hypo- and hyperthyroidism in breast cancer.

B502/P1626

Phenotypic Plasticity and Cell Number Cooperatively Regulate Proliferative Potential

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Carcinomas arise when epithelial cell proliferation exceeds cell death, and such cells become invasive. Most carcinoma-related deaths result from metastasis. One mechanism thought to promote metastasis is the epithelial-mesenchymal transition (EMT), whereby cells lose their adherent, non-invasive properties, thus enabling their escape from the primary tumor to a secondary site. Cells that have undergone EMT display reduced proliferation, and mesenchymal-epithelial transition (MET) is thought to be required for proliferation and formation of harmful macro-metastases at the secondary site. EMT/MET are not necessarily discrete, binary phenotypes, and carcinoma cells exhibit variable phenotypes along the EMT/MET spectrum. Studies suggest that invasive cells, including metastatic colonizing cells, can exist individually or in clusters. Cells within a cluster may display varying degrees of EMT/MET phenotypes, and such cells could differentially impact the proliferation and survival of neighboring cells. We aim to understand the dynamics of cell proliferation and survival depending on the number of cells present to model high-cell number macro-metastatic tumors compared to low-cell number micro-metastatic tumors, both in EMT and MET states. Moreover, we wish to determine the effects that cells undergoing EMT have on adjacent cells in a MET-state and vice versa. As a first step in this, we used MCF10A immortalized human epithelial cells as a model given its tractability in EMT studies. We treated cells with vehicle or TGFb1 to induce EMT and confirmed morphological and molecular EMT. We plated cells under low-, medium-low-, medium-high-, and high-cell number conditions, pulsed with EdU to label cells in S-phase, and detected proliferating cells using fluorescence microscopy. Under both control and EMT-inducing conditions, cell proliferation decreased under high-cell number confluent/near-confluent conditions. Low cell-number control vs. EMT cells exhibited similar proliferation rates, while the medium and medium-high cell number conditions exhibited lower proliferation in the EMT group. Interestingly, EMT-induced cells exhibited a higher proliferation rate at high cell numbers relative to control cells, suggesting that contact inhibition caused by high cell numbers may be attenuated in an EMT state. We conclude that cells in EMT vs. MET states exhibit different growth dynamics depending on the number of cells present, and this may reflect how carcinoma cells proliferate in vivo. Our future directions will employ multiple cancer models to quantify the proliferation and survival of cells under the conditions described above and to determine the proliferative effects that cells in EMT vs. MET states have on neighboring cells.

B509/P1633

Rnd3 Regulates Lung Cancer Cell Migration and Invasion via Integrin Alpha 5 Expression

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The purpose of this study is to investigate the pathways by which Rnd3, an atypical Rho GTPase, promotes lung cancer metastatic hallmarks, cell migration and invasion. Lung cancer is the most common and deadliest form of cancer. Most lung cancers metastasize resulting in poor prognosis, with lung-to-brain metastasis being the number one killer of lung cancer patients. Understanding the underlying mechanisms of metastasis is critical to identify new therapeutic strategies for lung cancer treatment. Our analysis of The Cancer Genome Atlas revealed that lung adenocarcinoma patients with low Rnd3 expression have better survival outcomes. To determine if there is a molecular basis for this correlation of improved survival for lung adenocarcinoma patients with low Rnd3 expression, we investigated the role of Rnd3 in tumor progression by knocking down Rnd3 expression in lung adenocarcinoma cell lines, and in patient-derived lung-to-brain metastasis (PDLBM) cell lines (601 and 620) as a proxy for advanced disease. Rnd3 depletion had no significant effect on cell viability or cell cycle progression but significantly reduced invasion and migration, two hallmarks of metastasis. We show that decreased Rnd3 expression decreases the ability of lung adenocarcinoma cell lines to migrate

and invade, and that this pathway is still functioning and targetable in advanced disseminated disease. Previous studies on Rnd3 in melanoma cells and fibroblasts have found that Rnd3 signals via the RhoA-ROCK1 pathway, which impacts the actin cytoskeleton, ultimately regulating cell migration and invasion. However, in lung adenocarcinoma and PDLBM cells, neither chemical inhibition of ROCK1 nor knocking down ROCK1 expression could rescue the invasion or migration phenotype back to wildtype levels in Rnd3 depleted cells, suggesting a novel mechanism of Rnd3 regulation of cell migration and invasion in lung adenocarcinoma cells. Cell adhesion to the extracellular matrix is vital for the cells ability to migrate and invade, we investigated the expression levels of integrins in our Rnd3 knocked down cells and observed a significant increase in integrin alpha 5 expression compared to control cells. We then tested if this increase in alpha 5 expression effected the cells ability to migrate and invade by performing double knockdown of Rnd3 and alpha 5 in lung adenocarcinoma cell line (A549), which restored both cell migration and invasion rates back to wildtype levels, whilst alpha 5 knockdown alone had no effect. These results revealed a novel pathway by which Rnd3 promotes lung cancer metastatic hallmarks via modulating alpha 5 integrin expression.

B500/P1624

Role of Profilin1 in regulation of tumorigenic response: Involvement of Angiomotin

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Malignant tumors are marked by their metastatic potential with cell migration being pivotal to tumor invasion. This migration relies on the dynamic remodeling of the actin cytoskeleton, orchestrated by various actin-binding proteins. Profilin1 (PFN1), a G-actin-binding protein is notably under-expressed in several cancers, including breast, pancreatic, and hepatic carcinomas. Mass spectrometry data suggested the PFN1 interaction with Angiomotin (AMOT). AMOT is a component of the Hippo pathway, where it inhibits the nuclear localization of Yes-associated protein (YAP) by cytoplasmic retention at tight junctions. YAP is the coactivator of the transcription factor TEAD, crucial in regulating the expression of genes controlling organ size control, tissue regeneration, and angiogenesis (including CTGF). Previous studies reported that PFN1 interacts with PTEN and Akt through its poly-L-Proline rich binding domain independent of its Actin binding domain, thereby rendering stability to the said proteins and also recognizing the additional role of PFN1. The interaction of PFN1 with AMOT was established by Co-Immunoprecipitation. AMOT+/+ and PFN1+/+ MDA MB-231 cells were generated and also ATRA (All Trans-Retinoic acid-FDA approved), a known inducer of PFN1 was used. Under PFN1 induced conditions of AMOT+/+ cells, YAP was arrested in cytoplasm. This was also reflected at transcript levels of CTGF-TEAD as shown by EMSA. Wound healing and Transwell assays were performed to understand the cell migration and invasion status respectively, which were observed to be attenuated in AMOT+/+ cells upon PFN1 induction and were comparable to that of PFN1+/+ cells. Immunoflourescence assay was performed to check the stability of AMOT in the absence and presence of MG132, where AMOT was visible in PFN1+/+ cells and also in MG132 treated conditions. Till now, findings suggest the PFN1 interaction with AMOT enables YAP cytoplasmic retention thereby restricting migration of MDA MB-231 cells. PFN1 might be protecting AMOT from proteasomal degradation. PFN1 might play a role in the Hippo pathway.

B504/P1628

Role of TET1 inhibition in IL-1 augmentation of TGF-B induced Epithelial to Mesenchymal transition in A549 lung adenocarcinoma cells

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Introduction: Lung cancer is one of the leading causes of cancer death worldwide. Epithelial-mesenchymal transition (EMT) plays a role in the fatality of lung cancer by decreasing epithelial characteristics and allowing for migration and invasion. IL-1 enhances TGF-β induced EMT in A549 lung adenocarcinoma cells. Previous experiments showed that IL-1 inhibits the induction of Ten-eleven-translocation-1 (TET1) by TGF-β and this is accompanied by additive inhibition of E-cadherin expression. TET1 is known to have a role in inhibiting the expression of zinc-finger-Ebox binding homeobox 1 (ZEB1), a repressor of E-cadherin. The current series of experiments aims to determine whether the inhibition of TET1 activity can enhance TGF-β induced EMT similarly to IL1. Methods: Real time qPCR was used to examine mRNA in A549 cells. Immunofluorescence was used to assess E-cadherin and demonstrate changing cell morphology in response to IL1 and TGF-β in the presence and absence of Bobcat339, a TET1 inhibitor. Results: TET inhibitor Bobcat339 had no effect on E-cadherin protein expression or progression to mesenchymal cell morphology on its own, but it did appear to enhance these effects of TGF-β in co-treated cells. Although IL-1 does enhance TGF-β induced expression of ZEB1 mRNA at 3 and 6 hours, inhibition of TET1 activity did not affect ZEB1 mRNA expression. Conclusion: Inhibition of TET1 activity may play at least a partial role in IL-1 enhancement of TGF-β induced EMT. However, the mechanism through which is acts is still undetermined. Increased expression of ZEB1 by IL-1 may play a role in its ability to enhance TGF-β induced EMT, but there is currently no evidence that this is achieved through inhibition of TET1 activity. Future experiments will examine expression of other EMT transcription factors.

B514/P1638

Role of the nuclear ATPase - BRG1 in nuclear mechanics and cell migration through confined spaces

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Deformation of the nucleus often acts as a barrier to cell migration through tight spaces, such as those encountered when cells move through tissues or across extracellular matrix barriers. Reorganizing the nucleus to pass through spaces much smaller than its resting diameter requires forces generated by the cytoskeleton, as well as active reorganization inside the nucleus driven by ATPases that crosslink or move chromatin. Here, we demonstrate that the nuclear ATPase BRG1/SMARCA4 motor of the BAF or SWI/SNF complex not only affects nuclear structure but also controls whole-cell motility in 3D. Inhibiting BRG1 makes the nucleus stiffer in karyoplasts from various cell types, including four different cancer cell lines and mesenchymal stem cells. The effects on nuclear stiffness match the effects of acute BRG1 inhibition on the ability of cells to migrate through tight spaces. Making the nucleus stiffer stops single cells from moving through tiny pores and prevents tumor cell spheroids from migrating outward into a collagen matrix. These findings highlight the importance of active motions inside the nucleus for its overall mechanics and how it deforms under external stresses.

B518/P1642

Serotonin Drives Amoeboid Plasticity in Melanoma via 5-HT7-PKA/VASP and Rho-ROCK Signaling

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Melanoma progression depends on migratory plasticity—the ability of tumor cells to switch between mesenchymal (protrusion-based) and amoeboid (bleb-based) modes to traverse confined spaces. Serotonin

(5-HT), a biogenic amine with broad immuno- and tumor-modulatory roles, has been implicated in cancer growth and invasion, yet how 5-HT shapes the cytoskeletal programs that enable melanoma dissemination is unclear. We hypothesized that serotonergic signaling tunes amoeboid behavior in confinement. Using A375-M2 melanoma cells, we find robust expression of the serotonin receptor 5-HT7 (HTR7). In microconfined assays, loss of 5-HT7—either by receptor depletion or by pharmacologic inhibition with dihydroergotamine (DHE)—significantly reduces the motility of amoeboid cells. Mechanistically, 5-HT7 couples to two pro-motility pathways. First, as a Gs-linked receptor, 5-HT7 elevates cAMP/PKA signaling: a PKA biosensor reports increased activity in serotonin-treated cells and decreased activity with DHE, and Western blotting shows enhanced phosphorylation of the PKA substrate VASP at S157/S239, modifications known to modulate actin dynamics. Second, 5-HT7 also engages Gα12 to activate Rho-ROCK; a ROCK biosensor reveals 5-HT–dependent increases in ROCK activity through 5-HT7, consistent with heightened contractility and bleb-based movement. Together, these data identify serotonin as a microenvironmental cue that promotes amoeboid migration of melanoma cells through 5-HT7-dependent activation of PKA/VASP and Rho-ROCK signaling. By positioning 5-HT7 as a dual-node regulator of cytoskeletal state, our work highlights serotonergic signaling as a potential therapeutic entry point to limit metastatic dissemination in confined tissue niches.

B511/P1635

STK11 loss induces stress-responsive programs supporting shear adaptation in circulating tumor cells from KRAS-driven lung adenocarcinoma

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Lung adenocarcinoma (LUAD), the leading cause of cancer mortality, is especially aggressive in tumors co-mutated in KRAS and STK11 (LKB1), which exhibit therapeutic resistance and high metastatic potential. Successful metastasis requires circulating tumor cells (CTCs) to survive the biomechanical and oxidative challenges of the bloodstream, yet how STK11 loss alters tolerance to fluid shear stress (FSS) remains unclear. Using an embryonic zebrafish xenograft platform with KRAS-mutant parental and STK11-null LUAD cell lines, we performed longitudinal imaging of CTC fate under physiologic circulation. Under glutamine-deprived conditions mimicking metabolic stress, STK11-null cells exhibited higher micrometastatic burden and more frequent extravasation at four days post-injection compared with parental controls. RNA-seq under glutamine deprivation, analyzed by Reactome GSEA, revealed enrichment of cellular response-to-stimuli pathways. An FSS-specific gene panel further demonstrated enrichment of shear-adaptive programs, notably NRF2-mediated antioxidant responses, highlighting the need to define these programs directly in vivo. These data support a model in which STK11 loss promotes CTC persistence through NRF2-driven adaptive survival under oxidative and mechanical stress. Ongoing work is quantifying the relationship between FSS and CTC survival using high-speed confocal imaging (200–500 fps) and Fiji/ImageJ-based velocity tracking, with planned pharmacologic modulation of cardiac output to experimentally vary shear forces in vivo. Future directions include defining molecular programs mediating shear-adaptive survival through candidate panels assessing NRF2 oxidative stress targets, YAP/TEAD outputs, and NF-κB/survival regulators. Collectively, this work identifies NRF2-mediated shear adaptation as a potential mechanism driving metastasis in KRAS/STK11 LUAD and a targetable vulnerability in shear-tolerant tumor cells.

B507/P1631

Stress granules induces tumor metastasis in oral squamous cell carcinoma.

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Stress granules (SGs) are non-membrane-bound protein-RNA aggregates assembled within the cytoplasm under various stress conditions, temporarily arresting transcription to prevent damage and enhance cellular recovery and survival. While SG-mediated translational arrest typically serves to repair stress-induced damage in mammals, it is known that SGs play a crucial role in carcinogenesis, tumor progression, and metastasis through interactions with diverse signaling pathways in cancer cells. Despite active SG research in a wide range of cancer diseases, the role of SG is still poorly understood in oral squamous cell carcinoma (OSCC). In this study, we investigated SG formation in OSCC and how SGs influence tumor progression and metastasis. At the tissue level, we confirmed that SG markers, including G3BP1, TIAR, and PABP, were significantly overexpressed in OSCC tissue compared to adjacent normal tissue. In an oral squamous cell carcinoma mouse model, pharmacologically inhibition reduced SG formation in OSCC xenograft tissue, leading to a significant decrease in tumor growth and metastasis.

B513/P1637

Stress Induced Signaling in Melanoma Progression and Survival: Role of NetrinG1

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Metastatic melanoma is a form of cancer with a 5-year survival rate of about 35% and it is highly therapy resistant. Melanoma progression, emergence from dormancy, and therapeutic resistance are influenced by alterations in the tumor microenvironment. Alterations in the tumor microenvironment are often driven by stressors such as DNA damage, aging and therapeutic treatment and result in an increase in non-canonical Wnt5A signaling in melanoma cells. Increased Wnt5A signaling is linked with therapy resistance and metastasis. We have identified a link between metastatic melanoma and increased expression of Netrin G1. Netrin G1, a protein originally identified in embryogenesis in the central nervous system, has recently been found to be expressed on cancer associated fibroblasts and linked with poor clinical outcome in pancreatic cancer. In this study, we explore the role of Netrin G1 in melanoma progression, with a focus on its following stress. Our data suggest that the expression of Netrin G1 correlates with AXL, a marker of metastasis and therapy resistance. We generated therapy resistant cells from early and late-stage human cell lines by treating cells with BRAFi/MEKi therapy for at least five weeks. We confirmed that the cells were resistant when we observed a decrease in apoptosis by flow cytometry following a rechallenge with drug. Following prolonged therapeutic stress, we observed an increase in WTp53 and Netrin G1 in cell lines, which before treatment had no or low WTp53 and Netrin G1 expression. Moreover, Netrin G1 expression increased in mouse melanoma tumors grown in aged (>52 wks.) compared to young (8 wks.) mice. Studies have previously shown that the aging microenvironment promotes metastasis, emergence from dormancy and alters response to therapy, suggesting that Netrin G1 plays a role in stress-induced melanoma adaptation. These findings reveal a novel

mechanism of melanoma resilience and identify Netrin G1 as a potential therapeutic target in therapy-resistant melanoma.

B505/P1629

Targeting SAE2 in non-small cell lung cancer to promote sensitivity to chemotherapeutics

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Lung cancer leads the list of annual cancer-related deaths, with non-small cell lung cancer (NSCLC) being the most common type, accounting for 80%-85% of cases. Acquired resistance to all the therapeutic approaches available seems inevitable and is one of the major contributors to treatment failure. It is widely accepted that SUMOylation, a post-translational modification in which SUMO is conjugated to proteins, plays a critical role in promoting cancer metastasis, proliferation, and chemoresistance. This justifies the need to search for new approaches to block the SUMOylation pathway. This project investigated a novel mechanism for inhibiting SUMOylation in cancer and enhancing sensitivity to chemotherapy. Using antisense oligonucleotides, we developed SAE2IN, an inhibitor that targets the SUMO Activating Enzyme 2 (SAE2), and explored its potential chemosensitizing activity using A549 and HCC827 cells, which encompass the most frequent mutations observed in NSCLC. SAE2IN's target specificity was evaluated using RT-qPCR and western blotting. Chemosensitizing effects were assessed by comparing cell responses to chemotherapy with and without SAE2IN. Our results demonstrate that SAE2IN specifically decreases SAE2 mRNA levels, downregulates SAE2 protein expression, decreases overall SUMOylation, enhances the cytotoxicity of cisplatin & etoposide, halts cell cycle progression, and reduces metastasis. This project bridges applied and basic science as it not only elucidates how targeting SUMOylation has therapeutic applications but also provides a new tool to further investigate SUMOylation regulation. Our results demonstrate SAE2IN's strong potential for clinical application in oncology. Furthermore, given the critical role of SUMOylation in overall cell physiology, SAE2IN may also provide therapeutic effects against other diseases.

B516/P1640

TRIM9 promotes melanoma tumor growth and metastasis and dampens immune response in vivo

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TRIM9 is a brain-enriched E3 ubiquitin ligase that acts as a potent regulator of actin dynamics, exocytosis, and membrane remodeling in developing neurons. Recent studies have documented TRIM9 expression outside the nervous system in several cancer types, including breast cancer, non-small cell lung cancer, and melanoma. However, the function of TRIM9 outside of the nervous system is unknown. Datamining of several scRNAseq studies indicates that TRIM9 is expressed in melanoma, but not its cell of origin, melanocytes. Melanomas undergo dynamic phenotype switching which influences treatment options and patient prognosis. TRIM9 appears enriched particularly in the neural crest like invasive melanoma subtype, which often correlates with poor prognosis. Here we investigate how loss of TRIM9 alters exocytosis in melanoma cells in vitro, and metastasis and immune responses in vivo to define the role of TRIM9 in melanoma progression. In neurons TRIM9 is a negative regulator of exocytosis. In a human melanoma cell line (1205Lu) cell, we show that loss of TRIM9 increased the rate of VAMP7-mediated exocytosis, particularly in starved conditions. Consistent with

increased secretion, we found increased levels of degradative enzymes, including MMP-2, in the conditioned media of TRIM9-/- cells compared to TRIM9+/+ cells. Interestingly we also found excess secretion of netrin-1, a classic axon guidance cue implicated in cancer progression. In addition, we found that loss of TRIM9 slows cell proliferation of 1205Lu cells in vitro. We next examined how loss of TRIM9 in a mouse model of melanoma altered disease progression. Primary melanoma were locally induced on the ear of PBT mice (harboring a floxed allele for PTEN tumor suppressor (P), a Cre-driven constitutively active BRAF (V600E) mutation (B), a Flox-Stop-Flox tdTomato reporter (T), and a tamoxifen- inducible melanocyte specific Cre (Tyr:CreER)) and Trim9F:PBT mice, to also specifically delete TRIM9 in tumor cells. Preliminary results indicate that TRIM9 loss reduces primary tumor growth and metastasis. Analysis of a panel of immune cells suggest that TRIM9 loss may slow disease progression by increasing systemic immune responses. This is consistent with examination of TRIM9 expression in human patients, which indicate that high TRIM9 expression correlates with poor response to current anti-PD1 and anti-CTLA immunotherapy approaches and poor patient prognosis. Together our findings suggest that TRIM9 regulates disease progression in vivo, likely by altering tumor cell proliferation, secretion, and systemic immune responses.

B523/P1647

Wnt-Driven Adhesion and Extracellular Matrix Dynamics in Breast Cancer Metastasis

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Triple-negative breast cancer (TNBC) is an aggressive subtype comprising 10–15% of U.S. cases and disproportionately affecting Hispanic and African American women. Its poor prognosis stems from high invasiveness and a lack of targeted therapies. TNBC is molecularly heterogeneous, with both canonical (β-catenin–dependent) and noncanonical (β-catenin–independent) Wnt signaling implicated in progression, though their roles in metastasis remain unclear. Using a TP53-null GEMM and a 7xTCF-eGFP Wnt reporter, we identified a shift from canonical to noncanonical Wnt signaling as metastases progress. Micrometastases exhibit high Wnt/β-catenin activity and lack Ror2, while macrometastases express Ror2 but lose canonical Wnt activity, suggesting a functional transition in Wnt states during metastatic progression. We hypothesize that Wnt signaling plasticity, driven by Ror2, regulates adhesion and extracellular matrix (ECM) remodeling to promote micro-to-macrometastatic progression in TNBC. To test this, we utilized spontaneous and experimental lung metastasis models to investigate Wnt signaling transitions in TNBC metastasis, integrating 7xTCF-eGFP reporter analysis, in vivo and in vitro vascular co-cultures, immunofluorescence, and histological analyses to map Wnt-driven alterations in metastatic progression, and functional assays to assess tumor cell adhesion and metastatic seeding. In our models, micrometastasis transitioned from Ror2-low/canonical Wnt-high to Ror2-high/canonical Wnt-low as they advanced to macrometastases. Ror2 loss enhanced seeding efficiency and tumor–vascular association, confirmed by in vivo metastasis assays and in vitro co-cultures. Bulk RNA-seq of Ror2-deficient cells showed an upregulation of integrin α5, implicating intrinsic tumor–ECM interactions in early lung colonization. Single-cell RNA-seq of metastasis-bearing lungs showed elevated ECM remodeling programs in pericytes and fibroblasts relative to healthy lungs, suggesting a broader stromal response to metastatic progression. We co-cultured mouse lung fibroblasts and/or endothelial cells with 7xTCF-eGFP labeled tumor cells to track Wnt/β-catenin activation. Fibroblasts showed strong 7xTCF-eGFP reporter induction, indicating that lung fibroblasts and CD31+ endothelial cells supply cues that boost tumor Wnt signaling responsiveness. This highlights reciprocal stromal–tumor signaling in tumor behavior. To test the contribution of ECM remodeling to seeding and survival, we found that blocking Wnt signaling dampened matrix changes within the lung metastatic niche. Our findings highlight Wnt signaling plasticity as a TNBC vulnerability and motivate future efforts to disrupt cell-matrix interactions during metastatic colonization.

Cancer Cell Biology - Cancer cell signaling and oncogenic pathways

B477/P1601
Adapter sequestration and EGFR suppression are widespread features among oncogenic RTK fusions
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Regulation of cancer cells by their environment contributes to tumorigenesis and drug response, though the extent to which the oncogenic state can alter a cell's perception of its environment is not clear. EML4-ALK is a receptor tyrosine kinase (RTK) fusion oncoprotein that forms condensates and suppresses transmembrane EGFR signaling in cancer cells. ALK inhibition restores EGFR signaling, thereby promoting survival and drug tolerance. Here we tested whether such modulation of EGFR activity was common among other RTK fusions, which collectively are found in ~5% of all cancers, and whether modulation is linked to oncoprotein condensation. Using live- and fixed-cell microscopy in isogenic and patient-derived cell lines, we indeed found widespread suppression of EGFR by active RTK fusions. However, suppression did not correlate with the formation of condensates but instead corresponded to the formation of low-order assemblies and oncogenic kinase activation. These assemblies triggered fusion activity and binding to Grb2, competitively inhibiting Grb2-mediated EGFR signaling from the membrane. Optogenetic probes confirmed that cytoplasmic sequestration of Grb2 was sufficient to suppress perception of EGFR stimuli, even in the absence of condensation or signaling downstream of the fusion. Our study uncovers shared functional consequences among RTK fusion oncoproteins that could be exploited for novel biomimetic therapies that suppress transmembrane RTK signals.

B466/P1590
Additional Mechanism for Xevinapant, an Inhibitor of IAPs, as an Enhancer for T2R14-mediated Apoptosis in Head and Neck Squamous Cell Carcinoma
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Background: Head and neck squamous cell carcinoma (HNSCC) has a high mortality rate with limited changes to the standard of care treatment over the past few decades. Inhibitors of apoptosis proteins (IAPs) are overexpressed in HNSCC and contribute to evasion of apoptosis and chemoresistance, making them plausible therapeutic targets. Xevinapant, an oral mimetic IAP inhibitor, has been shown to enhance the effects of cisplatin and radiation therapy in HNSCC cell lines and had some positive results in human clinical trials. Bitter taste receptors (T2Rs), a type of G-protein coupled receptor (GPCR), have also been shown to induce apoptosis in HNSCC. Specifically, lidocaine, a T2R14 agonist, has been shown to trigger a significant calcium response leading to mitochondrial depolarization, proteasome inhibition, and induction of apoptosis. Objective: We hypothesized that IAPs and T2Rs may share some apoptotic mechanisms and thus aimed to determine if Xevinapant 1) activates a T2R mediated apoptosis pathway and/or 2) enhances T2R14-activated apoptosis with lidocaine. Results: Using live-cell imaging in several HNSCC cell lines, we found that Xevinapant exposure causes a rapid, sustained intracellular calcium response that persists for up to 5 hours from extracellular stores and is not GPCR-mediated. This may indicate a novel mechanism of Xevinapant involved with ion channels and

calcium entry. Additionally, we found a significant reduction in cell viability (crystal violet), increased apoptosis (CellEvent), and increased calcium response (Fluo-4-AM) from lidocaine following Xevinapant pre-treatment compared to control, suggesting a potential cellular priming for T2R14-mediated effects. Exposure to Xevinapant for 24 hours significantly increased T2R14 mRNA measured via RT-qPCR, indicating that increased expression may contribute to the enhanced responses to lidocaine through upregulation of T2R14. Conclusion: This work suggests that IAP antagonists and T2R agonists, alone or in combination, may be novel therapeutics to treat HNSCC, warranting further investigation.

B465/P1589

Anandamide and arachidonic acid accelerate the development of androgen-independent adenocarcinoma in transgenic adenocarcinoma of the mouse prostate model

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The endocannabinoid anandamide exerts antitumor effects in different types of cancer, including prostate cancer cell lines. The excessive intake of its precursor, arachidonic acid, increases the risk of prostate cancer (PCa) development. However, the action of these lipids on PCa is influenced by several factors, such as the activity of fatty acid amide hydrolase enzyme, their derived bioactive lipids and the modulation of androgen signaling. Here we investigate the effects of dietary supplementation with arachidonic acid and anandamide treatment on tumor progression in transgenic adenocarcinoma of the mouse prostate mice (TRAMP). Animals (n=10/group) received daily oral gavage with either the vehicle (soybean oil only, C), 5mg/kg b.w. of anandamide (AEA) or 5mg/kg b.w. of arachidonic acid (AA), from 12 to 16 weeks of age, in accordance with local ethics committee guidelines (CEUA/IBILCE 259/2024; CEUA/UNICAMP 6606-1/2025). Histopathological analysis indicates that frequency of pathological lesions was unchanged, but the incidence of undifferentiated adenocarcinoma (UDAC) increased 30% in both AEA and AA groups. Furthermore, large primary tumors were found in the groups AEA and AA (C 0.05 ± 0.01g; AA 1 ± 0.02g; AEA 0.9 ± 1.7g). There were no changes in total cholesterol, LDL cholesterol, or triglycerides levels among the groups, but HDL cholesterol decreased in the AA group (C 79.4 ± 8.2mg/dL; AA 87.9 ± 24.6mg/dL, p=0.0231). Furthermore, triglyceride levels were 7-5-fold higher in animals with large primary tumors. Serum testosterone levels were dramatically reduced in the AA (0.4 ± 0.4ng/mL) and AEA (0.4 ± 0.2ng/mL) groups compared with C (5.1 ± 4.3ng/mL; p=0.0042). Based on these results, the subsequent analyses focused on the UDAC and the remaining prostate tissue. Immunohistochemistry for androgen-receptor (AR) indicated, as expected, that UDAC showed no AR-positive cells. The estimation of the proliferating cells frequency in UDAC, after immunolabeling for phosphohistone-H3 (pHH3) revealed no significant alterations. For the remaining prostate tissue it was observed an increase in frequency of AR-positive cells (C 84.7 ± 7.2%; AA 91.6 ± 4.9%; AEA 88.3 ± 4.9%; p=0.0016) without alterations in the frequency of proliferating cells (C 0.008 ± 0.002%; AA 0.007 ± 0.004%; AEA 0.009 ± 0.002%; p=0.75). Our results demonstrate exposure to anandamide and arachidonic acid lead to similar outcomes, with both compounds accelerating the development of androgen-independent PCa via suppression of the androgenic pathway. This similarity suggests that arachidonic acid may have been converted into anandamide, hereby contributing to the effects observed herein, although this possibility has yet to be clarified.

B472/P1596
Basal extrusion of Ras-transformed primary mammary luminal cells is associated with E-cadherin loss and reduced by EGFR inhibition
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Basal extrusion of tumor cells from the mammary duct is a defining event in the transition from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC). Despite its importance, the molecular and physical cues that drive this process remain poorly defined. Previous work in our lab showed that oncogenic HRasQ61L increases apical extrusion frequency in Eph4 mammary epithelial monolayers through MAPK and EGFR non-canonical signaling. Ras activation also triggered internalization of E-cadherin from junctions, and loss of E-cadherin is sufficient for cell extrusion. Here, we extend these findings to a physiologically relevant, lumen-forming cyst system from primary mouse mammary luminal epithelial cells that models ductal architecture ex vivo. Using this system, we observed that 60% of total Ras-transformed luminal cells extruded with a strong basal preference (96%). Treatment with the EGFR inhibitor Erlotinib reduced extrusion frequency by half, although extrusion directionality remained unchanged. Interestingly, extrusion frequency negatively correlated with cyst size, suggesting that macro-structural physical forces may modulate extrusion dynamics. Immunostaining revealed that Ras-driven extrusion was associated with loss of E-cadherin, suggesting that disruption of cell–cell adhesion is needed for dissemination. Together, these findings describe a distinct tumor cell invasion mechanism, advancing our understanding of how DCIS progresses to invasive breast cancer.

B480/P1604
Canonical NF-?B promotes B-cell differentiation to subvert RAS-driven B-cell leukemia
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B-cell acute lymphoblastic leukemia (B-ALL) is the most common childhood cancer, defined by a developmental arrest where immature B-cell precursors fail to mature. This arrest provides a survival advantage, enabling unchecked proliferation and evasion of differentiation. About 35–40% of pediatric B-ALL cases harbor RAS–ERK pathway mutations, driving constitutive signaling linked to drug resistance and relapse. Survival after relapse remains below 50%, underscoring the urgent need for new therapies. Background and Objective: Normal B-cell maturation proceeds through sequential expression of the precursor B-cell receptor (pre-BCR) and the mature B-cell receptor (BCR). The pre-BCR supports survival and expansion of early B-cell precursors. As B cells mature, the BCR enables antigen recognition. In B-ALL, leukemic cells are arrested before BCR expression. Cancers often progress through cooperating mutations; however, our prior work in B-ALL identified an alternative paradigm: pathway incompatibility, where co-activation of specific pathways disrupts rather than promotes malignancy. Here, we investigated whether RAS and canonical NF-κB form such an incompatible pair. Results and Conclusion: Analysis of 572 B-ALL cases showed strong mutual exclusivity between RAS- and NF-κB-pathway lesions. Experimentally, canonical NF-κB activation suppressed RAS-driven leukemogenesis, while NFKB1 knockdown enhanced it. NF-κB reduced pre-BCR programs and induced BCR expression, suggesting that progression to a differentiated, BCR-positive state antagonizes RAS-driven transformation. Consistently, pre-BCR was required for RAS-mediated leukemogenesis, whereas BCR expression was incompatible with it. Pharmacologic studies further supported this model: toll-like receptor agonists activated NF-κB, decreased ERK phosphorylation, and sensitized RAS-pathway mutated B-ALL cells to ERK inhibition. These effects were specific to RAS-pathway mutated B-ALL cells, with no impact on wild-type B-ALL. Together, these findings suggest that

canonical NF-κB suppresses RAS-driven leukemogenesis by disrupting a pre-BCR–dependent survival program and promoting progression to a BCR-positive state incompatible with oncogenic RAS. This developmental mechanism highlights a therapeutic opportunity for B-ALL.

B471/P1595

Comparing the effect of hydroxytyrosol on the protein expression of Proteinase Inhibitor 9 and Granzyme B in Estrogen Receptor-Positive and Negative mammary carcinoma cells

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Proteinase Inhibitor 9 (PI-9) affects the ability of immune cells to kill cancer cells and due to this, it is associated with poor prognosis in carcinomas. PI-9 targets Granzyme B (GrB) released by cytotoxic cells and protects cells from apoptosis. Estrogen upregulates the expression of PI-9 in Estrogen Receptor-Positive (ER+) MCF-7 breast cancer cells which evades the cells from apoptosis. Hydroxytyrosol (HT), a component in olive oil that strengthens the immune system, is known to suppress cancer cell growth. The structural feature of HT is similar to estrogen and has been shown to downregulate genes that are mediated by the estrogen receptor pathway. In this study, we examined whether HT regulates PI-9 and GrB in ER+ and ER- mammary carcinoma cells. Our results demonstrated that in ER+ MCF-7 and T47D cells, treatment with HT inhibited the protein expression of PI-9, while upregulating the expression of GrB in only T47D treated cells. In ER- MDA-MB-453 cells, we observed that HT suppressed the protein expression of PI-9 and did not alter the expression of GrB. These results show that the regulation of PI-9 is not mediated through the ER pathway, and the regulation of GrB by HT is not global among breast cancer cell lines. Future study will work to understand whether modulating the expression of PI-9 and GrB enhances immune targeted cell death in ER+ and ER- breast cancer cells.

B469/P1593

Crosstalk Between Circular RNAs, ER Stress, and Cytokines in Oral Squamous Cell Carcinoma

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Background: Oral squamous cell carcinoma (OSCC) poses a significant health challenge in India, characterized by poor survival rates due to delayed diagnosis and limited molecular understanding. Recent evidence indicates that circular RNAs (circRNAs) and endoplasmic reticulum stress (ERS) pathways influence tumorigenesis and immune signaling. However, their combined roles in oral cancer remain poorly understood. Objectives: To analyze the expression patterns and relationships among circular RNAs (circCDR1as, hsa_circRNA_101036), unfolded protein response (UPR) genes (GRP78, CHOP, PERK, ATF6, IRE1), and tumor-promoting cytokines (TNF-α, IL-6R, TGF-β, IL-12) in OSCC tissues and matched blood samples. Methods: Paired tumor and blood samples from OSCC patients(n=55) and matched controls (n=55) were analyzed. Total RNA was isolated and treated with RNase R to enrich the circRNAs. Gene expression was measured using SYBR Green-based qRT-PCR, normalized to β-actin, and cytokine proteins were validated by Western blotting. Results: Both circRNAs were significantly upregulated in tumor tissues (circCDR1as: 4.09-fold; has-circRNA_101036:3.5-fold) and blood (3.46- and 2.51-fold, respectively). Among the ER stress markers, PERK showed the highest increase (8.07-fold), followed by ATF6 (4.97-fold) and GRP78 (3.37-fold). Pro-inflammatory

cytokines were also elevated TNF-α (7.2-fold), IL-12 (5.65-fold), IL-6R (4.2-fold), and TGF-β (3.8-fold). Similar but milder expression trends were observed in the blood samples. Conclusion: The coordinated upregulation of circRNAs, ER stress genes, and inflammatory cytokines suggests a shared molecular axis contributing to OSCC progression.

B459/P1583

Decrotonylation of p62 K141 by SIRT1 promotes Lung adenocarcinogenesis via RIPK1/NF-?B pathway

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Lung adenocarcinoma (LUAD) is a leading cause of global cancer mortality. The SQSTM1/p62 plays a crucial role in tumorigenesis and is regulated by various post-translational modifications. However, the mechanisms of action underlying p62 elicited tumorigenesis remain elusive. Here we identified a signaling cascade in which p62 is crotonylated by acetyltransferase p300 while the crotonylated lysine 141 (K141) of p62 is decrotonylated by SIRT1. Mechanistically, decrotonylation of p62 K141 by SIRT1 regulates p62 and facilitates K63-linked ubiquitination of RIPK1 at K377 to ensure a robust activation of NF-κB signaling. Clinical samples analysis of LUAD tissues compared to adjacent normal tissues reveal decreased K141 crotonylation of p62, accompanied by elevated SIRT1, p62, and RIPK1 protein levels. These data reveal a non-conical role of SIRT1 in promoting LUAD progression via stabilizing p62 and RIPK1. Modeling crotonylation of p62 in LUAD mouse model recapitulated clinical samples which demonstrates the importance of crotonylation dynamics in lung epithelial homeostasis. Since SIRT1-p62-CrK141-RIPK1-NF-κB signaling axis functions in LUAD pathogenesis, these findings delineated a previously unrecognized role of crotonylation-dependent oncogenic signaling and offer a new avenue for synthetical lethal treatment of LUAD.

B481/P1605

Early-onset CRC, Retinoic Acid Signaling, Colonic Crypt Renewal

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The current rise in early-onset colorectal cancer (EOCRC) has been attributed to a Westernized lifestyle, but a definitive lifestyle factor or genetic etiology has yet to be discovered. Still, genetic studies do show that APC mutations are initiating events in both EOCRC and later-onset CRC (LOCRC). So, a key question is why do somatic APC mutations occur at a relatively young age in EOCRC patients? Hence, we surmise that something is retarding tissue renewal in colonic crypts so that somatic mutations are retained instead of being extruded during tissue turnover. Accordingly, we investigated vitamin A (retinol, retinoic acid), which is an essential vitamin requiring adequate dietary intake. Indeed, vitamin A is essential for tissue homeostasis because germline mutations in retinoid receptors and vitamin A deficiency during pregnancy can lead to fetal malformations. Moreover, population-based studies report vitamin A deficiency in pre-school-aged children. Consequently, young-aged individuals might be prone to cancer-initiating effects of vitamin A deficient diets. Our Goal is to determine how aberrant retinoid receptor expression might promote EOCRC development through dysregulated retinoic acid signaling. Rationale: Our previous studies show that retinoic acid (RA) signaling pathway plays an essential role in regulating cell differentiation in the colon. Moreover, we reported that CRC cells harboring mutations in RA receptors (RAR, RXR) have drug resistance to all-trans RA (ATRA). So, mutation or aberrant expression of RA receptors could lead to incomplete differentiation and promote CRC

initiation. Hypothesis: Aberrant RA receptor expression leads to dysregulated RA signaling that contributes to EOCRC development. Results: Our bioinformatics analyses show that retinoid receptors are often abnormally expressed and mutated in human CRCs. We found that up to 22% of CRCs have RA receptor mutations, but mutation status did not predict patient survival. However, we did find that frequency of retinoid receptor mutation was higher (>2X-fold) in EOCRC (age < 45) compared to LOCRC. Specifically, a high frequency of somatic mutations (2x-fold) occurred in receptors for vitamin A (STRA6) and retinoic acid (RARG, PPARG) in EOCRCs. We also found RARG hypermethylation occurs frequently in EOCRCs ($p<0.05$). Conclusions Since the rate of retinoid receptor alteration was increased in EOCRCs, aberrant RA signaling may be a predisposing factor for early CRC development and contribute to the etiology of EOCRCs. Based on this view, reduced retinoid signaling could lead to incomplete differentiation which provides a mechanism that may explain how delayed tissue renewal causes retention of acquired APC mutations in colonic epithelium:

B478/P1602

EGFR S442 Mutation Drives Cetuximab Resistance via Impaired Antibody Binding and Sustained Signaling, Reversed by ERBB2 Co-Targeting

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Resistance to EGFR-targeted therapies remains a major challenge in colorectal cancer (CRC) and head and neck squamous cell carcinoma (HNSCC). While EGFR ectodomain (ECD) mutations have been implicated in acquired resistance, their functional impact and therapeutic implications remain poorly defined. Here, we identify EGFR S442 mutations – particularly S442I – as recurrent alterations in cetuximab-treated CRC patients and demonstrate their mechanistic role in resistance. Using 3D collagen cultures, we generated a cetuximab-resistant CRC line (DiFi-CR) and identified an S442I mutation in EGFR ECD domain III. Inducible expression of S442I in CRC and HNSCC models conferred resistance to cetuximab and panitumumab in vitro and in vivo. FACS-based cell surface binding assays revealed reduced cetuximab binding in S442I-expressing cells, confirming impaired antibody engagement despite EGFR expression. These findings were supported by structural modeling and thermodynamic integration. Importantly, S442I also diminished EGF-induced EGFR activation, suggesting a neomorphic role in receptor activation and trafficking. In vivo, S442I tumors retained pERK activity and showed reduced intratumoral antibody enrichment, while WT EGFR-expressing tumors exhibited central necrosis and high cetuximab enrichment. To overcome S442I-mediated resistance, we co-targeted ERBB2 using trastuzumab deruxtecan (T-DXd). In cancer lines with EGFR–ERBB2 co-expression and physical interaction (co-immunoprecipitation), T-DXd restored cetuximab sensitivity. This synergy was absent in ERBB2-low HNSCC models, underscoring the importance of receptor context. Our findings thus establish EGFR S442 mutations as resistance drivers that can be specifically targeted. Co-targeting EGFR and ERBB2 offers a promising strategy to restore anti-EGFR efficacy in resistant tumors, while 3D culture systems provide a more physiologically relevant platform for modeling drug response.

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EGFR-driven Upregulation of Lymphocyte Cytosolic Protein 1 Promotes Oral Cancer Progression via the STAT3/IL-1ß Axis

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Oral cavity squamous cell carcinoma (OSCC) accounts for over 90% of oral cancer cases and represents the sixth leading cause of cancer-related mortality in Taiwan. Despite established standard therapies, OSCC remains highly prone to recurrence and metastasis, resulting in poor clinical outcomes. Tumor microenvironment (TME)-driven signaling abnormalities have been implicated in OSCC progression and relapse. However, the underlying mechanisms remain unclear. To elucidate the molecular determinants of OSCC progression, we applied iTRAQ-based mass spectrometry of primary and recurrent OSCC tissues. With integration of transcriptomic data from The Cancer Genome Atlas (TCGA), we identified lymphocyte cytosolic protein 1 (LCP1) as a candidate driver of OSCC progression. LCP1 was significantly upregulated in recurrent tumors, and elevated expression correlated with reduced patient survival. Functional assays revealed that LCP1 promotes OSCC cell proliferation, migration, and cisplatin resistance. Mechanistically, epidermal growth factor (EGF) stimulation enhanced LCP1 expression and Ser5 phosphorylation through PI3K/AKT and MAPK/ERK pathways. LCP1 upregulation and phosphorylation further engaged the JAK2/STAT3 axis, inducing IL-1β production and secretion, thereby facilitating tumor progression. In summary, our study reveals LCP1 as a novel oncogenic mediator in OSCC and highlights the EGFR/LCP1/STAT3/IL-1β signaling axis as a potential target for improving therapeutic outcomes in oral cancer.

B467/P1591
Expression of the MSK1 and STK11 genes in canine mammary cancer
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MSK1 and STK11 are key regulatory genes in the signal transduction cascade controlling cell proliferation in cancer cells. The open reading frames of the genes encoding MSK1 and STK 11 expressed in mammary cancers of domestic dogs, Canis lupus familiaris, were analyzed to find highly conserved sequences for designing an rtPCR reaction that can specifically amplify the targeted region of each of the coding sequences, as well as using gel electrophoresis and Sanger sequencing to verify the results, and designing a qRT-PCR. MSK1 encodes kinase, mitogen and stress-activated kinase 1, and STK11 encodes a serine/threonine kinase; both of these genes activate transcription factors that regulate the cell cycle and proliferation. To optimize the PCR protocol for amplifying the targeted sequences, first, rtPCR primers were created by using the genomic sequences of the MSK1 and STK11 genes in Canis lupus familiaris. The canine genome was then analyzed to identify primer pairs, also conserved in Homo sapiens, that were within the protein coding sequences and spanned an intron-exon junction. Once the successful primers were synthesized, they were utilized in an rtPCR reaction to amplify the targeted sequences. Canine mammary tumor cell lines 12, 27, 28, and 47 were cultured and the RNA extracted to use with each of the primers to assess expression among different cell lines. Fibroblast cell lines (CMEC) were also used for comparison against the canine mammary cell lines. A dilution series of RNA was assessed to confirm linearity of expression. Gel extraction along with DNA precipitation was performed to prepare samples for Sanger sequencing at an outside laboratory. The results were then further analyzed by comparing the sequences observed to target sequences expected. The results were then analyzed using Chromas software which looks at the flat files of the base pairs, ensuring accurate results and correct sequencing verification. Once verified, qRT-PCR was utilized to look at the varying levels of expression of each gene of interest, MSK1 and STK11, against the expressed sequences in each tumor cell line Along with normal canine mammary epithelial cells as a normal control cell group. The data suggests that MSK1 shows levels of suppression in all of the tumor cell lines in comparison to the normal epithelial cell line. STK11, on the other hand, shows both suppression and expression in the tumor cells. In contrast, CMT12 and CMT27 both

demonstrated elevated levels of expression compared to CMEC, while CMT28 and CMT47 were suppress for STK11 expression. This indicates that while STK11 is variable in terms of suppression or activation MSK1 is more consistently suppressed in the canine mammary tumor cells. These two genes could provide new therapeutic targets in the treatment of breast cancer.

B486/P1610
FGF19/ FGFR4 Axis: A Key Driver in Tumor Growth and Treatment Resistance in Colorectal Cancer
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Colorectal cancer (CRC) still has the high rate of treatment resistance, highlighting the need for novel therapeutic targets. Our study investigates the role of the Fibroblast growth factor (FGF) 19/ FGF Receptor (FGFR) 4 axis in CRC tumor growth and therapeutic response. This axis is particularly compelling because FGF19 and FGFR4 are overexpressed in human CRCs, with FGF19 exclusively found in tumor tissues. Importantly, FGF19/FGFR4 signaling regulates the MAPK and WNT/β-catenin pathways, key drivers of treatment resistance. Our preliminary data showed high endogenous FGF19 expression correlates with resistance to pan-FGFR inhibitors. We hypothesized that autoactivation of the FGF19/ FGFR4 axis promotes tumor proliferation and treatment resistance in CRC. We conducted comprehensive analyses using The Cancer Genome Atlas (TCGA), the Broad Institute's Single Cell Portal, and a CRC mouse model to evaluate expression and survival. Functional effects of FGF19/FGFR4 manipulation were determined by cell viability, apoptosis, and cell cycle assays, with molecular mechanisms elucidated through qRT-PCR and Western blot. Bioinformatic results demonstrated i) co-overexpression of FGF19 and FGFR4 in human CRCs, ii) with FGF19 expression increasing significantly from pre-cancer to advanced stages, and iii) both correlating with poor patient survival. Functionally, selective FGFR4 inhibition by BLU9931 in CRC cell lines decreased viability, induced G1/S cell cycle arrest, and overcame resistance to pan-FGFR inhibitors (Infigratinib, Pemigatinib). Conversely, FGF19 overexpression promoted cell proliferation by upregulating ERK1/2 and active β-catenin, inducing resistance to 5-fluorouracil (5 -FU). Interestingly, FGF19 treatment reversed pan-FGFR inhibitor effects but not BLU9931, indicating its specificity for FGFR4. These findings reveal that the dysregulated FGF19/FGFR4 axis contributes to colon tumorigenesis and drives tumor resistance in CRC. Its strong correlation with poor patient survival and tumor-specific expression suggests that the FGF19/ FGFR4 axis can be a highly promising and specific therapeutic target for CRC treatment.

B484/P1608
HYOU1 modulates ER stress for renal cell carcinoma progression
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Renal cell carcinoma (RCC) accounts for approximately 2% of all cancer cases worldwide and remains a major clinical challenge due to its limited treatment options along with high recurrence rates. These challenges highlight the need to identify novel molecular targets to tackle RCC. In recent years, the Endoplasmic Reticulum (ER) Unfolded Protein Response (UPR) has emerged as a promising pathway to target for cancer therapy. Under moderate ER stress, the adaptive UPR promotes tumour survival and growth by activating pro-survival signalling, whereas prolonged or excessive stress triggers terminal UPR, leading to apoptosis. For cancer cells, maintaining ER stress within an adaptive range is essential for survival and to prevent the shift

toward apoptosis by the terminal response. The duality of the UPR pathway makes it an attractive pathway for cancer therapy. However, the precise mechanisms through which cancer cells manipulate this pathway remains poorly understood, limiting its potential as a therapeutic target. In this study, we identified Hypoxia Upregulated Protein 1 (HYOU1), an ER-resident molecular chaperone, as one of the key modulators of the UPR pathway. Bioinformatic and micro-tumour array clinical data revealed that HYOU1 is overexpressed in RCC and that patients with elevated expression of HYOU1 had poorer overall survival rates. We found that the knockdown of HYOU1 decreases the growth and migratory ability while increasing RCC susceptibility to ER stress. Furthermore, we found that HYOU1, specifically through its nucleotide exchange factor (NEF), promotes the clearance of misfolded proteins via ER-associated degradation (ERAD) to mitigate ER stress thereby reducing ER stress. These findings shows that HYOU1 is a critical regulator of ER homeostasis and could serve as a potential novel therapeutic target for RCC in the future.

B460/P1584

Identification of a Novel Glioblastoma Proliferation Inhibitor using 3D Culture Screening with AI Image Analysis

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In conventional two-dimensional (2D) culture, it is difficult to mimic the structural and spatial tumor microenvironment. Therefore, three-dimensional (3D) spheroid culture has attracted attention as a method to mimic the tumor microenvironment in vitro. In 3D spheroids, factors such as structural barriers, cell density, nutrient gradients, and metabolic stress have been reported to significantly influence cancer cells. Therefore, the purpose of this study is to discover new treatments that target 3D-specific vulnerabilities that are difficult to identify in conventional 2D culture systems by analyzing the mechanisms of compound that specifically acts on the 3D tumor microenvironment in a glioblastoma spheroid model. In our previous study, we showed that the addition of large tumor suppressor kinase (LATS) inhibitors to serum-free medium supplemented with EGF and bFGF promoted spheroid-like growth to the U251MG glioblastoma cell line. Based on this finding, we developed a deep learning-based system to distinguish between adherent cells and spheroids. Using this AI-driven screening system, we identified a compound X that selectively inhibits cancer cell proliferation during spheroid formation. Since spheroid formation mimics microenvironmental feature of glioblastoma in vivo, compound X is promising candidate for new glioblastoma therapeutics. In this study, we found that the spheroid proliferation inhibitory effect of compound X may contribute to changes in protein expression in the cholesterol synthesis pathway through proteome analysis. The addition of compound X reduced the protein level of the rate-limiting enzyme HMGCR in glioblastoma spheroids. These results suggest that the reduction in cholesterol synthesis capacity acts as a spheroid-specific vulnerability and induces proliferation inhibition in glioblastoma spheroids. We are currently working to understand the mechanisms of action of compound X through omics analysis, with the aim of developing innovative treatments that specifically target vulnerability to three-dimensional cultured glioblastoma cells.

B474/P1598

Inhibitory Effect of Interleukin-24 on Programmed Death Ligand (PD-L1) Expression in Human Triple-Negative Breast Cancer

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Programmed death ligand 1 (PD-L1) is frequently overexpressed in triple-negative breast cancer (TNBC), the most aggressive type, where it suppresses anti-tumor immune responses and promotes tumor growth. In this study, we examined the effect of IL-24, a cytokine known for its tumor-suppressing properties, on PD-L1 regulation in breast cancer cells. We tested the breast cancer cell lines T47D, MCF7, MDA-MB-453, and MDA-MB-231, finding that the highly aggressive MDA-MB-231 cells had significantly higher PD-L1 levels at both mRNA and protein levels compared to the less aggressive T47D, MCF7, and MDA-MB-453 lines. IL-24 treatment reduced cell proliferation in MDA-MB-231 cells and significantly lowered both PD-L1 mRNA and protein expression. Furthermore, applying low doses of IL-24, combined with lower doses of doxorubicin, to TNBC cells induced apoptosis and decreased the levels of PD-L1 mRNA and protein. It also inhibited metastatic behaviors by blocking matrix metalloproteinases (MMP-9 and MMP-2), resulting in reduced migration in PD-L1–high MDA-MB-231 cells. In conclusion, IL-24 exhibits anti-cancer effects in TNBC by decreasing PD-L1 expression and reducing metastatic potential. These findings provide new mechanistic insights into the therapeutic potential of IL-24 for combating aggressive breast cancer.

B487/P1611

Investigating the Role of miR-27a-3p in the WNT Signaling Pathway and Chemoresistance in Colorectal Cancer Stem Cells

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Cancer stem cell (CSC) overpopulation drives colorectal cancer (CRC) development and growth, but molecular mechanisms are unclear. CSCs functional phenotypic characteristics include signaling pathway dysregulation and chemoresistance. CRC CSCs, in particular, are defined by their high WNT signaling activity and chemoresistance through several mechanisms, including the ability to evade apoptosis. A growing body of scientific evidence indicates that miRNAs, involved in post-transcriptional gene expression, play an important role in maintaining the CSC phenotype. However, the exact mechanism is still unknown. Our GOAL is to investigate how miRNAs contribute to the SC origin of CRC. My preliminary experiments show that miR-27a-3p is enriched in CRC and specifically in the leucine-rich-repeat containing G protein-coupled receptor 5-positive (LGR5+) CSCs. NanoString miRNA profiling of FACS-isolated LGR5+ CSCs from HT-29 CRC cells showed that miR-27a-3p is differentially upregulated in LGR5+ CSCs versus LGR5- cells. Analysis of available deep-sequencing data also shows that the canonical miR-27a-3p is the highest expressed isoform in human CRC and is significantly upregulated during the colonic adenoma-carcinoma progression. Additionally, miR-27a-3p has been shown to: i) be enriched in human CSCs in CRC utilizing other CSCs markers and methods; ii) activate or repress WNT signaling activity in various cancers; iii) increase chemoresistance in various cancers. My OBJECTIVE is to determine the mechanism by which miR-27a-3p functions in CRC to regulate WNT signaling and chemoresistance. HYPOTHESES: H1). miR-27a-3p promotes WNT signaling expression through repression of WNT antagonist targets; H2). miR-27a-3p induces chemoresistance via apoptosis evasion through target repression of pro-apoptotic genes. My bioinformatics analyses have identified key candidate mRNA targets RXRA, RARA, CSNK1D, and GSK3B, which are WNT antagonist. Ongoing experiments show a significant decrease in expression of these targets in SW480 or HT-29 cells post-transfection with miR-27a-3p mimetic. These findings provide a mechanism that could explain how miRNAs contribute to the SC origin of CRC

B461/P1585

Investigation of the Prognostic Impact of PIP4K2s And Its Pharmacological Citotoxicity in Hepatocellular Carcinoma

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Objetives: The PIP4K2 family play a significant role in cell proliferation, survival, and cytoskeletal organization, all processes reported to be dysregulated in cancer. Therefore, the aim of this project is to investigate the impact of PIP4K2 expression on survival outcomes, clinical features, and biological functions in patients with hepatocellular carcinoma (fifth most common type of cancer and one of the leading causes of cancer-related death worldwide) from the TCGA cohort, and to characterize the antineoplastic potential of its pharmacological inhibition in hepatocarcinoma-derived cell lines. Methods: RNA sequencing data, clinical characteristics, and outcomes of hepatocellular carcinoma patients from cBioPortal were analyzed. Survival rates were assessed using the Cox proportional hazards regression model. Gene Set Enrichment Analysis identified signaling pathways linked to PIP4K2 expression. In vitro experiments were conducted using hepatocellular carcinoma cell lines (HepG2 and Huh-7). The assays included cell viability (MTT assay), clonogenicity, protein expression (Western blotting), and cell cycle/proliferation analysis by flow cytometry. Results: The results indicated that PIP4K2s are more highly expressed in tumor tissue compared to adjacent normal tissue. High expression of PIP4K2s was associated with worse clinical outcomes in patients with hepatocellular carcinoma. In the GSEA analysis, the high-expression phenotype showed enrichment in the PI3K/AKT/mTOR signaling pathway, epithelial-mesenchymal transition, inflammatory response, KRAS signaling, mitotic spindle, and TGF-β signaling in hepatocellular carcinoma. High expression of these genes was associated with downregulation of the P450 epoxygenase pathway. In hepatocellular carcinoma cell lines, low doses of the inhibitors led to increased cell death and DNA damage (increased cleaved PARP1 and γH2AX), as well as reduced cell viability and colony formation. A decrease in p-YAP1/YAP and p-AKT/AKT expression was also observed. Flow cytometry showed that cells treated with the inhibitors had reduced proliferation. Cell cycle analysis indicated that treatment with the inhibitors induced G2/M arrest and cell death. Conclusion: The results obtained provide promising evidence that PIP4K2s may serve as potential prognostic markers and molecular targets for hepatocellular carcinoma.

B476/P1600

JAK/STAT in breast cancer dormancy and recurrence

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Breast cancer is the leading cause of cancer mortality in women worldwide, which is principally attributable to incurable recurrent tumors that arise from residual tumor cells (RTCs). Breast cancer RTCs can persist for decades following primary tumor treatment in what is thought to be a dormant state before re-entering the cell cycle and giving rise to recurrent tumors. However, the mechanisms that regulate dormancy maintenance are poorly understood. Given the central role of JAK/STAT signaling in normal mammary biology and cancer, we sought to determine if it plays a role in breast cancer dormancy and recurrence. We employed multi-omic sequencing of patient primary and recurrent tumors to examine the associations between JAK1/2 genomic alterations and recurrence-free survival (RFS). To determine if these associations have a functional basis, we

employed an extensively validated genetically engineered mouse model to conduct RFS assays in vivo. We used different murine hosts to assess the role of the immune system in RFS results. To mechanistically probe phenotypes, we used in vitro models of therapy-associated dormancy as well as microenvironment-induced dormancy. We used pharmacological inhibitors as well as inducible genetic systems to probe JAK1/2 function specifically during dormancy. We asked if pharmacological inhibition in vivo affects RFS. Lastly, we sought to determine if patient treatment with JAK inhibitors is associated with the probability of recurrence in a retrospective, case-control study. Patient primary and recurrent tumor multi-omic associations were functionally validated in vivo. Hypotheses about the roles of JAK1/2 and downstream effectors were confirmed in vitro in both therapy-associated and microenvironment-induced dormancy models. JAK1/2 inhibition consistently and significantly altered outcomes for both dormancy and recurrence. Our retrospective case-control study identified significant associations in breast cancer between JAK1/2 inhibitor treatment and post-treatment RFS, but not in lung, colorectal, skin, or ovarian cancers. JAK/STAT signaling plays an essential role in normal mammary biology and has also been implicated in cancer progression, especially hematopoietic malignancies where JAK1 and JAK2 function as bona fide oncogenes. This has motivated clinical trials testing the efficacy of JAK1/2 inhibitors in advanced breast cancer. Our work here focuses on understanding the role of JAK1/2 in dormancy and recurrence, since the propensity of breast cancers to recur following therapy is a critical determinant of clinical outcome.

B483/P1607

Low-order assemblies drive RTK fusion signaling without condensation

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Receptor tyrosine kinase (RTK) fusions are a diverse class of oncoproteins that have been identified in ~5% of cancers. RTK fusions are chimeric proteins where the intracellular domain of an RTK is fused to an oligomeric domain from an unrelated protein. Such fusions can drive oncogenic signaling from the RTK fragment despite their cytoplasmic localization, away from the plasma membrane where normal transmembrane RTK signaling occurs, thus posing mechanistic questions of how RTK fusions transmit oncogenic signals. Recent studies have suggested that condensation is essential for signaling from RTK fusions because condensates can organize the necessary signaling machinery in the cytoplasm. In our study we tested this hypothesis using live cell fluorescent imaging, quantitative analysis of condensation, single-cell analysis, and synthetic biology, focusing on condensates of EML4-ALK, a granule-forming RTK fusion that is found in ~5% of non-small cell lung cancer. Surprisingly, we found weak correspondence between condensate formation and downstream signaling. Although signaling (ERK activation) was a direct function of oncogene expression, condensation was maximal only at intermediate expression levels, resulting in high-expressing cells that had the strongest signaling but no condensation as assessed under high magnification confocal microscopy. Furthermore, increasing the magnitude of condensation through multiple methods did not result in increase in downstream signaling. We then constructed synthetic fusions to test the lower limit of multimerization that could trigger signaling. While monomers of the ALK domain did not signal, dimers triggered signaling to approximately the same magnitudes as EML4-ALK and promoted colony formation in soft agar, but notably did not form condensates. Finally, a survey of 9 distinct oncogenic RTK fusions found that only few other fusions formed condensates, while most appeared diffuse. Nevertheless, all fusions could strongly activate signaling, independent of condensate formation. Our work thus suggests that condensation is not essential for RTK fusion signaling and that strong signaling and cell transformation can result from fusions that appear diffuse under fluorescence microscopy, from both synthetic and natural fusions. These results suggest a more nuanced role of condensation in RTK

fusion signaling and call for further mechanistic studies to understand how cytoplasmic fusions can productively trigger oncogenic signals.

B462/P1586
Mapping Mutation-Specific Interaction and Signalling Networks Across FGFR1-4 to Understand Cancer-Associated Rewiring
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Fibroblast growth factor receptors (FGFR1–4) are essential receptor tyrosine kinases that play a crucial role in controlling cellular fate, guiding tissue development, and maintaining physiological balance. Cancer-driving mutations in FGFRs are found in many tumour types, but exactly how each mutation alters receptor behaviour, protein interactions, and signalling pathways is still not fully understood. In this study, we systematically analysed wild-type, kinase-dead, and two cancer driver mutants for each FGFR paralogue by generating stable, isogenic Flp-In™ T-REx™ 293 cell lines which were engineered under inducible expression for each FGFR variant. We performed affinity purification-mass spectrometry (AP-MS) and proximity-dependent biotinylation (BioID) to generate high-confidence mutation-level map of FGFR interactomes, integrating both stable and transient protein associations. To understand the functional implications of the altered interactome we performed phosphoproteomics on both global lysates and affinity-purified complexes. This enabled quantification of pathway-specific signalling changes and differentiation between the catalytic and scaffolding roles of mutant receptors. Distinct signalling rewiring patterns were observed between wild-type and mutant FGFRs such as preferred activation of MAPK versus PI3K–AKT signalling pathway and revealed mutation-specific signalling biases consistent with recent models of RTK signalling divergence. Functional assays in NIH3T3 and U2OS cells, including colony formation, immunofluorescence, cell cycle profiling, and reporter assays, validated the differential oncogenic potential and subcellular localisation of each variant. We further evaluated the effects of clinically relevant FGFR inhibitors on the viability and signalling pathway activity across all mutants, identifying both drug-sensitive and drug-resistant classes linked to specific mutations, including gatekeeper substitutions (e.g., FGFR4-V550L) and extracellular loop alterations (e.g., FGFR2-S252W). Together, our findings establish the first paralogue-wide, mutation-resolved signalling atlas of the FGFR family. This integrative approach of structural, functional and pharmacological analysis provides systematic insight into oncogenic mechanism and therapeutic sensitivity and establishes a broadly applicable framework for the functional annotation of kinase driver mutations in cancer.

B482/P1606
Multiomics-based assessment of KRAS mutation levels refines survival and recurrence predictions in patients with pancreatic ductal adenocarcinoma
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Current mutation profiling studies for pancreatic ductal adenocarcinoma (PDAC) remain confined to laboratory research without direct integration into clinical decision-making. For clinical use, it is essential to target clinically impactful mutations and combine their assessment with clinical indicators. We aimed to evaluate the prognostic value of the KRAS G12 mutation dosage, a key variant in PDAC, and to clarify the biological mechanism underlying its prognostic significance. In this retrospective cohort study, we analyzed clinical and

omics data from 193 PDAC patients who underwent pancreatectomy between 2009 and 2016 at Seoul National University Hospital. Using comprehensive RNA sequencing, whole-exome sequencing, and KRAS-targeted sequencing data, we quantified the KRAS G12 mutation dosage. The KRAS mutation dosage exhibited prognostic value comparable to clinical factors for survival prediction. Specifically, high KRAS mutation dosages were associated with the cell cycle pathway activation, leading to increased mutation rates and poor clinical outcomes. Additionally, we established a prognostic scoring system by combining the KRAS mutation dosage from targeted sequencing (> 0.195, 1 point), maximal tumor diameter at preoperative imaging (> 20 mm, 1 point), and carbohydrate antigen 19-9 levels (> 150 U/mL, 1 point). According to this system, patients categorized in the lowest-risk group (0 points) had a median overall survival of 97.0 months and 1-year, 3-year, and 5-year overall survival rates of 95.8%, 70.8%, and 66.4%, respectively. In contrast, the highest-risk patients (3 points) experienced markedly reduced median overall survival of 16.0 months and 1-year, 3-year, and 5-year overall survival rates of 65.2%, 8.7%, and 8.7%, respectively. Furthermore, patients scoring 0 or 1 experienced lower rates of early recurrence (25.0% and 51.0%, respectively) compared to those scoring 2 or 3, among whom 73.9% (68/92) experienced early or very early recurrence. Incorporating the KRAS G12 mutant dosage variable significantly improves clinical variable-based survival and recurrence timing prediction, highlighting the feasibility and clinical significance of this integrated scoring system.

B468/P1592

Novel Mechanisms that Promote Chemoresistance in High-Risk Neuroblastoma

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This project aims to identify novel signaling pathways that promote chemoresistance in high-risk Neuroblastoma. High-risk Neuroblastoma (HRNB) is an extracranial solid tumor that has one of the least favorable outcomes of all pediatric cancers. Despite the plethora of treatment options available for HRNB, up to 65% of patients are unresponsive (refractory) or exhibit an initial response to treatments that inevitably transition to therapy-resistant relapse. Finding an effective treatment strategy for patients with HRNB, particularly relapsed HRNB, continues to be a major challenge due to the aggressive phenotypes of these tumors and a myriad of complex mechanisms that promote resistance to treatments and recurrence. To develop more effective treatment strategies for this devastating disease, it is critical to elucidate the underlying signaling anomalies that drive therapy resistance and relapse in HRNB. A key feature that promotes HRNB progression is aberrant calcium (Ca2+) signaling. Ca2+ signaling is regulated by several druggable channel proteins, which offers a tremendous therapeutic potential. Unfortunately, due to the fact that many of the Ca2+ channels in HRNB also perform fundamental functions in normal healthy cells, targeting them increases the potential for adverse effects. To overcome this challenge, we sought to identify novel Ca2+ signaling pathways that are observed in HRNB but not normal non-cancerous cells with the intent that these novel pathways may serve as potential therapeutic targets. The current study utilized cell proliferation assays and western blot analysis of whole cell lysates to screen a library of novel compounds to identify compounds that disrupt the dynamic signaling between SOCE, ER and mitochondria pathways and induce cell death in HRNB. In addition, quantitative and phospho-proteomic analysis of paired patient-derived high-risk NB cells were performed. These paired cells consist of tumor samples from patients at diagnosis (chemo-naïve) and tumor samples from the same patients after relapse (chemo-resistant). Our results found that compounds 248 and 249 activated SOCE that is unique to drug sensitive HRNB and resulted in mtCa+ overload and cell death. However, drug resistant HRNB activated autophagy to promote survival. Pre-treatment with autophagy inhibitors overcame this pro-survival response, resulting in mtCa+ overload and cell death in relapsed HRNB.

Quantiative and phospho-proteomic analysis identified key signaling pathways that regulate this mechanism. These findings may lead to an effective treatment strategy for relapsed HRNB.

B464/P1588

NSFL1C Upregulation Promotes Cancer Progression through the LAMC2/THBS1 Axis in Oral Cancer

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Oral squamous cell carcinoma (OSCC) is one of the most common malignancies worldwide and is the sixth leading causes of cancer-related death in Taiwan. OSCC, due to its proximity to lymphatic vessels, is prone to metastasis, resulting in a poor prognosis for OSCC patients. Identifying biomarkers and therapeutic targets associated with metastasis is crucial for improving patient outcomes. To investigate molecular mechanisms underlying OSCC metastasis, we performed proteomic profiling of tissue interstitial fluids (TIFs) from paired cancerous and adjacent noncancerous tissues of 10 OSCC patients. Among dysregulated proteins, NSFL1 cofactor p47 (NSFL1C) was not only upregulated in cancer TIFs, but also elevated in cancer TIFs from patients with lymphatic metastasis. Immunohistochemical and RNA sequencing analyses further confirmed that NSFL1C expression was increased in OSCC tissues compared with adjacent noncancerous counterparts. Importantly, higher expression of NSFL1C was observed in lymphatic-metastasis tissues. Functionally, NSFL1C overexpression enhanced OSCC cell migration and invasion, whereas knockdown (kd) reduced these abilities. To further elucidate the functions mediated by NSFL1C, we performed iTRAQ-based proteomics using NSFL1C-kd OSCC cells. Pathway enrichment analysis of proteins downregulated in NSFL1C-kd cells highlighted biological processes involved in focal adhesions. We selected two focal adhesion-associated proteins—laminin subunit gamma-2 (LAMC2) and thrombospondin-1 (THBS1)—and further validated their expression using qPCR and Western blot. In OSCC cells, NSFL1C kd resulted in downregulation of LAMC2 and THBS1, whereas NSFL1C overexpression led to upregulation of both. Furthermore, LAMC2 or THBS1 kd inhibited OSCC cell migration and invasion. NSFL1C-mediated promotion of mobility was abrogated by LAMC2 or THBS1 kd in OSCC cells. Notably, LAMC2 knockdown reduced THBS1 levels, whereas THBS1 knockdown did not affect LAMC2 expression in OSCC cells. In summary, NSFL1C promotes migration and invasion of OSCC cells through the LAMC2/THBS1 axis. This study provides novel insights into the oncogenic role of NSFL1C in OSCC, and identifies NSFL1C as a promising target for therapeutic intervention.

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Nuclear Translocation of EPRS1 is Required for AKT-Dependent Activation of PARP-1

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Glutamyl-prolyl-tRNA synthetase (EPRS1) is a bifunctional aminoacyl-tRNA synthetase (aaRS) essential for decoding the genetic code. EPRS1 resides, with seven other aaRSs and 3 non-catalytic proteins, in the cytoplasmic multi-tRNA synthetase complex (MSC). Multiple MSC-resident aaRSs, including EPRS1, exhibit stimulus-dependent release from the MSC to perform non-canonical activities distinct from their primary function in protein synthesis. Here, we show that EPRS1 is present in both the cytoplasm and the nucleus of breast cancer cells with constitutively low PTEN expression. EPRS1 is primarily cytosolic in PTEN-expressing cells, but chemical or genetic inhibition of PTEN, or chemical or stress-mediated activation of its target, AKT induces EPRS1 nuclear localization. Likewise, preferential nuclear localization of EPRS1 was observed in

invasive ductal carcinoma that were also P-Ser473-AKT+. EPRS1 nuclear transport requires a nuclear localization signal (NLS) within the linker region that joins the catalytic GluRS and ProRS domains. Nuclear EPRS1 interacts with poly(ADP)-ribose polymerase 1 (PARP1), a DNA-damage sensor that directs poly(ADP-ribosyl)ation (PARylation) of proteins. EPRS1 is a critical regulator of PARP1 activity as shown by markedly reduced ADP-ribosylation in EPRS1 knockdown cells. Moreover, EPRS1 and PARP1 knockdown comparably alter the expression of multiple tumor-related genes, inhibit DNA-damage repair, and reduce tumor cell survival in breast cancer cells. In the presence of an EPRS1 knockdown, the potency of a current PARP1 inhibitor was increased 3-fold. EPRS1-mediated regulation of PARP1 activity provides a new mechanistic link between PTEN loss in breast cancer cells, PARP1 activation, and cell survival and tumor growth. Targeting this non-canonical activity of nuclear EPRS1 provides a new clinically relevant approach, potentially supplementing existing PARP1 inhibitor treatments.

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Rational design of an AXIN2-based chemotherapy combination treatment to selectively target APC-mut CRC cells with minimal damage to APC-wild-type ones

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Most colorectal cancers (CRC) initiate from biallelic APC mutation which constitutively activates WNT/β-catenin signaling activity and promotes cancer stem cell (SC) overpopulation. For most (> 80%) CRCs this mechanism leads to the distinction between normal colonic cells carrying wild-type APC, and cancerous tumor cells carrying two alleles of mutant/inactivated APC. Our GOAL is to develop a treatment utilizing this distinction between normal and tumor cells, to therapeutically target APC-mutant SCs with minimal damage to APC-wild-type normal cells. Our HYPOTHESES are: (1) AXIN2 knockdown differentially promotes SC self-renewal and apoptosis only in APC-mutant CRC cells but not in APC-wild-type ones. And due to these phenotypical differences between APC-mutant and APC-wild-type cells, (2) AXIN2 knockdown selectively sensitizes APC-mutant CRC SCs to 5-FU treatment, but not APC-wild-type ones. METHODS AND RESULTS: we conducted AXIN2 knockdown and overexpression in CRC cells that are either APC-mutant (HT29, SW480) or APC-wild-type (RKO) and NanoString transcriptome profiling on these AXIN2 modulated cells. Pathway enrichment results show that AXIN2 inhibits apoptosis pathway activity in APC-mutant CRC cells, which is confirmed by our annexin V/PI staining results. But this effect is not significant in APC-wild-type cells. Similarly, pathway enrichment suggests that AXIN2 inhibits S-phase protein and DNA synthesis during cell cycle for APC-mutant CRC cells, and PI/RNase staining results confirmed that finding. But this effect is not significant in APC-wild-type cells. Additionally, we found that AXIN2 increases both ALDH+ and LGR5+ SC populations in APC-mutant CRC cells, but only LGR5+ SC population in APC-wild-type CRC cells. Accordingly, we knocked down AXIN2 in APC-mutant human CRC organoids (HCM-CSHL-0257-C18) and conducted organoid formation assay upon 5-FU treatment over a range of concentrations. Results indicate that organoid regeneration (measuring stemness) decreases significantly when AXIN2 is knocked down, compared to negative controls. This is to be compared with previously reported and our experimental observations that 5-FU treatment promotes stemness in CRC cells. CONCLUSION: We found AXIN2 is a candidate target that can be incorporated in CRC treatment to improve 5-FU's efficacy on selectively killing APC-mutant CRC SCs. Since the same mechanisms enabling this selective killing does not significantly happen in APC-wild-type cells and most normal colon cells carry wild-type APC, reducing AXIN2 level would not damage APC-wild-type SC as significantly. Most likely, decreased AXIN2 promotes normal colon tissue turnover and recovery since it might boost WNT/β-catenin signaling more in APC-wild-type cells than in APC-mutant ones.

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SNX2 Upregulation by the TGF-beta1/AKT/beta-catenin Cascade Drives Cisplatin Resistance in Oral Cancer Cells through Met Signaling

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Oral cavity squamous cell carcinoma (OSCC) is the most prevalent type of oral cancer and a significant cause of cancer-related mortality worldwide. Although surgery combined with chemo- and/or radiotherapy is typical treatment for OSCC, development of chemoresistance remains a major challenge to effective treatment and long-term survival of OSCC patients. To investigate molecular mechanisms underlying chemoresistance, we performed transcriptomic and proteomic analyses using OSCC cell lines with acquired cisplatin resistance (CR). Among molecules upregulated in the CR cells, sorting nexin 2 (SNX2) was selected for further validation because its increase was cisplatin dose-dependent and its high expression was associated with poor survival in OSCC patients. Moreover, SNX2 knockdown sensitized CR cells to cisplatin treatment, suggesting that SNX2 contributes to drug resistance. Mechanistically, SNX2 expression was regulated by the transforming growth factor beta 1 (TGF-β1)/AKT/β-catenin axis in OSCC cells. SNX2 promoted Met phosphorylation, likely by enhancing Met receptor recycling to the plasma membrane. Furthermore, inhibition of Met signaling enhanced the cytotoxic effect of cisplatin in CR cells. In summary, SNX2 is upregulated by the TGF-β1/AKT/β-catenin axis and promotes cisplatin resistance via Met signaling in OSCC cells. These findings identify SNX2 as a key mediator of cisplatin resistance in OSCC and suggest that targeting the SNX2/Met signaling axis could provide a promising therapeutic approach to overcome chemoresistance in oral cancer.

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STK11 C-terminal domain variants impact lung cancer biology independent of kinase activity

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KRAS-driven lung adenocarcinomas (LUADs) with concurrent STK11 loss of function (LoF) mutations are associated with aggressive tumors, increased risk of metastasis and lower survival. In LUAD, STK11 LoF impacts many aspects of coordinated cell motility and promotes alterations characteristic of metastasis. STK11 is a serine-threonine kinase that is comprised of an N-terminal, kinase, and C-terminal (CTD). Aiming to develop a method for identifying pathogenic STK11 mutations, we assessed the functional impact of genetic variants identified from patients at the University of Vermont Medical Center by evaluating STK11 kinase activity through in-vitro kinase assays and luciferase reporter assays. Results revealed that CTD variants retain kinase activity and the ability to form the STK11 heterotrimeric complex. Although the CTD has no catalytic activity, it is thought to be integral for STK11 localization to the plasma membrane, where it negatively regulates focal adhesion kinase (FAK) activity. Recent work by another group identified a region of the CTD, the polybasic motif (PBM), (aa403-426) as key for localization of STK11 to the plasma membrane. We reasoned that STK11 CTD variants would demonstrate altered localization and cytoplasmic function. We show in LUAD cell lines through immunofluorescent microscopy that expression of a truncated STK11 construct with a CTD deletion results in full nuclear sequestration. Additionally, point mutants in 3 of the polybasic motif residues (R409W, K416E, and K423E) retain kinase activity, but lead to partial nuclear sequestration despite their ability to bind heterotrimeric complex partners STRADa and MO25. This suggests a novel mode of dysregulated localization, independent of the known mechanism of STK11 export into the cytoplasm through STRAD-a binding. This

phenotype is exacerbated in a double mutant within the PBM: K416E/K423E. To determine whether this partial nuclear retention of STK11 leads to altered cell function, 2D migration assays were conducted using a Boyden chamber system. Here we observed that both the single PBM mutants, and the K416E/K423E double mutant display an increased migratory phenotype that mimics a total loss of STK11. Taken together, these findings suggest that point mutations within the C-terminal polybasic region disrupt its localisation, and lead to functional alterations within the cell. These data highlight the importance of examining both kinase dependent and independent functions when evaluating genetic variants of STK11. Current experiments aim to determine whether these CTD variants also display a more aggressive phenotype in 3D invasion assays. Overall, this work highlights the need to utilize a multipronged approach when assessing the pathogenicity of STK11 missense mutants.

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STK11-dependent NF?B Localization Drives Characteristic Transcriptional Signatures in Lung Cancer

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Lung cancer remains the leading cause of cancer-related deaths worldwide. Recent therapeutic advances, including check point inhibitor therapies (ICIs), have improved 5-yr survival for patients with lung cancer but these rates are still low (~25%) and therapeutic options to counter metastatic disease are few. One strong genotype/phenotype correlation predicting general therapy resistance and poor overall survival in KRAS-driven lung adenocarcinomas (LUADs) is Serine/Threonine kinase 11 (STK11) loss. Patients harboring tumors with this mutational signature present with later stage disease, have higher rates of metastasis, and exhibit ICI resistance (~95%). Critically, the molecular mechanisms responsible for these clinical correlations remain undefined. We performed whole transcriptome and pathway enrichment studies comparing a KRAS-driven/STK11-competent LUAD (K) cell line with KRAS-driven/STK11-null (KS) cell line and identified STK11-loss-dependent activation of the NF-kappa B (NFκB) signaling pathway in response to glutamine deprivation. Kmeans clustering of the RNA transcripts highlighted 36 genes induced upon glutamine depletion in KS cells. These genes were subsequently downregulated in the absence of the NFκB transcription factor (p65) through genetic ablation or pharmacological inhibition (using PS-1145), with corresponding decreases in protein expression of candidate inflammatory cytokines and anti-apoptotic markers. These findings indicate that NFκB activation is essential for transcriptional induction of survival, proliferation, and inflammatory genes under glutamine stress. We then showed that p65 consistently accumulates in the nucleus of KS cells following the removal of glutamine, suggesting transcriptional regulation through NFκB signaling. Following pharmacological inhibition of NFκB and glutamine depletion, p65 was unable to translocate into the nucleus and was sequestered in the cytoplasm of KS cells. To assess whether glutamine stress and NFκB signaling promote metastasis, we used 3D spheroid and embryonic zebrafish xenograft models. Spheroid models more closely mimic the microenvironment of in vivo tumors, in which nutrients and oxygen become restricted. Glutamine deprivation enhanced ameboid-like single-cell invasion in KS spheroids, an effect markedly reduced by p65 knockout or PS-1145 treatment. In vivo, glutamine-depleted KS cells exhibited increased metastatic events compared to K cells, which was abolished by p65 knockout. Using a combination of transcriptomic analyses, in vitro invasion assays, and an in vivo zebrafish model of metastasis, these results suggest that STK11 loss in KRAS driven lung adenocarcinoma promotes an NFκB-dependent metastatic phenotypes.

Cancer Cell Biology - Engineered models of cancer

B558/P1682
Adipose-Mimetic Granular Hydrogels Reveal Biophysical Cues Driving Breast Cancer Invasion
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Approximately one in eight women will develop breast cancer in their lifetime, and metastatic breast cancer is the leading cause of cancer-related death in women worldwide. In the first steps of metastasis, cancer cells invade out of the glandular structures of breast tissue and into surrounding adipose tissue-rich mammary stroma. While mechanical properties of the tumor microenvironment are known to regulate invasion, the role of adipose tissue mechanics, which are shaped largely by adipocytes and their surrounding extracellular matrix (ECM), remains poorly understood. This knowledge gap arises, in part, from a lack of appropriate model systems which replicate the mechanical properties of primary adipocytes. Moreover, no methods currently exist to decouple the effects of adipocyte physical and biochemical properties for investigation. To address these shortcomings, we developed an in vitro model system to identify how adipocyte size and stiffness affect ECM microarchitecture and leveraged this system to evaluate breast cancer cell migration through structurally heterogenous engineered tissue conditions. We characterized the stiffness of primary adipocytes and recapitulated these properties using adipocyte-sized polyacrylamide (PAAm) beads with tunable Young's moduli. Embedding these beads in type I collagen, i.e., the principal fibrillar ECM component of breast adipose tissue, yielded a 3D granular hydrogel that mimicked key features of adipose tissue architecture. PAAm bead inclusion facilitated the assembly of collagen networks resembling native ECM structure and enhanced breast cancer cell invasion relative to bead-free hydrogels. This effect was mediated by bead-induced collagen alignment and hierarchical matrix organization. Notably, breast cancer invasion was more pronounced in soft bead hydrogels, whereas stiffer beads physically restricted tumor cell migration. Live cell imaging of cancer cell invasion through granular hydrogels further demonstrated the rate-limiting role of cell nuclei, which deform and generate recoil force as they squeeze between PAAm beads. Lastly, bulk RNA sequencing of breast cancer cells pre-conditioned in mechanically variable granular hydrogels revealed differing transcriptional responses to adipose tissue characteristics. These findings suggest that adipocyte physical properties regulate tumor invasion by coordinating ECM architecture and cellular confinement, and they highlight the utility of tunable PAAm bead-collagen composites as a micromechanical model of adipose tissue.

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Extracellular matrix stiffness promotes cell division errors that lead to chromosome loss and genetic heterogeneity in cancer spheroids
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In cancer, progressive stiffening of the extracellular matrix (ECM) is associated with chromosomal instability, as solid tumors often display aberrant chromosome numbers. Although chromosome loss is a hallmark of solid tumors, it remains unclear whether ECM stiffness drives mitotic errors that lead to heritable chromosome loss. Using cancer spheroids carrying a live chromosome reporter, we found that stiff three-dimensional matrices

distort spindles, increase micronucleus formation, and trigger rare but heritable chromosome losses that persist across generations. Inspired by classic Luria-Delbrück fluctuation analyses, we show that stiff matrices increase the stochastic rate of chromosome loss, amplifying variation across spheroids and fueling population heterogeneity. In vivo, tumors surrounded by collagen-rich stroma displayed similar chromosome losses, underscoring the physiological relevance. To capture the principles underlying these trends, we applied physics-based machine learning models of evolving chromosome numbers, which reproduced the statistical features of both experiments and theory. Together, these results demonstrate that ECM stiffening is not merely a hallmark of tumor progression but a driver of "mechano-evolution," destabilizing chromosome segregation and generating diversity that complicates therapy. By linking extracellular mechanics, stochastic genetic variation, and quantitative modeling, this work reveals a new physical dimension of tumor heterogeneity.
Keywords: mechanobiology; extracellular matrix; chromosomal instability; cancer; genome integrity; tumor heterogeneity; quantitative modeling; fluctuation analysis

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Investigating the Impact of Cancer-Associated Fibroblast Contractility on the Activation of Nuclear Mechanosensing Pathways in Cancer Cells

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Cancer arises from the accumulation of mutations, yet its progression is significantly influenced by the non-mutant stromal cells in the tumor microenvironment (TME). Cancer-associated fibroblasts (CAFs) are among the most abundant stromal cell types within the TME and play critical roles in matrix remodeling, immunosuppression, and cancer invasion. Notably, CAFs spatially organize around cancer cells, forming a contractile, compressive capsule. This encapsulation generates radial traction forces that increase cancer cell density and solid stress. The nucleus has been recently viewed as a critical mechanosensory organelle. However, the specific effects of CAF-mediated compression on the activation of nuclear mechanosensing pathways in cancer cells remain elusive. To investigate this, we established a 3D co-culture model where cancer spheroids are embedded with CAFs at high-density in collagen spheres. In this model, CAFs self-organize into a core-shell architecture, aligning perpendicularly to the spheroid to generate compressive forces that mimic in vivo tumor-stroma interface. CAF encapsulation reduced nuclei volume of cancer cells by over 30% compared to control spheroids in collagen without CAFs. Interestingly, spatial analysis revealed that in control spheroids, cells at the core exhibited a ~10% reduction in nuclear volume compared to cells at the edge. This gradient was abolished in CAF-encapsulated spheroids as nuclear volume globally reduced across the entire spheroid diameter. This suggests CAF encapsulation uniformly increases and evenly distributes solid stress across the spheroid. In agreement, nuclear circularity globally increased by ~50% under CAF encapsulation compared to control, indicating that cancer cells experience isotropic compression by CAFs. Strikingly, we observed nuclear envelope rupture (NER) events in the co-culture model (by cGAS immunostaining, which binds to exposed cytosolic DNA after NER). Collectively, these findings show that CAF contractility exerts compressive forces on tumor spheroids that impact nuclear volume, shape and integrity. Critically, these nuclear deformations, together with NER events, are known to influence cancer cell invasion. However, a limitation of our model is that the excessively dense CAF layer physically obstructs invasion, preventing assessment of how CAF contractility influences cancer cell invasion. To address this, we are engineering a droplet-based microfluidic system to generate core–shell spheroids where cancer cells form the core and are encapsulated by a CAF shell of tunable thickness. This system will enable investigation of the relationship between CAF-induced nuclear mechanosensing pathways in cancer cells, and the regulation of cancer cell invasion.

B553/P1677
Organoid and 3D Bioprinted primary Cancer tissues for Chemotherapeutic Testing reveals Method and Cell Type matters for success
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Much evidence now exists that animal models have problematic translation to the human system, but human models also have limitations. Organoids, though an important advance, were originally developed using homogeneous cell lines where self-aggregation is relatively uncomplicated. This, however, is not always sufficient with primary human tissues. Testing advanced chemotherapeutic strategies requires primary cancer models. For this reason, we have investigated primary epithelial ovarian (OVA), breast (BRE) and pancreatic (PAN) cancer for cohesive reproducible organoid structures and 3D bioprinted macrotumor models. Having already developed protocols to harvest and cryopreserve primary cancer cells and associated fibroblasts and cryopreservation, we investigated organoid reproducibility on ethically biobanked ovarian, breast and pancreatic patient-derived tumor cells using both ultralow attachment (ULA) plates and 3D Bioprinting. ULA plates were seeded with 2, 3, 5, 7, 10 and 15 thousand cells per well. 3D bioprinting was set up by computer-aided model generation for 3D sliced g-code production before cells were mixed with bioink containing alginate and nanofibrillar cellulose and printed in multiwell plates (15M/ml, viscosity 3–20,000 Pa/s, shear rate 0.002–500 1/s). Models were incubated with media optimized for epithelial growth and maintained for up to 5 weeks and evaluated (Alamar Blue metabolic, Live-Dead survival staining and immunofluorescence for proliferation, apoptosis and hypoxia). Where tumors were significant, they were challenged with dose-response chemotherapeutic. OVA and PAN organoids (all concentrations) failed to form consistently cohesive structures on ULA plates, with micro-tumor reformation probably due to migration. BRE formed highly cohesive organoids with clear 3D development of outer live and inner hypoxic tissue (> 220 µm), typical of the cancer type with size related to the dose of cells, 21 days being optimal. 3D printed macro-tumors could be formed from PAN and BRE tissues. Chemo-response of 3D structures: PAN Erlotinib & Gemcitabine (GEM) and BRE GEM & Rapamycin, was dose-responsive (10-100 µM). Although 3D cancer models were not always possible, it demonstrates the promise of preserving patient-specific tumoral cells to facilitate treatment selection and test new therapeutics without animal testing.

B552/P1676
Overcoming Drug-Resistant Non-Small Cell Lung Cancer with Proteolysis-Targeting Chimeras (PROTACs)
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Lung cancer is the leading cause of cancer-related deaths in the U.S. Non-small cell lung cancer (NSCLC) makes up >80% of lung cancer cases, 5% of which result from chromosomal inversion in the gene for Anaplastic Lymphoma Kinase (ALK), a receptor tyrosine kinase that activates the MAP kinase pathway. One common form is the EML4-ALK fusion leading to a constitutively active tyrosine kinase. While competitive ALK inhibitors have been approved by the FDA, spontaneous suppressor mutations in the drug binding site limit their efficacy, with median duration of response of 11 months. To address this limitation of inhibitor therapeutics, we have generated a series of proteolysis-targeting chimeras (PROTACs) to induce the degradation of the EML4-ALK protein. These PROTACs contain an EML4-ALK targeting motif, a flexible linker, and a ligand that recruits an E3

ubiquitin ligase. This complex induces the polyubiquitination and subsequent proteasome-mediated degradation of the target protein. In early stage PROTAC development, we used the FDA-approved crizotinib, alectinib, or brigatinib as the targeting motif, and varied the length and chemical constituency of the linker. Using a cell model of EML4-ALK-dependent lung cancer, we found that brigatinib-based PROTACs showed the highest degree of growth inhibition and degradation of the EML4-ALK protein, with our lead compounds showing IC50 and DC50 values in the single nanomolar range. To test the ability of our PROTACs to degrade EML4-ALK with therapeutic-resistant suppressor mutations, we constructed a series of Ba/F3 cell lines expressing EML4-ALK containing suppressor mutations commonly found in NSCLC patients. In these cells where FDA-approved therapeutics lose efficacy, we see dose-dependent decreases in growth inhibition and decreases in EML4-ALK protein with IC50 and DC50 values at these same single nanomolar concentrations. To test the in vivo efficacy of our lead PROTACs, we generated xenograft models in mice with our Ba/F3 cell lines followed by oral PROTAC administration. In each model, we observed a significant decrease in tumor volume resulting from PROTAC administration compared to either equivalent doses of FDA-approved therapeutic or vehicle. Pharmacokinetics analyses of bioavailability show significant plasma levels of PROTACs following oral administration at doses used in xenograft studies. This work points toward the potential therapeutic efficacy of PROTACs in drug-resistant forms of NSCLC.

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Scalable Microfluidic DACIT Platform Enables Sequencing and Time-lapse 3D Imaging at Cellular Resolution

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Rationale: There is increasing interest in studying the role of peripheral innervation in tumor growth and metastasis. However, quantitative studies of interactions between cancer cells and axonal projections are technically challenging, as mimicking the environments where neuron soma and axonal terminals reside requires different chemical components. Moreover, in depth mechanistic studies of cancer-axon interactions require high-resolution dynamic imaging, as well as cell harvest and single cell sequencing. Optimization of the two applications require significantly different number of cells i.e. device sizes. Towards this goal, we designed, fabricated and validated multi-compartment microfluidic Devices for Axon-Cancer cell Interaction (DACIT) that mimic peripheral soma–axon anatomy and depending on the scale of fabrication, allow for either real-time imaging of 2D and 3D cell cultures, or cell harvest for downstream single cell sequencing. Methods: Two-layer device designed in LayoutEditor and fabricated via photolithography. PDMS was then cast on the molds, cured, and plasma-bonded to coverslips. The imaging-optimized chip (DACIT-I) has compact chambers and stringent fluidic isolation; the harvest-optimized chip to be used for downstream sequencing (DACIT-S) preserves the geometry but increases chamber volume and access for gentle dissociation and single-cell RNA-sequencing workflows. Results: Microgroove geometry reliably permitted axonal ingress while excluding somata and cancer cell bodies, yielding stable soma–axon separation with no observed cell-body crossover during multi-day cultures. Axon trajectories were aligned by the grooves, enabling consistent pathfinding and reproducible region-of-interest placement for imaging. Microchannel height and footprint accommodated intact 3D tumor spheroids without compression or loss of architecture, while maintaining optical access for confocal imaging in DACIT-I. Fluidic isolation enabled localized delivery to the axon compartment, with sustained gradients confirmed by tracer profiles. At larger scale, DACIT-S preserves compartment fidelity and yields viable, compartment-resolved cell suspensions for downstream single-cell library prep. Conclusion: The scalable DACIT platform translates a shared, two-layer design into complementary use cases: rigorous, imaging-grade compartmentalization and guidance for dynamic assays in DACIT-I, and scaled geometry for high-yield, compartment-resolved cell harvest in DACIT-S. Together, they provide an integrated pipeline that couples

anatomically faithful separation of somata and axons with either real-time readouts or single-cell molecular profiling, while ensuring spheroid compatibility and robust fluidic isolation.

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The Lipid Chaperone FABP5 is a Druggable Driver of Metastatic Prostate Cancer
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Large scale genome sequencing efforts have demonstrated that lethal, therapy-resistant, metastatic prostate cancer (PC) is enriched for loss of function alterations in PTEN and TP53 tumor suppressor genes. Unfortunately, patients with metastatic PC do not respond to PI 3-Kinase pathway inhibitors and invariably present with lethal disease that is resistant to androgen receptor blockade and taxol-based chemotherapy. This underscores the impending need for new targetable avenues in the metastatic setting. Altered metabolism is a cancer vulnerability, and several metabolic pathways have been shown to promote PC. Through a cross-species cancer genomics and transcriptomics analysis, we nominate the lipid carrier protein FABP5 as an actionable target in PC. Oncogenic signaling pathways that drive prostate tumor progression independently converge on FABP5 via transcriptional activation and post-translational regulation. CRISPR-based genetic perturbation of FABP5 hampers proliferation of human and mouse-derived prostate cancer cell lines that highly express the gene. Pharmacological screening in isogenic Pten/Trp53-deficient cells that have or lack FABP5 revealed marked sensitization to ferroptosis inducers upon Fabp5 loss, thus highlighting a gatekeeping role for this lipid carrier in limiting ROS in membrane compartments. This led us to further validate this dependency in RapidCaP, our autochthonous mouse model of metastatic prostate cancer. We delivered Cre-expressing lentivirus into the prostates of Ptenfl/fl;Trp53fl/fl or Fabp5fl/fl;Ptenfl/fl;Trp53fl/fl mice to evaluate the functional consequences of Fabp5 ablation on Pten/Trp53 deficient PC initiation, progression, and overall survival of tumor-bearing animals. Fabp5 loss significantly delays prostate tumor progression, blunts metastatic colonization, and extends the lifespan of prostate tumor-bearing mice. Together, our findings reveal FABP5 as a druggable vulnerability that prostate cancer cells rely on to evade ferroptosis during metastatic tumor progression.

Cancer Cell Biology - Extracellular vesicles and exosomes

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Extracellular vesicle 5'half-GlyGCC links ovarian cancer platinum resistance to T cell MAPK pathway inhibition
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Introduction Tumor-infiltrating T cells are known to play a crucial role in the acquisition of platinum resistance in ovarian cancer, but the underlying mechanisms remain unclear. We previously identified 5’half-GlyGCC, highly enriched in extracellular vesicles (EVs) derived from platinum-resistant tumors, as a candidate T cell inhibitor. Here, we investigated the effects of 5’half-GlyGCC on T cells and platinum resistance, aiming to elucidate its role as a T cell suppressor. Methods Clinical specimens: Ovarian cancer tissues were obtained from patients who underwent primary resection at Osaka Medical and Pharmaceutical University, Japan. Written informed consent was obtained from each patient, and the study was approved by the institutional review board. Tissue-derived EVs: Tissue-immersed media from normal ovaries and platinum-sensitive or -

resistant ovarian cancer tissues were centrifuged at 2,000 g for 30 min, followed by ultracentrifugation (100,000 g × 2) to isolate tissue-exudative EVs (Te-EVs). Proteomic analysis: Whole-cell lysates of EV-treated T cells were extracted with RIPA buffer. Proteins were digested with trypsin and analyzed by UHPLC-Tribrid-MS. Results Compared with Te-EVs from normal ovaries or platinum-sensitive tumors, platinum-resistant Te-EVs were preferentially taken up by CD4 T cells and markedly suppressed their activity. Overexpression of 5'half-GlyGCC in normal ovarian Te-EVs reduced T cell activation and IFN-γ secretion. In a mouse ovarian cancer model, EVs promoted platinum resistance in a 5'half-GlyGCC-dependent manner, accompanied by reduced activity and infiltration of tumor-infiltrating T cells. Proteomic profiling revealed enrichment of MAPK suppressor proteins in T cells exposed to 5'half-GlyGCC-containing EVs. Consistently, phosphorylation of ERK1/2 and p38 was downregulated in T cells treated with ovarian cancer EVs overexpressing 5'half-GlyGCC.Conclusion Ovarian cancer–derived EVs containing 5'half-GlyGCC contribute to the acquisition of platinum resistance by suppressing MAPK signaling in T cells.

B543/P1667
Unveiling the Significance of Midbody Remnants: A Novel Class of Translating Extracellular Vesicles
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Our lab identified and characterized the role of the midbody (MB)-associated RNA-binding protein ATX-2 in early C. elegans embryos. One striking phenotype observed following ATX-2 knockdown was a significant delay in abscission, leading us to propose a radical hypothesis: translation may play a critical role in the final stages of mitosis. To explore this, we investigated RNA localization and activity within mammalian MBs. Historically considered vestigial remnants of cytokinesis, MBs and midbody remnants (MBRs) have recently emerged as central players in cell communication, fate determination, and extracellular vesicle (EV) biology. We discovered that the MB matrix functions as a hub for ribonucleoprotein assembly, enriched with mRNAs encoding proteins involved in cell fate, oncogenesis, and pluripotency—collectively termed the "MB granule." Translation occurs within both MBs and post-abscission MBRs in a spatiotemporally regulated manner, initiating as nascent daughter cells transition into G1 and continuing after extracellular release. RNA localization to the MB dark zone is regulated by MKLP1 and ARC, while ESCRT-III modulates translation levels. Next, we established that MBRs represent a novel class of EVs by demonstrating that all commonly used EV isolation methods recover MBRs. Furthermore, inhibiting cytokinesis or cell cycle progression reduces MBR production as well as EV generation across all size classes, suggesting that proliferative cells are major contributors to EV biogenesis. Based on these findings, we propose a model linking EV production to cell cycle dynamics, highlighting mitotic cells as key sources of these actively translating large EVs. Our discovery of MBRs as translating EVs provides new insights into cancer diagnostics and therapeutic engineering while redefining our understanding of post-mitotic cellular processes.

Cancer Cell Biology - Genome Instability, Epigenetics, and DNA Damage

B449/P1573
Aneuploid evolution in human cells reveals fitness-based and genetic-background-dependent selection following chromosomal instability
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Chromosomal instability (CIN)—the persistent mis-segregation of chromosomes during mitosis—is a hallmark of cancer and a major source of aneuploidy. A central question is what governs fitness-based selection, including the environment and genetic background. Distinguishing these factors is critical because karyotype selection confounds CIN measurement and shapes tumor evolution and therapy response. We first tested CIN induction in CAL51 p53-WT cells, where aneuploid cells emerged transiently but failed to persist, consistent with p53-mediated elimination of chromosomally unstable cells. We therefore performed all subsequent experiments using p53-deficient CAL51 and MCF10A derivatives that tolerate aneuploidy and enable evolutionary tracking. Karyotype evolution was monitored at the single-cell level (FISH validation; scDNA-seq at Days 2 and 14). From arm-level frequency shifts, we derived chromosome-specific fitness coefficients and simulated clonal expansion to evaluate how selection drives aneuploid persistence. Specific CNAs were preferentially retained from Day 2 to Day 14—declining less than other states—indicating selective tolerance rather than neutral drift. We compared chromosome-arm CNAs across both models and classified them as concordant (shared between models) or discordant (cell-line–specific). Several concordant CNAs overlapped with recurrent alterations in breast-cancer datasets, highlighting generalizable selection (e.g., gains of 1q and 20q, losses of 5q and 17q). Discordant CNAs reflected genetic-background–dependent selection, including CAL51-specific losses on 7q and 16p and MCF10A-specific losses on 18q. Model simulations accurately reproduced the observed Day-14 landscapes, supporting selection on specific CNAs as the driver of clonal outcomes. Subcloning under low oxygen and in human plasma–like medium did not alter clonogenic survival, confirming that the observed selection reflects intrinsic karyotypic fitness rather than environmental effects. Conclusion: Aneuploid evolution after CIN follows reproducible fitness rules that combine genetic-background–dependent and generalizable selection, providing a quantitative basis for how CIN shapes stable aneuploid states in cancer.

B441/P1565

Aneuploidy Shapes Nucleolar Morphology via Dosage of Nucleolar Organizer Regions

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Nucleolar enlargement and aberrant morphologies are pathognomonic of cancer cells. Given that aneuploidy, defined as abnormal chromosome number, is pervasive in human cancers and frequently involves acrocentric chromosomes carrying nucleolar organizer regions (NORs), we investigated how aneuploidy-driven changes in NOR dosage affect nucleolar architecture. Using high-resolution 3D imaging and quantitative analyses in reversine-induced aneuploid DLD1 colorectal cancer cells and patient-derived normal colon organoids, we demonstrate that aneuploidy significantly increases nucleolar number and volume in a genome dosage-dependent manner. Surprisingly, nucleolar enlargement occurs independently of global protein synthesis, challenging prevailing views that nucleolar size primarily reflects increased biosynthetic demand, and our 3D analyses reveal a direct linear correlation between nucleolar volume and NOR number. These findings establish NOR dosage as a structural determinant of nucleolar remodeling, providing a new mechanistic framework to understand how chromosomal instability reshapes nuclear architecture and spatial chromosomal constraints in cancer.

B450/P1574

Autoimmune regulator (AIRE) suppresses keratinocyte apoptosis through coordinated regulation of transcription and the DNA damage response

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Autoimmune regulator (AIRE) is a stress-inducible transcriptional regulator whose loss limits pre-cancerous growth in mouse models of non-melanoma skin cancer. Although AIRE interacts with DNA damage response (DDR) proteins, its functional role in the DDR in stressed keratinocytes remains poorly defined. We engineered WT (non-targeting small guide RNA) and AIRE-/- HaCaT keratinocytes using CRISPR/Cas9 and treated them with ultraviolet B (UVB; 25-100 mJ/cm2). AIRE-deficient cells exhibited increased sensitivity to cell death as assessed by sustained DNA damage (comet assays, unscheduled DNA synthesis) and elevated apoptotic markers (annexin V/7-AAD, cleaved caspases 3/8/9, cleaved PARP) relative to control cells. Additionally, 5-ethynyl uridine labeling revealed reduced transcriptional recovery post-UVB in AIRE-/- cells, suggesting a defect in re-initiating RNA synthesis. Using BioID proximity labeling, we identified that AIRE-interacting proteins were enriched in nuclear bodies, transcriptional regulation, and DDR machinery. Live-cell imaging of mCherry-AIRE following laser microirradiation revealed rapid foci formation within seconds that was sensitive to PARP and transcriptional inhibition, but independent from canonical DDR pathways (ATM, ATR). These foci were spatially adjacent, but distinct from, DDR (γH2AX, 53BP1, RPA) and transcriptional (RNA pol II, BRD4) foci. Domain analysis demonstrated that damage-inducible mCherry-AIRE foci assembly required AIRE's caspase activation and recruitment domain (CARD), but was independent of other key domains. In vivo, Aire-/- mice exhibited elevated γH2AX staining in back skin following UVB exposure, indicating increased DNA damage in an AIRE-deficient tissue context. These results position AIRE as a spatial and temporal coordinator of DDR resolution and transcriptional restart that suppresses apoptosis in stressed keratinocytes.

B454/P1578

Biochemical dissection of histone synthesis for cancer therapeutics

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Introduction: Most cancer therapies rely on cytotoxic drugs that inhibit DNA replication, damaging rapidly dividing cells but causing severe side effects and secondary malignancies. During DNA replication, newly synthesized DNA must be packaged into chromatin by incorporating newly made histones into nucleosomes. While more than 50 FDA-approved chemotherapies target DNA synthesis, no existing drugs inhibit nucleosome production—an untapped opportunity for safer, more selective therapies. Histone synthesis occurs almost exclusively during S-phase and is absent in quiescent cells, offering a wide therapeutic window. Disrupting nucleosome synthesis could induce cell cycle arrest without the genotoxicity of conventional chemotherapy. However, pharmacologically actionable targets for blocking nucleosome production remain undefined. Methods: We focus on Histone Acetyltransferase 1 (HAT1), a key regulator of nascent histone stability and chromatin assembly. Using chromatin immunoprecipitation and promoter mapping, we identified HAT1 recruitment to histone H4 promoters. We developed biochemical assays to characterize and inhibit HAT1 enzymatic activity, designed labeling and capture methods to measure histone biosynthesis in cancer versus normal cells, and performed tumor xenograft studies with genetic HAT1 depletion to assess biological consequences of impaired histone production. Results: HAT1 binds directly to H4 promoters, facilitating transcription and histone stabilization. Its loss disrupts histone synthesis, impedes DNA replication, and suppresses tumor growth. HAT1 deficiency reduces nucleosome assembly, induces replication stress, and halts proliferation. Biochemical studies confirm HAT1 acetylation is essential for H4 maturation. Histone production quantification reveals tumors have significantly elevated synthesis compared to matched normal tissue, validating HAT1 as a cancer-specific vulnerability. Conclusion: This proposal pioneers cancer therapies targeting chromatin assembly rather than DNA synthesis. Inhibiting HAT1-driven histone production offers a

strategy to selectively block tumor proliferation while minimizing toxicity. We will define HAT1 recruitment mechanisms, validate its essential role in tumor growth, and measure histone production in patient-derived samples. These studies could establish histone biosynthesis inhibition as a broadly applicable, less toxic therapeutic paradigm, paving the way for new anti-cancer drugs that improve outcomes and quality of life.

B447/P1571

CRISPR screen identifies a cancer-testis gene as a key regulator of DNA damage repair

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Cancer-testis genes (CTGs) are normally expressed exclusively in germline tissues but are aberrantly reactivated in a wide spectrum of human cancers. Although CTGs have been primarily investigated as immunogenic antigens in cancer, the biological basis for their widespread reactivation in tumors remains unclear. Tumor cells possess robust DNA repair capacity, which enables their survival under chemotherapy and other genotoxic stress. Notably, we observed that a substantial subset of CTGs is upregulated in chemotherapy-resistant persister cells, prompting us to perform a CRISPR-Cas9 screen to identify CTGs functionally involved in the DNA damage response (DDR). Using a library targeting CTGs, our screen identified CTG1 as a top candidate that may play a critical role in DDR. We found that depletion of CTG1 exacerbates DNA damage in tumor cells under chemotherapy, whereas CTG1 overexpression reduces their sensitivity to genotoxic stress. Functional assays indicate that CTG1 promotes the repair of DNA double-strand breaks (DSBs) via the non-homologous end joining (NHEJ) pathway. Mechanistically, CTG1 localizes to the nuclear envelope, where it interacts with Lamin-A/C to maintain nuclear envelope structural stability. Upon DNA damage, CTG1 associates with the Ku70/Ku80 heterodimer, a core NHEJ factor, and is specifically recruited to DSB sites. Tracking of DNA damage foci further revealed that CTG1 is required for the immobilization of broken DNA ends and for efficient NHEJ repair at the nuclear periphery. In conclusion, our findings identify CTG1 as a novel regulator of DNA repair and tumor cell survival under DNA damage stress, providing a mechanistic explanation for CTGs reactivation in cancer and highlighting CTG1 as a potential therapeutic target to improve the efficacy of chemotherapy.

B453/P1577

DUSP12 integrates nucleolar function and DDR through structural remodeling, phase separation and bimolecular interactions in Hepatocellular Carcinoma.

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Dual-specificity phosphatase 12 (DUSP12) is a conserved atypical phosphatase with a zinc finger-like domain, suggesting functions beyond enzymatic catalysis. This enzyme plays relevant roles in protecting cells from oxidative stress injuries and apoptosis conditions and has links to hepatocellular carcinoma (HCC) tumorigenesis, which mechanisms are still unclear. Proteomic analysis revealed DUSP12 partners in maintenance of nucleolar and chromatin pathways, including NAT10, HP1BP3 and TCOF1 proteins. Here, we investigated DUSP12 in nucleolar organization and DNA damage response (DDR) using biochemical, structural and cellular approaches. In HuH-7 cells, genotoxic stress by cisplatin (CP) or doxorubicin (DX) activated DDR, with DX strongly inducing p53 (Ser15) and γH2AX (Ser139) phosphorylation and increasing DUSP12–NAT10 colocalization. NAT10 relocalized to the nucleoplasm, while DUSP12 localization remained stable. CRISPR-Cas9-mediated DUSP12 knockout (KO) did not impair proliferation but reduced the proportion of cells in S and M

phases, indicating cell cycle alterations and genomic instability corroborating bioinformatics data. DUSP12-KO clones showed augmented DX sensitivity in both monolayer and spheroid cultures, which correlated with reduced DUSP12 levels. This sensitivity was not due to oxidative damage, as ROS levels remained unchanged, but was associated with stronger DDR activation and delayed repair of both single- and double-strand breaks, as shown by comet assays. Recombinant DUSP12 underwent liquid–liquid phase separation (LLPS) under oxidative conditions (e.g., TBHP) and in presence of RNA or DNA, forming reversible condensates. Redox conditions and nucleic acids also modulate phosphatase activity. Circular dichroism and XL-MS revealed stress induced conformational changes and intramolecular contacts between catalytic and C-terminal domains, potentially regulating active-site accessibility. Pull-down assays showed that DUSP12–NAT10 interaction depends on phosphatase activity, and its inhibition abolishes the binding. Preliminary data support interactions with HP1BP3 and TCOF1, implicating DUSP12 in broader chromatin and stress scenarios. NAT10 immunoprecipitation from DUSP12-deficient cells showed increased phosphotyrosine levels, suggesting functional regulation by dephosphorylation. Altogether, our findings identify DUSP12 as a redox-sensitive phosphatase integrating nucleolar function and DDR through structural reorganization, LLPS formation and protein-protein interactions, highlighting novel roles in genomic homeostasis and potential therapeutic target in HCC.

B442/P1566

Endogenous retroviral element expression drives extracellular matrix remodeling and immune evasion in prostate cancer

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Endogenous retroviral elements (ERVs) are a subclass of transposable elements that make up 8-10% of the human and mouse genomes. ERVs are epigenetically repressed in normal differentiated cells, but can be derepressed in cancer cells. ERVs can also become derepressed in prostate cancer cells after treatment with second generation anti-androgens. However, how sustained ERV expression affects in vivo prostate cancer growth is unclear. Recent work from our lab showed that deletion of Trim28, a known epigenetic co-repressor of ERVs, in an immunocompetent mouse model of prostate cancer with Pten and p53 deletion results in accelerated tumor progression and a shift to a pro-tumorigenic tumor microenvironment (Yende, Williams, et al., Oncogene 2023). The objective of this study was to characterize ERV expression in these Trim28 deleted tumors. We performed bulk RNA sequencing on tumor tissues and analyzed the expression of transposable elements and protein-coding genes. Additionally, we utilized immunostaining to assess double-stranded RNA (dsRNA) formation and immune cell localization. Our results indicated that Trim28 deletion in prostate tumors derepresses ERVs independent of androgen signaling. ERVs are known to regulate expression of neighboring genes, and we detected increased expression of several protein-coding genes near derepressed ERVs. We also observed dsRNA staining and increased activation of the innate immune system in Trim28-deleted tumors compared to controls. Despite evidence of increased immune cell activation, T cell and CD206+ macrophage infiltration was not increased in Trim28-deleted tumors compared to controls. Gene expression analysis revealed an enrichment for genes involved in collagen formation and tumor extracellular matrix remodeling in Trim28-deleted tumors, which we confirmed by picrosirius red staining. Overall, our provide new insights into how ERV derepression affects the tumor microenvironment to promote immune evasion.

B452/P1576

Epigenetic Chemotherapy Leads to Heritable Genetic Change

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Epigenetic drugs are used clinically but anti-cancer effects are limited. Here we use a fluorescent chromosome reporter 'ChReporter' to show that a common epigenetic drug leads to heritable DNA loss. We focus on the DNA methyltransferase inhibitor Azacytidine (AZA) and Eprenetapopt (APR) that can restore P53 function as they are clinically used in combination. Although AZA has previously been shown to also induce double strand breaks and micronuclei, heritability of any genotoxic events is unexplored. We also inhibited the spindle assembly checkpoint with reversine as a positive control for chromosome missegregation. 2D cultures focused on 'M2' mouse ovarian cancer cells with P53/PTEN knockpout, B16 mouse melanoma with functional wild-type P53, and H23 human lung adenocarcinoma cells with P53 mutation. Flow cytometry and/or microscopy were used to measure GFP-ChReporter loss and micronuclei formation. Visible colonies of GFP negative nuclei can be seen after AZA and APR stress. Both micronuclei counts and ChReporter loss increased for M2 treated with AZA, whereas APR showed no effect. Reversine led to the expected increases, and M2 cells with BRCA defects amplified the increases. B16's showed similar increases with AZA and Reversine but no effect of APR. H23 were affected by APR as well as AZA, with APR increasing ChReporter loss. Heritable ChReporter loss tends to increase with micronuclei counts increase and that this loss is heritable, and APR did not affect cell lines with normal or no P53. Additional epigenetic drugs and tests on 3D tumoroids will further clarify some of the cancer-relevant effects and possible limitations in the clinic.

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Epigenetic Reprogramming of Triple-Negative Breast Cancer via HDAC Inhibition Enhances Chemotherapy Response

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Background: Triple-negative breast cancer (TNBC) is an aggressive subtype with poor prognosis and limited response to therapies such as Atezolizumab and Nab-paclitaxel. Epigenetic modulation represents a promising strategy to overcome resistance. Methods & Results: Droxinostat, an HDAC 3, 6, and 8 inhibitor, was evaluated alone and in combination with Atezolizumab and Nab-paclitaxel in TNBC (MDA-MB-231) and normal breast (MCF-10A) cells. IC50 values for Droxinostat were 60 µM in cancer cells and 190 µM in normal cells; for Nab-paclitaxel, 0.58 µM and 0.16 µM, respectively. The Droxinostat (48 µM) + Nab-paclitaxel (0.28 µM) combination showed moderate synergy (CI=0.808), yet this was associated with a marked biological effect, including a 5–6 fold increase in apoptosis in TNBC cells while sparing normal cells from apoptosis and inducing only necroptosis. Atezolizumab (100 nM) alone did not show significant overall cytotoxicity, but induced specific nuclear anomalies (increased nuclear bridges) in micronucleus assay. Cell cycle analysis revealed S/M phase arrest with Droxinostat and M phase arrest with Nab-paclitaxel, with no significant changes in normal cells. Comet assay demonstrated that the triple combination significantly enhanced oxidative DNA damage, with tail comet scores approaching positive control levels. Micronucleus assay showed Droxinostat-induced nuclear budding and additive effects in combinations. Gene expression analysis revealed strong inhibition of HDAC8 and upregulation of caspase-3/-8 by Droxinostat. Nab-paclitaxel showed similar but weaker effects, while PD-L1 expression remained unchanged with Atezolizumab. HDAC activity assays confirmed direct target inhibition

by Droxinostat, enhanced in combinations. Oxidative stress assays indicated elevated SOD activity and increased GSH levels with the triple combination, reflecting a compensatory antioxidant response to heightened oxidative stress. Conclusion: Droxinostat emerges as a promising agent for TNBC therapy, particularly in combination with Nab-paclitaxel and Atezolizumab, enhancing selective cytotoxicity via multiple complementary mechanisms. This work was financially supported by the Health Institutes of Türkiye (TUSEB).

B439/P1563

Epitranscriptomic Regulation Analysis of the RNA Demethylase ALKBH3 in Pancreatic Cancer

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Introduction AlkB homolog 3 (ALKBH3), an RNA demethylase enzyme cloned in our laboratory, is highly expressed in pancreatic cancer, one of the most difficult cancers to treat, and it has been shown that patients with high ALKBH3 expression have poor prognosis. Furthermore, in analysis using pancreatic cancer cell lines, ALKBH3 siRNAs demonstrated cell proliferation inhibitory effects and antitumor effects. Based on these results, ALKBH3 is expected to be a promising molecular target for pancreatic cancer. In this study, we aim to verify the effects of the newly developed ALKBH3 demethylase inhibitory compound as a tool compound in pancreatic cancer cells and to investigate the molecular mechanisms of epitranscriptomic regulation by ALKBH3. Methods and Results The ALKBH3 demethylase inhibitory effect of the compound was evaluated by measuring the residual m1A level using an anti-m1A antibody when the compound was applied to silkworm recombinant ALKBH3 using oligonucleotides containing 1-methyladenosine (m1A) as a substrate. The results showed that the compound inhibited the m1A demethylation activity of ALKBH3. Additionally, the compound significantly suppressed the proliferation of pancreatic cancer cells and induced apoptosis. The effect of this compound was shown to be due to inhibition of ALKBH3 demethylase activity by immunocytochemical staining of pancreatic cancer cells and measurement of m1A levels in extracted small RNA by dot-blot analysis. Furthermore, we found that the compound-induced increase in m1A levels in small RNAs leaded to decrease protein synthesis capacity in pancreatic cancer cells. To elucidate the molecular mechanism underlying the compound's inhibitory effect on pancreatic cancer cell proliferation, proteomics analysis was performed. As a result, 378 proteins with reduced expression were identified upon compound addition, and these were enriched in pathways related to mitochondria functions. Furthermore, evaluation of mitochondrial functions upon compound addition revealed increased ROS production, decreased membrane potential, and reduced ATP production. Conclusion In pancreatic cancer cells, ALKBH3 may contribute to cell proliferation promotion via regulation of mitochondria functions by demethylating m1A in small RNAs such as tRNA.

B446/P1570

Evaluating Phosphaplatin Efficacy in Breast and Lung Cancer Cells

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Platinum-based chemotherapies, while effective, are limited by systemic toxicity and the development of resistance in many cancers. Phosphaplatins, a novel class of platinum-containing compounds, are currently being investigated to overcome these limitations. Unlike traditional agents such as cisplatin, which rely on labile chloride ligands to form DNA adducts, phosphaplatins lack these reactive groups, potentially reducing direct DNA binding and resistance via DNA repair pathways. Prior studies suggest they may exert their effects

through alternative mechanisms including induction of apoptosis via extrinsic signaling, oxidative stress, and mitochondrial dysfunction, but they may act via additional mechanisms as well. This study assessed the in vitro effects of three phosphaplatin compounds—(1R,2R-diaminocyclohexane)(dihydropyrophosphato)platinum(II) (RRD2), dicyclobutylaminepyrophosphatoplatinum(II) (cBuAm-2), and dicyclopentylaminepyrophosphatoplatinum(II) (cPnAm-2)—in two molecularly distinct human cancer cell lines. MDA-MB-231 cells, derived from triple-negative breast cancer, display mesenchymal characteristics and therapy resistant properties. A549 cells, derived from non-small cell lung carcinoma, exhibit epithelial characteristics and are used as a standard lung cancer model. Cells were treated with increasing concentrations of each compound (cisplatin for 48 hours; phosphaplatins for 72 hours), and viability was measured using MTT assays. IC50 values were calculated from dose-response curves, with phosphaplatins showing a broad range of efficacy (1.34 μM to 98.0 μM). Among the tested phosphaplatins, RRD2 showed the strongest dose-response effect, with IC50 values of 3.07 μM in MDA-MB-231 and 1.34 μM in A549 cells, compared to cisplatin's IC50 values of 14.4 μM and 6.81 μM, respectively. While these findings are promising, differences in treatment duration limited direct comparisons between cisplatin and the phosphaplatins. Nonetheless, the cytotoxic activity of RRD2 highlights its potential as a lead phosphaplatin drug for further study. Future directions aim to determine the specific mechanisms by which phosphaplatins exert their effects on cancer cells.

B457/P1581

Heat and fat slow the growth of cancer cells but drive genetic change

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Tumor heterogeneity can cause increased resistance to treatment, and so understanding whether physical stresses contribute to heritable changes can help with therapies. Here we focused on two distinct physical stresses – heat and fat - to study their effect on chromosome instability. To visualize and quantify heritable genetic changes at single cell resolution, we use our chromosome reporter (ChReporter) system, such that loss/gain in GFP signal indicates loss/gain of all or part of the chromosome. Heating at 42C followed by recovery at 37C mimics hyperthermia treatments in the clinic, and using U2OS osteosarcoma ChReporter cells we find unique losses of ChReporter signal per division are higher despite delayed division. A similar result is obtained from the stress of lipid droplets (LDs, induced with oleic acid) that accumulate in various cancer cell types and increase malignancy. Loss and gain colonies colocalize across conditions, but the gain-to-loss ratio of cells is <1, indicating a lag in the gains. Although micronuclei harbor gains and often suffer extensive DNA damage, GFP-negative cell counts correlate with micronuclei counts across heating times (R2 =0.998). 3D tumoroid models also showed heat and fat stress decrease growth, consistent with our 2D findings of slowed proliferation and again suggesting genomic instability.

B440/P1564

Identification of functionally important residues and domains in XRCC3 through cancer variant analysis

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The RAD51 paralogs are a group of ATPases that play a key role in regulating homologous recombination (HR) repair of DNA double-stranded breaks (DSBs). Important in maintaining genome stability, mutations in several of these paralogs have known links to tumorigenesis, particularly in breast, ovarian, and prostate cancers. Notably, the RAD51 paralog XRCC3 is relatively poorly characterized and has a debated role in tumorigenesis. To elucidate its function, we characterized 23 cancer-identified XRCC3 missense variants found in breast, ovarian, and prostate cancer patients. We first used the deep learning tool Alpha Missense to predict the pathogenicity of missense mutations based on sequence conservation. The tool predicted high pathogenicity for mutations of residues in XRCC3's conserved Walker A and Walker B ATPase motifs. We then examined how well the in silico analysis predicted loss of XRCC3 function by performing functional analysis of the individual variants. By complementing an XRCC3 CRISPR/Cas9 U2OS knockout cell line with a plasmid that expressed the XRCC3 variant of interest, we examined each variant's ability to proficiently perform HR using a sister chromatid recombination assay. We also examined the proficiency of the XRCC3 variants to interact with XRCC3's binding partner RAD51C and the central recombinase RAD51 by yeast-two-hybrid. Thus far, we have identified residues needed for XRCC3 DNA binding as critical for HR. In addition, we identified a potential separation-of-function allele that disrupts XRCC3's interaction with RAD51 while maintaining its interaction with its binding partner RAD51C. Together, our results identified functionally important residues and activities of XRCC3 needed for its biological function during DNA repair and suggest that these residues may be important for cancer prevention.

B456/P1580

Lamin B loss in nuclear blebs is rupture dependent while increased DNA damage is rupture independent

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The nucleus must maintain shape and integrity to protect the function of the genome. Nuclear blebs are deformations identified by decreased DNA density that commonly lead to rupture. Lamin B levels often vary drastically between blebs. We tracked rupture via time lapse imaging of NLS-GFP into immunofluorescence of lamins and known rupture markers. We find that lamin B1 loss consistently marks ruptured nuclear blebs better than lamin A/C, emerin and cGAS. Visualizing post-rupture lamin B1 loss and emerin enrichment reveals that cell lines display widely different propensities for nuclear bleb rupture. To determine how rupture affects DNA damage, we time lapse imaged ruptured and unruptured blebs, then conducted immunofluorescence on the same cells for DNA damage markers γH2AX and 53BP1. We find that DNA damage is increased in blebbed nuclei independent of rupture. This was verified in blebbed LNCaP nuclei, which do not rupture and maintain lamin B1, but still show increased DNA damage. Thus, we confirm that lamin B is the most consistent marker of nuclear rupture, and that blebbed nuclei have increased DNA damage regardless of rupture.

B448/P1572

Long non-coding RNA TERRA overexpression after particulate matter PM10 exposure leads to telomeric instability

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Background: Telomeres are repeated DNA sequences whose function is to protect the chromosomal ends and distinguish them from double-stranded DNA breaks, thus preventing fusions between chromosomes. Loss of telomere integrity can lead to a telomere crisis, a state of extensive genomic instability that can promote cancer progression. TERRA is a long non-coding RNA (lncRNA) involved in maintaining telomere length, regulating telomerase activity in protecting chromosomal ends. Alterations in TERRA levels are associated with the generation of genetic instability, a feature of tumor development. Air pollution is considered an environmental problem with a high impact on human health. Exposure to air pollutants smaller than or equal to 10 micrometers (PM10) is associated with respiratory diseases and the development of lung cancer. Although exposure to PM10 has been associated with the generation of genomic instability, the impact on the mechanisms that control telomere regulation has not been determined. Objective: To determine the effect of PM10 particulate matter on TERRA expression, as well as its impact on telomere regulation and genetic instability, in the A549 cell line. Methods: Cultures of A549 cells (lung adenocarcinoma) exposed to 10 µg/cm2 of PM10 for 24 h were performed to evaluate the sub-telomeric expression of the TERRA lncRNA (5p and 10q). In addition, telomerase (hTERT) expression was estimated under the same conditions. The frequency of cells with anaphase bridges was analyzed, to evaluate DNA damage as a function of telomerase expression and telomere length. Finally, relative telomere length (RTL) after 120 hours of PM10 exposure was analyzed by qPCR. Results: Cells exposed to PM10 increased expression levels of TERRA 5p (36.4%) and TERRA 10q (32.9%) compared to the control group ($p<0.05$). In addition, an increase in HTERT expression levels (36.0%) was observed in cells exposed to PM10. The frequency of anaphase bridges was higher in cells treated with PM10 compared to the control group (31.3/1000 cells vs. 16.3/1000 cells; $p<0.05$). Finally, the RTL was higher in cells treated with PM10 compared to the control group (1.38 vs. 1.00; $p<0.05$) Conclusion: In vitro overexpression of both long non-coding RNA TERRA and telomerase (hTERT) after particulate matter PM10 exposure leads to the generation of telomere and genomic instability, a feature required for tumor progression. This work was supported by SECIHTI Grants: CF-2023-I-2624 (MS-A) and FPIS-INCAN-4352 (RG-B)

B451/P1575

Loss of nuclear integrity at persistent DNA bridges contributes to the anti-tumor response

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BRCA mutant tumors, deficient in DNA repair, exhibit some of the highest levels of tumor cell-intrinsic interferon(IFN)-stimulated gene (ISG) expression among The Cancer Genome Atlas primary tumors, consistent with preclinical studies tying DNA repair deficiency to increased ISG expression through an as yet poorly understood mechanism. The goal of my ongoing studies is to examine the contribution of mitotic errors to IFN signaling in BRCA-deficient cancer. We find that DNA bridges that persist beyond the abscission checkpoint into the following interphase occur more frequently with BRCA1 deficiency. Treatment with PARP inhibitors (PARPi), which cause targeted tumor cell death of BRCA-deficient cells, further increases the frequency of persistent bridges. Most persistent bridges recruit the cytosolic DNA sensor cGAS, suggesting a loss of nuclear integrity that exposes the bridging DNA to the cytoplasm. Indeed, performing the first correlative light and electron microscopy (CLEM) of persistent DNA bridges, we find nuclear envelope (NE) ruptures at PARPi-induced bridges. cGAS is maximally activated by non-nucleosomal DNA, and we find the bridging DNA to be highly accessible as assessed by Tn5 transposition (ATAC-see). Surprisingly, our CLEM data reveals efficient NE repair at persistent bridges, which we tie to the robust recruitment of the NE repair factors BAF, LEM2 and CHMP7. This NE repair network may influence cGAS activation by the bridging DNA thereby influencing ISG expression. Indeed, knockdown of the most upstream NE repair factor, BAF, increases ISG expression and synergizes with PARPi. Taken together, our results demonstrate that PARPi treatment increases the frequency of persistent

DNA bridges that are over-stretched, non-nucleosomal, and defective in nuclear integrity, making them potent activators of cGAS-dependent IFN signaling. Further elucidating the mechanisms by which persistent DNA bridges induce pro-inflammatory signaling will provide novel insights into strategies for maximizing the therapeutic potential of PARPi treatment in DNA repair-deficient cancer.

B443/P1567
Microenvironmental effects of BRCA1/2 haploinsufficiency on chromosome loss processes and immune signaling in 2D and 3D tumoroids
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Ovarian cancer survival is just 5 years, with most tumors evolving resistance to therapy. BRCA1/2 proteins are frequently involved in ovarian cancer and normally help maintain genome stability via DNA repair, but any effects of the microenvironment seem underexplored. Ovarian cancer indeed has among the highest levels of chromosome losses and gains and also the highest expression of collagen-I, which we generally find correlates across cancers. We find BRCAheterozygous mouse ovarian cancer cells (P53, PTEN null) harbor as many micronuclei as BRCA-deficient cells and more than control cells. Chromosome loss is also high for BRCA-deficient cells as measured by GFP-Chromosome Reporters, and missing chromosomes are generally harbored in micronuclei that are susceptible to rupture and DNA damage. Although all cell types grow similarly in 2D despite increased DNA damage in the BRCA heterozygous and fully deficient cells, these latter cells form larger 3D tumoroids compared to control cells that also increase in size upon addition of a DNA-damaging agent. Past results have indicated 3D is better than 2D at revealing cancer growth upon deletion of tumor suppressors [Han Nature 2020] and that 3D is much less sensitive than 2D to p53 signaling from genome instability [Narkar Cell Rep 2021]. Intriguingly, our results so far suggest that DNA damage helps ovarian cancer overcome growth limits typical in a 3D context.

B455/P1579
Sage1: a Novel Regulator ofAlternative Lengthening of Telomeres via Interaction with SLX1
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Cellular immortality, a hallmark of cancer, requires the maintenance of telomeres to bypass replicative senescence. While the majority (~85%) of cancers achieve this by reactivating telomerase, a significant subset (approximately 15%) utilizes a telomerase-independent mechanism termed Alternative Lengthening of Telomeres (ALT), which relies on homologous recombination. However, specific molecular targets with therapeutic potential for ALT-positive cancers are still lacking. Here, we identify SAGE1 (Sarcoma antigen 1) as a novel and critical regulator of the ALT pathway. SAGE1 is a simian-specific gene that belongs to the Cancer-Testis Antigen (CTA) family, typically restricted to the testis but frequently aberrantly activated in various malignancies. We demonstrate that SAGE1 directly interacts with the structure-specific endonuclease SLX1, a key component of the SMX (SLX4-MUS81-XPF-SLX1) complex responsible for resolving telomeric recombination intermediates. This interaction is mediated by a crucial eight-amino-acid motif in SAGE1, which is essential for suppressing SLX1's enzymatic activity. Depletion of SAGE1 in ALT-positive cells causes ALT telomere shortening and alterations in ALT-associated features, including elevated telomere-sister chromatid exchange (T-SCE) and reduced ALT-associated PML Bodies (APBs) and C-circles. The altered ALT phenotype

cannot be rescued by the SAGE1 mutant that is unable to bind and inhibit SLX1, underscoring the essential role of the SAGE1-SLX1 interaction in ALT regulation. We next extended our analysis to in vivo models through patient-derived xenograft (PDX) experiments. Knockdown of SAGE1 in TERT-negative ALT-dependent PDX tumors significantly inhibited tumor growth and proliferation, while increasing the proportions of senescent and apoptotic cells, accompanied by marked telomere shortening and a pronounced reduction in C-circles. These results reveal the therapeutic potential of SAGE1 for ALT-positive tumors. Collectively, our datas define SAGE1 as a novel and critical player in telomere recombination, with potential therapeutic implications for ALT-positive cancers.

B445/P1569

The role of DNA methylation in CENP-A stability and centromere function

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The formation and maintenance of a functional centromere require the deposition of CENP-A, a histone H3 variant which epigenetically marks centromere identity and is essential for kinetochore assembly and faithful chromosome segregation. CENP-A-containing chromatin is embedded within a distinct epigenetic environment, defined not only by specific histone modifications but also by unique DNA methylation patterns. Notably, centromere dip regions (CDRs), domains of hypomethylated DNA, are associated with CENP-A enrichment. Our preliminary data suggest a dynamic relationship between CENP-A binding and the DNA methylation landscape, with changes in CENP-A occupancy accompanied by corresponding shifts in DNA methylation. Whether CDRs stabilize CENP-A chromatin or CENP-A actively contributes to the establishment and maintenance of CDRs remains unclear, representing an important gap in knowledge.To investigate the role of DNA methylation in CENP-A assembly and maintenance and for centromere function, we treated cells with Decitabine (5-aza-2'-deoxycytidine), a DNA methyltransferase (DNMT) inhibitor that irreversibly traps DNMT1, leading to progressive reduction in DNA methylation. Decitabine treatment reduced cell viability in a dose- and time-dependent manner, with higher concentrations associated with decreased survival. DNMT1 protein levels were reduced by ~50-70% after 15 days of treatment, as confirmed by western blot. Immunofluorescence staining further revealed increased intensity of CENP-A foci following treatment with high concentrations of Decitabine. Lastly, CENP-A CUT&RUN analysis, that defines CENP-A binding genome wide, showed higher CENP-A occupancy at centromeric regions and expansion of CENP-A domain following Decitabine treatment. Our results suggest that DNA methylation confines CENP-A to the CDR region and that reduced DNA methylation allows CENP-A to expand beyond its original site.

B444/P1568

The role of SFKs in resolving DNA damage response in MCF7 cells

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Src Family Kinases (SFKs) are known to be upregulated in cancer backgrounds, specifically in breast cancers. SFKs and SFK inhibitors (SFKis) have been implicated in DNA damage response however, the exact mechanisms remain largely explored. SFKs have been previously shown to translocate to the nucleus to both positively and negatively modulate DNA damage response related apoptosis. We hypothesize that SFKis, at optimum concentrations, will enhance the DNA damage response and lead to DNA damage being resolved faster. To explore this we induced DNA damage in MCF cells and followed the recovery in the presence of SFKis. We have

used apoptotic markers to analyze cells through flow cytometry. We have supported our work with mRNA and protein expression analysis. We have shown that SFKis have the capacity to modulate the DNA damage response. In the future we will further investigate the molecular mechanisms involving SFKs in DNA damage response to elucidate SFKs play a much broader role in DNA damage response related to both single and double strand DNA breaks.

B458/P1582
Understanding chromosomal copy number variations via evolutionary and physical learning approaches
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Chromosomal copy number variations (CNVs) are among the most prevalent mutations in tumor cells, yet their distributions across cells, patients, and cancer types remain poorly understood. We present a physical learning framework to uncover the origins of CNV distributions, by modeling cancer regulatory networks that adapt their reaction constants to minimize a cost function, given as the difference between targeted and actual concentrations of certain regulated species. These reaction constants are updated via optimization algorithms, including evolution dynamics that emulate selection pressures in tumors, as well as simulated annealing, contrastive learning, and gradient descent. Under evolutionary optimization and in the limit of large tumor size, we find that CNV distributions across patients with different cancer types follow a sub-binomial form: the variance for any given cancer is reduced relative to a binomial distribution by a prefactor ~0.34, matching clinical data. This prefactor is independent of network topology but is smaller for nonlinear tasks and non-evolutionary based optimization strategies. These results sharply contrast with predictions from neutral genetic drift models such as Wright-Fisher, highlighting the role of strong selection in CNV statistics. Our findings suggest that CNV distributions encode signatures of underlying learning dynamics in cancer evolution. Insight from this work may inform therapeutic strategies that target multiple mutations in accordance with predictable CNV patterns, offering an informed approach to multi-site intervention.

Cancer Cell Biology - Immune System Interactions

B546/P1670
Denatonium benzoate enhances tumoricidal macrophage reprogramming in head and neck squamous cell carcinoma
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The immunosuppressive microenvironment of head and neck squamous cell carcinoma (HNSCC) limits treatment efficacy, with tumor-associated macrophages (TAMs) playing a central role. Their plasticity makes TAMs a promising target for reprogramming toward a tumoricidal phenotype. Macrophages and TAMs express bitter taste receptors (T2Rs), G-protein-coupled receptors activated by bitter compounds. Upon activation, they undergo metabolic reprogramming, shifting from oxidative phosphorylation to glycolysis to sustain cytokine release and phagocytosis. This study aimed to determine how T2R activation by the bitter agonist denatonium benzoate (agonist for ~8 T2Rs) influences macrophage function and metabolism in HNSCC. Human monocyte-derived macrophages (MDMs) were generated from peripheral blood mononuclear cells from healthy donors. Macrophages were stimulated with the bitter agonist denatonium benzoate, and phagocytosis was assessed via co-culture of CellMask™-labeled MDMs and DAPI-labeled tumor cells (SCC47).

Cytoskeletal changes were evaluated with CellMask™ Actin staining, mitochondrial activity was measured using MitoTracker™, Biotracker ATP, and TMRE dyes. Reactive oxygen species (ROS) were assessed using CellROX Green dye, and cytokine expression was quantified by qPCR. Denatonium benzoate treatment increased macrophage phagocytosis of SCC47 cells by 143% compared to controls. This effect was accompanied by a 2.5-fold increase in F-actin and the formation of tunneling nanotubes, which mediated intercellular mitochondrial transfer. Analysis of mitochondrial ATP production revealed a 3.4-fold reduction in response to denatonium benzoate. Furthermore, a 2.5-fold decrease in mitochondrial polarization was detected by TMRE. In addition, elevated ROS production was observed in denatonium benzoate-treated macrophages. Further analysis via qPCR showed IL1B was up-regulated 13.2-fold in response to denatonium benzoate treatment. These results demonstrate that the activation of macrophage T2Rs with denatonium benzoate enhanced tumor cell phagocytosis while driving cytoskeletal remodeling, mitochondrial programming, ROS generation, and pro-inflammatory cytokine expression. These findings suggest that T2R signaling reprograms macrophages toward a more activated phenotype and may represent a novel immunomodulatory mechanism in HNSCC.

B549/P1673

Dual Immune Checkpoint Blockade of PD-L1 and B7-H3 as a Novel Therapeutic Strategy in Head and Neck Squamous Cell Carcinoma

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Head and neck squamous cell carcinomas (HNSCCs) remain difficult to eradicate with current treatment modalities, with high recurrence rates and frequent development of therapeutic resistance. Here we investigates an innovative immunotherapeutic strategy by targeting programmed cell death ligand 1 (PD-L1) and B7 homolog 3 protein (B7-H3), two immune checkpoint molecules implicated in tumor immune evasion. We hypothesize that dual blockade of PD-L1 and B7-H3 will be more effective than monotherapy in overcoming resistance in HNSCC. To address this, we employed bioinformatic analyses of publicly available clinical datasets to evaluate PD-L1 and B7-H3 expression as predictive indicators of immunotherapy response. Correlation analysis demonstrated a significant association between PD-L1 and B7-H3 expression (Pearson's r = 0.1020, p = 0.02257). Clinical data revealed that patients with low PD-L1 and B7-H3 expression exhibited longer survival compared with those with high expression levels. In vitro, coculture assays with NK cells were performed using HNSCC cell lines sorted to four subtypes: double-positive, double-negative, or single-positive for PD-L1 or B7-H3. Functionally, coculture experiments showed that double-negative cell lines were more resistant to NK cell–mediated killing, suggesting that high checkpoint expression may suppress immune clearance. These findings suggest that elevated PD-L1 and B7-H3 levels may contribute to treatment failure by promoting immune suppression. Future studies will evaluate the in vivo efficacy of dual checkpoint inhibition and delineate underlying molecular mechanisms. Collectively, this work provides a rationale for considering combined anti–PD-L1 and anti–B7-H3 therapy as a novel approach for HNSCCs expressing both immune checkpoints.

B545/P1669

Proteomic analysis of immune synapses by high-precision microscopy-guided photo-biotinylation

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A proper immune synapse (IS) is critical for a lymphocyte to recognize and eliminate tumor cells. While a broad set of proteins involved in IS formation, stability, and signaling have been identified, its full proteome remains unclear. A major challenge is to purify proteins at such a nanoscale organization with sufficient specificity and sensitivity. Here, we employed optoproteomics, an advanced technology integrates microscopy-guided automated photo-biotinylation, pulldown and mass spectrometry, to isolate and identify proteins localized between T-lymphocytes (Jurkat T cell line) and B cells (Raji B cell line). IS formation between T-B cells were recognized using a convolutional neural network-based deep learning algorithm to segment the locations of the interface between CD3 (T-lymphocyte surface marker) and the CMTPX dye (B cells). Automated photo-induced biotinylation was performed across thousands of fields of view so that sufficient proteins were biotinylated for the subsequent pulldown and LC-MS/MS-based proteome identification. We successfully identified canonical proteins associated with key components of T-cell receptor (TCR) signaling pathways such as the TCR/CD3 complex, Src and Tec family tyrosine kinases, and pivotal NF-kB signaling proteins, supporting the specificity of our spatial proteomics approach. Additionally, we observed enrichment of proteins that have not been associated with the T-B IS, generating the testable hypotheses for further functional understanding of the IS. Together, optoproteomics enables the precise enrichment of the IS proteins at the Raji-Jurkat interface, and can be readily applied to studies of other types of ISs or ISs from patient tissue samples.

B548/P1672

Regulating type I interferon signaling through temporal control of tyrosine kinase 2 in mouse melanoma cells

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Controlling immune cell signaling has become a prominent research field, yielding significant clinical advancements such as CAR-T cells and immune checkpoint therapies. One major challenge of all immune therapies is T cell exhaustion, which causes T cells to stop responding to antigens, thus limiting the efficacy of all existing immune cell-based therapies. Although the cause of T cell exhaustion remains elusive, chronic activation of type I interferon (IFN-I) has been identified as a significant contributor to T cell dysfunction. Inhibition of IFN-I signaling in hyperinflammatory cancer cells through genetic knockouts has been shown to improve T cell functions and reduce exhaustion; however, in vivo administration of IFN-I inhibitors has shown controversial outcomes, potentially due to the pivotal role of IFN-I in the early stage of immune responses. To address the issue, I proposed to use protein switches to temporally sequester and inhibit tyrosine kinase 2 (TYK2), a Janus kinase that is important for IFN-I but not for IFN-II signaling, to rescue T cell functions in the presence of inflammatory cancer cells. In my previous work, I engineered synthetic protein switches that can inhibit endogenous proteins by forming inhibitory subcellular compartments, with fast temporal control and reversibility compared to conventional genetic or cell-biology strategies. I have generated TYK2 knockout (KO) melanoma lines and validated the loss of IFN-I signaling. Afterwards, I added back TYK2 with a recruitment tag to the KO lines and fully restored the IFN-I signaling. I have also successfully deployed drug-inducible protein switches to sequester exogenous and native-tagged TYK2 in both T cells and melanoma cells, achieving over 50% recruitment efficiency within 12 hours of induction. The upcoming studies will test the loss of IFN-I by TYK2 sequestration and investigate the epigenomic and transcriptomic alterations by temporal regulation of IFN-I in melanoma cells and T cells. By harnessing the power of synthetic protein switches, this study illustrates new and versatile tools to reprogram immune responses in the cancer environment for next-generation immunotherapies.

B547/P1671
Spaceflight-Induced Circadian Disruption: Exploring the Cosmic Impact on Immunoglobulins, T-Cell Suppression, and Colonic Inflammation
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As astronauts embark on missions through the expanse of space, it is crucial to address health challenges that could arise. While previous research has examined the effect of spaceflight conditions and disruption of circadian rhythms on astronaut health, the impact of circadian rhythm dysregulation on the colon immune system remains not fully understood. Through the transcriptomic analysis of Mus Musculus colon tissue from NASA's RR-6 mission (OSD-247) using Galaxy software, significant changes in circadian and immunoglobulins gene expression were observed. Additionally, the data also showed suppression of the T-cell activation pathway, indicating compromised immunity in space. Suppression of T-cell activation and dysregulation of the circadian rhythm in colorectal cells have both been linked to cytokine production and inflammation in the colon, and in extreme cases, to inflammatory bowel disease and even colorectal cancer. However, inflammatory diseases in the colon have been understudied in spaceflight conditions. To address this knowledge gap, we propose an experiment to investigate the relationship between spaceflight-induced circadian dysregulation, decreased immune function, and increased colon inflammation. We hypothesize that spaceflight-induced circadian dysregulation causes downregulation of immunoglobulins, leading to the suppression of T-cell activation, and ultimately, inflammation in the colon. To test this, three groups of twenty Mus musculus will be exposed to altered light-dark cycle conditions, and immunoglobulin and cytokine levels will be quantified through a set of three experiments. Immunohistochemistry, western blotting, RNA interference, flow cytometry, and enzyme linked immunosorbent assay techniques will be implemented to investigate the link between circadian rhythm and inflammation in the colon. This research aims to bridge the gap between circadian rhythm disruption and immune system dysfunction in space, which is critical for the mitigation of health risks in astronauts during long duration space missions. Understanding the mechanisms of inflammation in space can also provide valuable insights into the progression of inflammatory bowel diseases as a whole, advancing current medical knowledge and contributing to the development of preventative treatments on Earth.

B550/P1674
Validation of High-Throughput Imaging Assays to Characterize CAR-T-Mediated Migration and Targeted Cell Killing
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By directing the exquisite potency and molecular specificity of the immune system toward cancer cell destruction, immunotherapy in its various forms has permanently changed the landscape of clinical oncology. Among the frontrunners ushering this new paradigm are adoptive cell therapies such as chimeric antigen receptor (CAR) and T cell receptor (TCR) T cells, genetically engineered effector cells that drive T cell activation, proliferation, and target cell killing. While CAR T cell therapies offer significant promise for treating solid tumors, considerable challenges remain. One of the most pressing is overcoming the influence of the tumor microenvironment on lymphocyte function. A major obstacle is the navigation of acellular regions densely packed with the structural scaffolding of the extracellular matrix (ECM) during lymphocyte migration and

invasion. The ECM is highly heterogeneous, and its diverse components can modulate a wide range of cellular responses in both resident tumor cells and infiltrating lymphocytes. This study demonstrates an automated, high-throughput imaging approach for measuring cytotoxicity in 3D cancer cell models, specifically designed to address the challenges of working with matrix-embedded samples. Using T cells genetically engineered to express a variable fragment (Fv) targeting epithelial cell adhesion molecule (EpCAM, also known as CD326), we evaluated their cytotoxic effects on T-47D tumor spheroids embedded in a 3D extracellular matrix (ECM). EpCAM is a transmembrane glycoprotein involved in cell-to-cell adhesion and is expressed in various epithelial tissues, carcinomas, and progenitor or stem cells, but not in non-epithelial cells or cancers of non-epithelial origin—making this CAR T construct well-suited for targeting solid tumors. Using this imaging-based assay, we demonstrated that EpCAM-targeted CAR T cells exhibit dose-dependent cytotoxicity against T-47D tumor spheroids embedded in a 3D extracellular matrix. The matrix environment required individual T cells to actively migrate through the ECM to reach their targets, resulting in a delayed cytopathic response compared to suspension models. This delay was reflected in higher IC50 values observed under embedded conditions. Additionally, we showed that antigen-stimulated CAR T cells not only bind to the spheroids but also infiltrate them, indicating deep penetration and engagement with tumor cells. These results highlight the utility of high-throughput 3D imaging for capturing dynamic cell interactions and quantifying therapeutic efficacy in physiologically relevant models, offering valuable mechanistic insights that can inform the development of more effective cell-based cancer therapies.

Cancer Cell Biology - Tumor Microenvironment

B534/P1658
Adenosine and Lymphatic Endothelial Function: Implications for Tumor Progression in Gastric Cancer
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INTRODUCTION: Gastric cancer remains among the leading causes of mortality attributable to cancer on a global scale. Lymphatic metastasis signifies a pivotal point in the disease's prognosis and is intricately associated with tumor lymphangiogenesis. The VEGF-C/VEGF-D pathways enable the tumor to stimulate the development of new lymphatic vessels, thereby facilitating the dissemination of cancer cells to lymph nodes, an event correlated with poorer survival outcomes. Recent research has highlighted adenosine as a molecule of notable interest within this context. Nevertheless, its function within human lymphatic endothelial cells (HLECs) remains insufficiently elucidated. Emerging evidence indicates that activation of the A2A receptor may promote lymphangiogenesis. A comprehensive understanding of how adenosine influences HLEC behavior is essential, as it will not only deepen insights into tumor biology but also potentially unveil new therapeutic strategies to impede lymphatic metastasis in gastric cancer. In this context, our aim is to investigate the effects of adenosine on HLECs. METHODS: The study used a commercial line of human lymphatic endothelial cells (HLECs). The expression of LYVE-1 (Lymphatic Vessel Endothelial Hyaluronan Receptor-1) served as a marker for this cell type. RT-PCR was performed to measure the transcript levels of adenosine gene metabolism. Western blot and immunofluorescence assays were then conducted to evaluate protein levels. Migration assays were subsequently performed. Finally, levels of VEGF-C were measured using ELISA. RESULTS:Regarding adenosine metabolism, we determined through RT-PCR that HLECs express transcripts of the genes encoding the adenosine receptor A2B, ENT1, ENT3, PAP, ADA, ADK, and CD73. Using Western blot, we found that these cells express the proteins A2B, ENT1, and CD73. Subsequently, via Western blot and ELISA assays, we found that adenosine stimulates VEGF-C production in HLECs. Additionally, we observed that this nucleoside promotes migration and reduces LYVE-1 protein expression in HLECs. These findings were confirmed in transwell assays

with gastric cancer stem cells (GCSCs). Conclusion: Our findings demonstrate that adenosine activates human lymphatic endothelial cells, enhancing their migration and stimulating VEGF-C production; both key processes in lymphatic vessel development, also known as lymphangiogenesis. These effects suggest that adenosine may facilitate lymphatic metastasis in gastric cancer. Therefore, targeting adenosine signaling could represent a novel therapeutic strategy to limit tumor spread through the lymphatic system. Funding: FONDECYT INICIACIÓN N°11230446 (Etapa 2025); Agencia Nacional de Investigación y Desarrollo (ANID) -Chile.

B528/P1652

Age-associated changes to the extracellular matrix drive YAP signaling and melanoma invasion

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Melanoma is the most lethal form of skin cancer and is disproportionately diagnosed in older patients, often resulting in higher rates of metastasis and therapy resistance. In older patients, damaged and senescent fibroblasts in the skin are known to build and maintain an extracellular matrix (ECM) that is stiffer and more aligned. However, how that affects the invasiveness of melanoma in older patients is unknown. YAP is a mechano-sensitive transcriptional coactivator that responds to tissue mechanical environments. YAP translocates to the nucleus under stiff ECM conditions and is known to drive melanoma invasion. Using cell-derived matrices (CDMs), which are created using dermal fibroblasts collected from healthy young and aged donors, we found that melanoma cells plated on aged CDMs have higher YAP nuclear enrichments. We will understand how nuclear YAP in melanoma cells plated on aged CDMs lead to higher invasiveness using cancer cell assays and genomic approaches. These studies will lead to a deeper understanding of the mechanisms through which melanoma becomes more invasive and therapy resistant in the aged tumor microenvironment and allow us to create better treatments targeting novel aspects of cancer progression that occur in older patients.

B536/P1660

ANTIPROLIFERATIVE AND ANTIOXIDANT EFFECTS OF SCHINUS AREIRA L. EXTRACTS ON GASTRIC CANCER STEM-LIKE CELLS

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Introduction: Gastric cancer is one of the leading causes of cancer-related deaths worldwide. Its recurrence and resistance to therapy are partly driven by gastric cancer stem cells (GCSCs), which possess strong self-renewal and tumor-initiating capacities. Natural compounds with antioxidant and antiproliferative properties have emerged as promising adjuvants in cancer prevention. Schinus areira L., a Chilean native species rich in bioactive compounds, shows high antioxidant activity; however, its effects on GCSCs remain unexplored. This study aimed to evaluate the antioxidant and antiproliferative effects of methanolic extracts from S. areira leaves and fruits on GCSCs derived from the MKN74 cell line. Methods: Extracts were obtained by maceration and analyzed using DPPH and ABTS assays, with total phenolic content expressed as Trolox equivalents. Bioactive metabolites were identified by thin-layer chromatography and GC–MS. GCSCs were treated with varying extract concentrations for 24 h. Cell viability (MTT), spheroid morphology (light microscopy), and KI67/CD54 expression (Western blot, RT-qPCR, immunofluorescence) were evaluated. Results: Both leaf and

fruit extracts exhibited high antioxidant capacity and contained piperine and oxygenated terpenes. Treatment significantly reduced spheroid integrity and viability in a dose-dependent manner, along with a marked decrease in KI67 and CD54 expression, suggesting reduced proliferation and stemness. Conclusion: Methanolic extracts of Schinus areira L. exert potent antioxidant and antiproliferative effects on gastric cancer stem-like cells, likely mediated by piperine and terpenoid compounds. These results highlight S. areira as a promising natural source of bioactive molecules for developing complementary strategies in gastric cancer prevention and therapy. Funding: FONDECYT INICIACIÓN N°11230446 (ETAPA 2025); Agencia Nacional de Investigación y Desarrollo (ANID) – Chile.

B540/P1664

Centrosome overduplication and cell competition define the predicted evolution of newly formed tetraploid cancer cells in mouse xenografts.

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Extra centrosomes are often observed in cancer cells and are believed to contribute to tumorigenesis, potentially through promoting aneuploidy and chromosome instability (CIN). These supernumerary centrosomes can arise through a variety of processes, including spontaneous centrosome overduplication and incomplete cell division. The latter process, also known as whole genome doubling (WGD), produces daughter cells with twice the amount of DNA (tetraploidy, 4N) and typically twice the number of centrosomes. Importantly, WGD has been reported in over 30% of tumors and was shown to induce tumorigenesis in mouse models, where evidence indicated a potential role of extra centrosomes in promoting tumorigenesis. These observations have led to the idea that, at least in some cases, 4N cells may promote tumorigenesis due to the extra centrosomes acquired during WGD. However, in lab cultures, experimentally induced 4N cells tend to rapidly lose their extra centrosomes while maintaining roughly 4N chromosome numbers. This supports the hypothesis that the in vivo tumor microenvironment (TME) may impose selective pressures that differ from those in the in vitro environment and that influence the evolution and fate of extra centrosomes in 4N cancer cells. In recent work (Sweet et al., in press), we induced tetraploidy in 2N colorectal cancer cells and injected the newly formed 4N cells into nude mice. We found that the newly formed 4N cells, the dominant population at the time of injection, were outcompeted by 2N cells in these tumors, but a substantial fraction of the remaining 4N cells harbored extra centrosomes. Because the in vivo cell population can only be analyzed prior to injection and after resection, it is not possible to dissect the evolutionary processes of the cell population. To address this issue, we developed a mathematical model to track the co-evolution of ploidy and centrosome numbers. We further utilized Bayesian Inference to infer which parameters within the model were altered between the in vitro “control” and in vivo “target” conditions. Our data showed that the extra centrosomes found in the 4N cells within the tumors were most likely arising via spontaneous centrosome overduplication, rather than through further WGD events or retention of extra centrosomes from bipolar symmetric divisions, while the loss of 4N cells was likely due to 2N cells having higher fitness and outcompeting the 4N cells over time. Our model analyses help narrow down the possible mechanistic pathways of tetraploid cancer evolution, focusing the following experimental studies on identifying specific TME factors that promote centrosome overduplication, and environments that reduce the relative fitness of tetraploid cells.

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Comparative efficacy of cancer therapy between a Del1 fragment and Docetaxel enhanced in Squamous cell carcinoma

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Background Cancer gene therapy using the E3 and C1 (E3C1) domains of developmental endothelial locus-1 (Del1) has been investigated. Cancer gene therapy using E3C1 domains has exhibited significant therapeutic effects, including tumor volume reduction and improved survival in mice with transplanted tumors. Thus, we hypothesized that cancer therapy combining cancer gene therapy using E3C1 domains and anticancer agents would enhance treatment efficacy. Docetaxel (DTX), cisplatin (CDDP), and 5-fluorouracil (5-FU) are commonly used in standard chemotherapy for head and neck squamous cell carcinoma (HNSCC). Previously, combination cancer therapy with the E3C1 domain and cisplatin (CDDP) showed promising antitumor effects. Therefore, the aim of this study was to examine the effects of combination cancer therapy using the E3C1 domain and DTX. Methods Mice with transplanted tumors (A431 or SCCKN cells) were injected intraperitoneally with DTX and locally with non-viral plasmid vectors encoding E3C1. Histochemical analyses of the transplanted tumors were performed to assess their effects on prognosis. The effects on tubulin and the cell cycle in SCCKN and A431 cells treated with DTX or E3C1 peptide were analyzed using in vitro studies. Results The DTX, E3C1, and DTX+E3C1 injected groups had reduced tumor growth and longer survival than the control group. However, no synergistic effect was observed with the DTX+E3C1 treatment. In contrast, structural changes in tubulin were observed in the tumors of the DTX, E3C1, and DTX+E3C1 groups. Furthermore, the DTX and E3C1 groups showed increased Cyclin B1 protein expression compared to that in the control. The DTX group showed decreased Cyclin D1 expression, whereas the E3C1 group showed no apparent effect on Cyclin D1 protein expression. Conclusions The DTX+E3C1 treatment resulted in improved survival and structural changes in tubulin in mice with transplanted tumors. In vitro studies revealed that DTX affected the G2/M phase of the cell cycle, whereas E3C1 affected both the G2/M and G1 phases of the cell cycle. These findings indicated that DTX and E3C1 exert different antitumor effects on tubulin. Elucidation of the detailed mechanisms underlying the effects of cancer gene therapy using E3C1 on tubulin is warranted.

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Exposure to arsenic trioxide lowers gene expression in five DNA repair pathways in breast reduction mammoplasty cultures from women of South Florida ancestries

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We are studying pro-carcinogenic exposures in an attempt to prevent environmentally driven breast cancer. Breast cancer (BC) continues to be a significant U.S. public health concern, with 310,720 new invasive cases in 2024. Disparities in incidence and mortality disproportionately impact women of African ancestry (AA), who exhibit a higher incidence before age 40 and a 40% increased mortality rate relative to women of Non-Hispanic White (NHW) ancestry. In Florida, several zip codes in Broward and Miami-Dade counties have advanced BC diagnosis rates of 35-55%, which are above the national average of 30%. Within these areas live higher proportions of populations with African ancestry. Environmental exposures, including environmental carcinogens, may contribute to these disparities of BC outcomes. Arsenic levels are elevated in the tap water of

seven Broward county zip codes and in Miami Dade which also shows increased rates of advanced BC diagnoses. We hypothesized that different ethnicities have different pharmacogenomic sensitivities to environmental exposures, leading to distinct responses to environmental agents. Unique patient-derived culture explants (PDCEs) generated in the NSU AutoNation Institute from AA (N=3) and NHW (N=3) women were exposed to 0.3 uM arsenic trioxide for 14 days. Arsenic exposure in AA PDCEs resulted in a significant reduction in gene expression in all 5 DNA repair pathways [base excision repair, nucleotide excision repair, mismatch repair, homologous recombination, and non-homologous end joining] vs. NWH. Total RNA was analyzed using Illumina TruSeq Stranded Total RNA library preparation. Sequencing was completed on a 2 Å~ 150 bp paired-end run using the NextSeq 500 High Output kit. AA cultures manifested dysregulation in 66–86% of genes vs. 25–38% in NHW. This work shows clear pro-carcinogenic changes in breast reduction PDCEs in both ancestries. However a greater number of genes in each pathway was impacted, in the AA population relative to NHW. Although arsenic has been implicated in skin and lung cancers epidemiologically, this is one of the few studies indicating that breast cancer could be an outcome of arsenic exposure. This study is also unique in that exposures were performed on novel multi-cellular patient derived cultures using tissue engineering techniques.

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Intracellular pH modulates cancer migration phenotypes under compressive environments.

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Cancer cells exhibit an increased intracellular pH (pHi) compared to normal cells which promotes a variety of cancer cell behaviors. In addition to dysregulated pHi, cancer cells also experience an abnormal mechanical tumor microenvironment, including increased mechanical compression (solid stress). Under compression, Rho/ROCK are part of the central regulators of the actomyosin cytoskeleton, allowing cell movement, invasion and survival to new environments. Both increased pHi and solid stress are associated with aggressive cancer phenotypes, including increased cell migration, however their co-regulation and the molecular mechanisms that link the two are still unknown. Understanding this connection could uncover potential molecular targets that enable cancer cells to adopt aggressive phenotypes. Here, we show that pHi dynamics of cancer cells are sensitive to mechanical compression. Using a 2D in vitro compression model, we show that mechanical compression of 0.14 kPa for 24 hours raises pHi, increases pHi heterogeneity, and causes cells to shift from a mesenchymal to an amoeboid morphological phenotype. We further assessed how compression altered cell behaviors and pHi during cell migration using a wound healing assay. We demonstrated that uncompressed cancer cells exhibited controlled leading lamellipodium protrusive motion and slight retraction on the opposite side, while compressed cells (with high pHi) adopted blebbing, an amoeboid migratory phenotype, with both increased cell speed and migration compared to uncompressed cells. To test whether pHi is required for amoeboid morphology and movement under compression, we used CRISPR knockout cells, lacking the sodium proton exchanger NHE1 (NHE1 K.O.). We found that knocking out the sodium proton exchanger (NHE1) lowers pHi under compression and abrogates amoeboid morphology and migration phenotypes. Importantly, experimentally raising pHi in NHE1 K.O. cells partially restored amoeboid blebbing formation, demonstrating that high pHi is a necessary mediator of the mechanosensitive amoeboid migration under compression. Pharmacological inhibition of Myosin II and ROCK similarly suppressed the amoeboid phenotype, even though pHi was still increased under compression. This implicates RhoA signaling as the downstream regulator of the pH-dependent amoeboid phenotype. Together, these findings suggest high pHi is a necessary regulator of RhoA/ROCK-dependent amoeboid migration phenotypes under compression. By establishing pHi as a regulator of compression-induced amoeboid migration, future work could therapeutically target pHi to limit aggressive and mechanically adaptive cancer behaviors.

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Matrix Matters: Uncovering Collagen VI-Dependent Stromal Vulnerabilities in Pancreatic Cancer

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Pancreatic ductal adenocarcinoma (PDAC) is characterized by a dense desmoplastic stroma, with cancer-associated fibroblasts (CAFs) as the main source of the collagen-rich extracellular matrix (ECM) defining this tumor. While CAF-generated ECM supports PDAC cell survival in a nutrient-poor environment, mechanisms underlying this support remain unclear. This study aimed to identify key drivers of ECM-dependent PDAC survival and elucidate the mechanistic basis of the CAF-driven niche. Using six syngeneic human CAF/ECM functional unit models, previously established and characterized by their ability to support or restrict PDAC cell survival under nutrient deprivation, we performed comparative ECM proteomic profiling. Nine matrisome proteins consistently differed between tumor-supportive and tumor-restrictive ECMs, including two essential collagen VI subunits and a third linked to collagen IV heterotrimers. Given that loss of COL6A1 or COL6A2 should disrupt collagen VI assembly, we employed CRISPRi to suppress COL6A2 transcription (the most downregulated subunit) in the tumor-supportive CAF control. COL6A2 downregulation produced ECM that no longer supported PDAC growth, with in vitro and in vivo assays confirming loss of tumor-promoting function. Mechanistic analyses showed COL6A2 suppression did not affect expression of other COL6 subunits but prevented collagen VI incorporation into polymerized ECM fibers, altering assorted cytokine and chemokine storage and release, including IL-8 and IL-6. Loss of collagen VI also caused accumulation of inactive TGFβ, blocking its activation and CAFs' ability to form a tumor-supportive ECM. This was reversed by exogenous activated TGFβ but not by recombinant collagen VI lacking ECM integration. COL6A2-deficient CAFs exhibited a normalized, anti-tumor phenotype, mirroring effects observed with integrin αvβ5 inhibition, substantiating our previous reports and proposing this integrin as a key modulator of collagen VI-dependent tumor support. Because low collagen VI expression correlates with improved PDAC patient outcomes, these findings reveal this protein and their fibrillogenesis modulators as stromal vulnerabilities and highlight ECM composition as a promising therapeutic target in pancreatic cancer.

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Mechanotransduction Signals from Cancer-Associated Fibroblasts Regulate Nuclear Structure and Invasive Characteristics in Cancer Cells

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Emerin is an inner nuclear membrane protein that maintains nuclear structure and rigidity by binding to nucleoskeletal partners at the nuclear envelope. In breast cancer cells, the size and rigidity of the nucleus is inversely correlated with metastasis. Our lab showed triple-negative breast cancer (TNBC) cells had 50% less emerin protein and smaller, dysmorphic nuclei, resulting in increased cell invasion and tumor formation compared to normal breast cells. However, the mechanism of how emerin regulates cancer cell invasiveness and metastasis is unclear. We hypothesize this occurs by mechanotransduction from the tumor microenvironment (TME), as stiffening of the TME decreases nuclear rigidity. We predict emerin detects and/or transduces mechanical signals from the extracellular matrix (ECM) via the linker of nucleoskeleton and

cytoskeleton (LINC) complex to alter nuclear structure by reducing emerin levels. Cancer-associated fibroblasts (CAFs) modulate the TME by depositing ECM and secreting soluble factors. To begin testing whether soluble, CAF-secreted factors affect emerin expression and nuclear structure, we incubated multiple cancer cell lines in CAF-conditioned media (CAF-CM) from both wild type (wt CAF-CM) and Yap/Taz KO CAFs (Yap KO CAF-CM). We found that there was a significant decrease in the nuclear size of cells grown in CAF-CM in at least two of these cell lines. Interestingly, we observed differences in impeded cell migration for cells grown in wt CAF-CM compared to Yap KO CAF-CM. Further, emerin expression levels were not significantly different between cells grown in unconditioned media versus CAF-CM (wt or Yap KO), suggesting that an alternate mechanism is driving the changes elicited by the CAFs. To further investigate the mechanism by which nuclear structure is regulated by the CAFs and TME, we are using verteporfin, a Yap/Taz inhibitor, and Y-27632, a ROCK inhibitor, to block biochemical signaling pathways. LINC involvement is also being analyzed using DN-KASH and CRISPR. Collectively, our data show that soluble and/or mechanical signals from CAFs play an important role in regulating nuclear structure to impact cancer cell invasion and metastasis.

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M-Sec (TNFAIP2) is a Negative Regulator of Invadopodia Function in Breast Carcinoma Cells

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Cells make many different types of actin-rich cellular protrusions that are used for various cell functions such as migration, phagocytosis, invasion and matrix degradation. Some of these actin protrusions are highly specialized to perform specific cellular functions, such as podosomes or invadopodia, often referred to collectively as invadosomes, that mediate matrix degradation and cell invasion. Other thin protrusions connect cells together to form signaling networks that have been called by various names such as membranous nanotubes, tumor microtubes, or tunneling nanotubes (TNTs), known collectively as Thin Membranous Connections (TMCs). M-Sec, or TNFAIP2, mediates TNT formation in several different cell types but doesn't appear to inhibit other cellular functions, such as chemotaxis in breast tumor cell lines (Carter et al., 2019). We investigated whether M-Sec also played a role in the function of invadopodia. We found GFP-M-Sec co-localized with endogenous cortactin and Tks5, two well-established invadopodia markers. Endogenous M-Sec also co-localized with cortactin-rich puncta that was associated with matrix degradation, in alignment with invadopodia structures. Reduction of endogenous M-Sec by shRNA decreased the number of invadopodia precursors with no effect on mature invadopodia. This shift in invadopodial dynamics resulted in enhanced matrix degradation, which may be due to the increased in invadopodium lifetime in shM-Sec cells. Additionally, over-expression of GFP-tagged M-Sec reduced tumor cell matrix degradation. Previous work demonstrated that Rac1 enhanced invadopodia turnover in a Rac1-dependent manner and M-Sec is known to binds to Rac1. We found that the association of M-Sec with Rac-1 is enhanced following treatment of tumor cells with macrophage conditioned media. Overall, our data indicates a role for M-Sec in inducing invadopodial turnover possibly by regulating Rac1 localization to invadopodia. We speculate that M-Sec may be recruited to invadopodia by binding to cortactin, a protein essential for invadopodium maturation. Furthermore, our data also suggests that M-Sec levels could regulate the balance of TNTs and invadopodia in tumor cells.

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Oxidative stress and serum deprivation influence the evolution of newly formed tetraploid cells during

tumorigenesis in mice

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Cells with extra centrosomes, a state known as centrosome amplification, are frequently detected in cancers. Previous studies have shown that extra centrosomes can promote aneuploidy and are sufficient to induce tumorigenesis in some contexts. One mechanism that leads to centrosome amplification is whole genome doubling (WGD), an event that occurs in over 30% of all cancers and is associated with genomic instability and disease progression. As a result of WGD, newly formed tetraploid (4N) cells have twice the number of chromosomes and centrosomes as diploid (2N) cells. Since 4N, but not 2N, mouse mammary cells were able to form tumors in mice and many still had extra centrosomes, it has been proposed that centrosome amplification contributes to the tumorigenicity of 4N cells. Newly formed 4N cells, however, lose extra centrosomes within the first few cell divisions following WGD in cell culture models. These paradoxical observations suggest that centrosome number evolution following WGD may be influenced by the in vivo tumor microenvironment (TME). To address this question, we induced WGD in 2N colorectal cancer cells and injected newly formed 4N cells subcutaneously into the flanks of nude mice. Although ~80% of the population is 4N at the time of injection, we found that recovered cells were primarily 2N, suggesting 4N cells are outcompeted by 2N cells in vivo. Despite the proliferative deficit in the 4N cells, the tumors grew to the same size and at the same rate, suggesting the small fraction of 4N cells may be promoting tumor growth and/or cell survival. To understand this, we performed immunohistochemistry staining of xenograft tissue sections for stromal components and an apoptosis marker. We found that the 4N tumors had a higher number of cancer associated fibroblasts and intra-tumoral macrophages, while the 2N tumors had higher levels of cleaved-caspase-3, a marker of apoptosis. Moreover, we found that 25-35% of 4N cells recovered from xenograft tumors had extra centrioles, while 2N cells did not have extra centrioles. Follow-up in vitro experiments demonstrated that lack of growth factors and oxidative stress in the TME can explain, respectively, the proliferation deficit of the 4N cells and extra centrioles in vivo. Overall, our data demonstrate that specific aspects of the TME can shape the ploidy and centrosome numbers of cancer cell populations after WGD.

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Pep A promotes tumor cell apoptosis likely via macrophage activation in a zebrafish xenotransplant model

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Cancer is a disease characterized by the uncontrolled proliferation of cells that do not follow the normal mechanisms of cell growth regulation. These cells can invade surrounding tissues and spread to other parts of the body through metastasis. Tumors are composed of various immune cell populations, including lymphocytes, dendritic cells, myeloid cells, and different macrophage phenotypes, known as tumor-associated macrophages (TAMs), which can either promote or suppress tumor growth depending on their functional polarization. Recent studies from our group have shown that the conditioned medium from prostate tumor epithelial cells, in the absence of androgens, releases soluble factors capable of inducing the differentiation of RAW 264.7 and BMDM macrophages into a killer phenotype. One of these factors is a progranulin derived peptide (PepA), which mimics the effect of the conditioned medium. In co-culture, macrophages activated by PepA induce apoptosis in p53-positive epithelial tumor cells. PepA has no direct effect on tumor cells, but acts activating the killer capacity of macrophage precursors. These findings suggest that PepA may have therapeutic potential in cancer treatment. Given this, we used the zebrafish (Danio rerio) experimental model, widely used in cancer research due to its transparency, rapid tumor development, and sensitivity to therapeutic compounds, to evaluate the in vivoeffect of PepA. The aim was to investigate whether PepA, when co-injected

with tumor cells, increased tumor cell death mediated by macrophages present in the tumor. A total of 100 zebrafish embryos at 48 hours post-fertilization were maintained in E3 medium at 28–30 °C. Thyroid tumor cells (TPC1 lineage) were cultured in high-glucose RPMI medium until 90% confluency and labeled with CellTracker CM-DiI. Cells were divided into two groups: one treated with PepA and the other with a scrambled version of PepA (PepAs) as a negative control. Following xenotransplantation into the yolk sac, embryos were maintained for 24 hours and then fixed for caspase-9 immunofluorescence analysis. The results showed that TPC1 cells were capable of forming localized tumors in the zebrafish. Co-injection of PepA or PepAs did not cause apparent toxicity to the embryos within the first 24 hours. Caspase-9 expression was strongly detected within the tumors of PepA-treated animals, while in the control group, staining was poorly localized. These data indicate that the PepA peptide promotes tumor cell apoptosis mediated by caspase-9, likely via activation of macrophage precursors in the TMA. These findings highlight PepA as a promising therapeutic agent for use in immuno-oncological strategies. Keywords: Macrophages, zebrafish, apoptosis, tumors.

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Plasma Membrane Blebbing Promotes Melanoma Cell Survival Under Tryptophan Deprivation

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Tryptophan (Trp) is an essential amino acid required for protein synthesis and cell proliferation. Tumor cells often express elevated levels of Trp-catabolizing enzymes, resulting in Trp depletion in the local microenvironment. How tumor cells sense and adapt to Trp deprivation remains poorly understood. Here, we observed that Trp deprivation induces non-apoptotic plasma membrane blebbing and activates pro-survival Akt signaling in melanoma cells. Functionally, previous research show blebbing allows melanoma cells to evade anoikis by assembling scaffolds for pro-survival signaling and is involved in bleb-based amoeboid migration, mediated by the small GTPases Rac and Rho. Under Trp deprivation or in cells overexpressing a Trp-catabolizing enzyme, inhibition of bleb formation leads to increased cell death, indicating that blebbing is critical for melanoma cell survival under these conditions. To gain mechanistic understanding of membrane blebbing, we carried out a series of proteomics analyses. We found that Trp deprivation activates the GCN2-mediated stress response, which downregulates the Akt phosphatase PHLPP, thereby sustaining Akt signaling. We further show that the membrane blebbing is driven by the small GTPase Arf6, which is activated by its guanine nucleotide exchange factor, ARNO, a direct phosphor-substrate of Akt. Together, our study delineates a signaling pathway in which Trp deprivation promotes membrane blebbing, and identifies a previously unrecognized stress-adaptive mechanism that enables tumor cell survival under amino acid deprivation.

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Regulation of brain metastasis by glial interferon response

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Brain metastasis from primary tumors is invariably lethal, and patients have median survival of less than 2 years. To successfully colonize a distal organ, cancer cells must adapt to their new microenvironment, a concept known as the "seed and soil" theory. In the brain, cancer cells must leverage advantageous

relationships with the resident stromal cells, such as astrocytes and microglia. Our results demonstrate that the local brain tumor microenvironment sustains a chronic, low-level type I interferon response. This neuroinflammatory response by the glia ultimately shapes the tumor immune microenvironment and tumor growth. The goal of this project is to identify the underlying mechanisms behind interferon-responsive glia in the progression of brain metastasis. These novel insights will guide the design of new therapeutic strategies targeting cancer-glia interactions with the goal of improving clinical outcomes.

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Streamlined spatial analysis of the tumor microenvironment and associated pathological states using multiplex immunohistochemistry

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Spatial studies of the tumor microenvironment are crucial for understanding the complex interactions between different cell types and how these interactions contribute to disease progression. This poster presents a streamlined approach to spatial analysis of the tumor microenvironment in brain tissues, focusing on astrocytoma and oligodendroglioma. We employed primary antibody conjugates to perform immunohistochemistry (IHC) on human tissue samples, utilizing an 8-plex panel of markers. The study incorporated IHC-verified antibody conjugates supplemented by an antibody labeling kit for pathology-specific markers, facilitating high spatial resolution imaging without the need for lengthy workflows commonly associated with secondary amplification or cyclic stripping methods. Our findings demonstrate a significant increase in astrocytes and oligodendrocytes in the tumor tissue, as revealed by the increased presence of GFAP and Olig2, respectively, compared to healthy controls. The use of the EVOS S1000 imaging system made image capture and analysis remarkably straightforward, further simplifying the workflow. This approach provides clear advantages over traditional methods, highlighting its potential for broad application in immunological and neuropathological studies.

B531/P1655

The role of NetrinG family proteins in modulating the pancreatic cancer microenvironment

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Pancreatic Ductal Adenocarcinoma (PDAC) is the most common type of pancreatic cancer with only 13% of patients surviving beyond five years following diagnosis. This high lethality is mainly attributed to late diagnosis and a lack of effective therapy. Surgical tumor resection remains the only curative intended therapy, yet only a small percentage of patients are eligible and many recur soon after surgery, while advanced-stage patients present with even worse prognoses. These limitations underscore the urgent need for improved therapeutic approaches. A major contributor to poor outcomes is the characteristic PDAC fibrous microenvironment shaped by cancer-associated fibroblasts (CAFs), which generate a collagenous extracellular matrix (ECM) and actively modulate disease progression, including response to therapy. Given that eliminating CAFs often accelerates tumor progression, identifying strategies to inhibit pro-tumorigenic fibroblastic functions while restoring their natural tumor-suppressive properties may lead to improved therapeutic interventions. We reported that Netrin-G1 (NTNG1), typically expressed in neurons, is ectopically expressed in PDAC. This protein

and its binding receptor NGL1 (LRRC4C) promote tumor growth by facilitating glutamate/glutamine exchange between CAFs and cancer cells, potentially forming oncogenic synapses and providing metabolic support while enhancing immunosuppression. To investigate this mechanism, we optimized a pipeline utilizing one of the most physiologically relevant ex vivo models for studying human fibroblast function: the fibroblast/ECM multilayered functional unit. Our medium-throughput assay employs multi-well plates for CAF/ECM units' production, followed by multi-acquisition confocal imaging and semi-automated digital analysis of key unit traits including nuclei layer analysis, ECM thickness, fiber orientation, and sub-cellular localization of selected biomarkers' expression using established FIJI utilities, Python-based libraries, and AI tools such as StarDist. Obtained data demonstrated that blocking NetG1/NGL1 signaling protects animals from PDAC onset and significantly reduces tumor size. To further explore this pathway, comparative analysis of conserved Netrin-G family members and their binding partners (NetG2/NGL2, NGL3/LAR) suggests these axes may also regulate fibroblast functional states. Notably, NetG2 expression is inversely associated with pro-tumorigenic programs in publicly available and our own datasets, suggesting an opposing role and motivating mechanistic investigation across the Netrin-G network. These results support the hypothesis that the Netrin-G family modulates fibroblast/ECM unit activity.

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Using 3D Spheroid Coculture Assays to Model Glioma-Macrophage Interactions

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Glioblastoma Multiforme (GBM) is the most aggressive form of adult brain tumor with a median survival time of twelve months. GBM is highly resistant to conventional therapy which includes surgical resection of the tumor, radiation treatment and chemotherapy. GBM cells are highly motile and invasive resulting in infiltrative tumors with poorly defined borders. GBM tumors are heavily infiltrated with microglia cells which are known to stimulate glioma cell invasion. Our laboratory has previously demonstrated that macrophages strongly stimulates glioma invasion both in-vitro and in orthotopic animal models. This interaction was found to be dependent on CSF-1R which is expressed on all tumor infiltrating macrophages/microglia. Blockade of the CSF-1R using compounds such as pexidartinib (PLX3397) can inhibit microglia/macrophage-stimulated glioma invasion in-vitro and in vivo. Here we generate glioma cell spheroids using the "hanging drop" method on a 96 well U bottom plate. After 24 hours, spheroids are embedded in matrigel containing GFP-expressing macrophages. Time lapse imaging is used to measure glioma cell- macrophage interactions including trafficking and stimulation of invasive protrusions. A variety of chemokines are upregulated in the GBM tumor microenvironment and facilitate "cross-talk" between macrophages and glioma cells eliciting a chemotactic response. Using novel CCR-1 specific antagonists, we show that macrophage-stimulated glioblastoma invasion in-vitro is dependent on CCR-1. Furthermore, using quantitative-PCR, immunofluorescence and ELISA assays we observe that treatment of microglia with glioblastoma conditioned media strongly induces the expression of CCR-1 and several ligands for CCR-1 including CCL-3 and CCL-5. Finally, we investigated potential effectors of CCR-1 signaling which mediates glioblastoma invasion. Among these, the matricellular protein osteopontin (OPN) emerges as a promising candidate. OPN is highly upregulated in glioma conditioned media stimulated macrophage cell lines and human monocyte derived macrophages. Furthermore, the upregulation of OPN is dependent on CCR-1 as shown using two different pharmacological inhibitors of CCR-1.

B530/P1654
Vascular Dysfunction in High-Grade Tumors
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Glioma and melanoma are highly aggressive tumors with a significant risk of recurrence and death. Since angiogenesis contributes to tumor progression and treatment resistance, we aimed to analyze the structure and function of intra-tumoral blood vessels in tissue from patients with high-grade gliomas (protocol DI/25/403/03/19, General Hospital of Mexico) and in C57BL/6J mice inoculated with B16 melanoma cells. To determine vascular morphology, immunostaining for the endothelial cell marker IB4 was performed on 18-µm-thick tissue sections. Glioma samples showed a vascular density of 3,553 µm ± 694.5 (mean ± SE), which was slightly higher than control tissue (autopsies), but not statistically significant (P=0.0649). Notably, although the number of branches did not differ between these groups, glioma samples showed thicker vessels that were 3.58 times more fragmented than those in autopsies. When we measured the area of fluorescence positive for the albumin marker in the tissue, we found 16 times higher permeability in gliomas, as compared to autopsy samples. Consistent with this, the localization of claudin-5 protein, which regulates vascular permeability, was lost from inter-endothelial junctions of gliomas, while total protein expression showed no difference between groups. Similar to gliomas, B16 melanoma tumors formed in WT mice showed high vascularization, with 20 µm diameter vessels that were thicker than the peri-tumoral ones. These tumors also showed permeability to albumin, and only a portion of the total claudin-5 expressed was localized at the endothelial borders. Importantly, inoculation of B16 cells in mice lacking mast cells (B6.Cg-KitW-sh) resulted in 65% smaller tumors than in WT mice and with 28% less vessel length. Moreover, vascular alterations were more pronounced, with vessels twice as thick and more fragmented. Permeability to albumin was 1.83 times higher than in WT mice, and claudin-5 expression was decreased by more than 50%, indicating that mast cells contribute to angiogenesis and tumor growth. Together, these results demonstrate that high-grade tumors, including gliomas and melanoma, exhibit a pathological vessel network with endothelial barrier dysfunction, which is regulated by mast cells. This work was supported by Conahcyt Grants: CF-2023-G-1516 (MDC) and CF-2019-51488 (CGE); and Scholarships: 806461 (JGG) and 118851 (RZG).

Cell Adhesion, Migration, and the Extracellular Environment - Cell Migration

B267/P0260
A Tale of Two Tissues: Investigating Tissue Interface Dynamics via Antagonistic Migration Assay
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The confluent and homeostatic cellular monolayer, upon presentation with an empty space via wounding, migrates collectively into the cell-free region. The resulting two cell sheets eventually collide, effectively “healing” the wound. The biological and biophysical characteristics of individual cells yield such collective migratory behavior at the tissue-scale monolayer. In contrast, when two opposing tissues with different properties collide, they form either a mixed or a distinct visible boundary at the tissue interface. Only a few studies have employed such a competition assay between different cell types, and a significant gap remains in

understanding the factors that govern the dynamic changes at these boundary interfaces. In this study, a barrier-lift assay was used to simulate wound creation, with each side containing different cell types. The collective migration of the opposing monolayers was observed through time-lapse microscopy as the cells move, collide, and display post-collision interface dynamics. We find that the interface dynamics are determined by the properties of the constituent monolayers. First, we control the overall tissue dynamics by overexpressing the endocytic protein Rab5a, which fluidizes the cellular collective. When fluidized Rab5a-expressing MCF10A, a human non-tumorigenic breast cell line, collide with solid-like, control MCF10A cells, they shift the boundary interface, pushing the control cells away from the boundary. We further explore interface dynamics by creating antagonistic migration assays between monolayers of cell lines with distinct biological and biophysical properties. We find that, across a wide range of experimental conditions, a similar boundary displacement occurs, such that one monolayer appears to “win” the interaction, displacing the opposing monolayer and shifting the tissue boundary. For example, a metastatic prostate cancer cell line, DU-145, can shift the boundary interface shared with MCF-7, a breast cancer cell line. The ability of DU-145 to “win” the interaction is robust and does not depend on relative cell density between the opposing monolayers. Our current research focuses on systematically quantifying the cellular properties that collectively regulate and govern tissue-scale heterotypic interface dynamics.

B257/P0250

Analysis of Adhesion Protein Dynamics in Fast Moving Fish Epithelial Keratocytes

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The movement of fish keratocytes is the fastest among eukaryotic cell types. Their rapid motility and broad, fan-shaped lamellipodium has made keratocytes ideally suited for studying the integration of molecular and biomechanical processes that drive cell movement. Although much has been learned about the interplay between force and actin filament dynamics, little is known about the dynamics of keratocyte adhesions. To determine how adhesion turnover is coordinated with cycles of protrusion and retraction, we have used Tol2 transposase mediated transgenesis of zebrafish embryos to express different fluorescently tagged adhesion proteins in keratocytes. Using TIRF microscopy and FRAP we measured the respective adhesion turnover rates and molecular kinetics of specific adhesion proteins during cell movement. Analysis of adhesion dynamics was performed using the Focal Adhesion Analysis Server (FAAS). Preliminary data shows that the rates of adhesion assembly and disassembly in GFP-vinculin containing adhesions were ~ 68 and ~95 times faster, respectively, than previously published rates for adhesions in slower moving, fibroblasts. In addition, the lifetime of keratocyte adhesions is at least 10 times shorter in duration than that of fibroblasts. FRAP experiments in keratocytes expressing RFP-zyxin or GFP-vinculin reveal that the half-life of fluorescence recovery to be ~ 12 secs and was completed to ~ 80% of the pre-bleach value, within 25 to 40 seconds. By contrast, FRAP experiments in fibroblasts expressing fluorescent adhesion proteins occurs over a period of 2 to 5 mins, which is ~10 times slower than in keratocytes. The rapid turnover of keratocyte adhesion proteins is consistent with the fact that these cells move ~ 50 times faster (30-60 um/ min) than fibroblasts (~ 1 um/min). Since keratocytes possess the same types of adhesion proteins as other fibroblastic cell types, we suggest that differences in the regulatory mechanisms governing the rate of adhesion turnover may be a major determinant of cell speed.

B248/P0241

ATGL-mediated lipid droplet lipolysis promotes collective cell migration in Drosophila

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Lipid droplets (LDs), dynamic organelles central to lipid and energy homeostasis, have emerged as a novel factor contributing to cell migration. In vitro studies reveal LDs promote invasion and migration in several cancer types, but the precise role of LDs during in vivo collective cell migration remains poorly understood. To address this, we use Drosophila border cell migration as an in vivo model of invasive, collective cell migration. Using high-resolution microscopy, we find LDs within the border cells undergo dynamic changes in both size and number as migration proceeds. Specifically, border cell LDs decrease in volume by the end of migration, suggesting lipids are being mobilized to promote invasion and migration. To test whether lipid mobilization from LDs is important for border cell migration, we characterize the role of Adipose Triglyceride Lipase (ATGL), a conserved LD lipase responsible for triglyceride hydrolysis and release of free fatty acids from LDs. Loss of ATGL increases LD volume in the border cells. By contrast, overexpression of ATGL within the border cells depletes LDs. Importantly, either genetic loss, border cell knockdown, or border cell overexpression of ATGL leads to migration defects, including delays in migration and failures to detach (aka delaminate) from the follicular epithelium. Together these results are, to the best of our knowledge, the first to demonstrate that tight regulation of mobilization of stored lipids from LDs is essential for successful in vivo detachment and collective migration, as either stunted LD lipolysis or LD depletion in the border cells prevents their invasion and migration. Future work will aim to determine how ATGL-mediated lipolysis is regulated during border cell migration, as well as how mobilized lipids act to promote invasion and migration. The released fatty acids could serve as substrates for energy production or as precursors for membrane lipids and/or signaling molecules. Indeed, we find loss of ATGL disrupts normal mitochondrial morphology in the border cells, an indication of mitochondrial stress and a possible bioenergetic defect. Taken together, these results support that LDs are critical organelles for mediating the detachment, invasion, and migration of cells in vivo. Our findings not only have the potential to inform how metastasizing cancer cells exploit LDs to promote their invasive behaviors, but also highlight the crucial role of LDs in the migration of non-cancerous cells during development, hinting at their broader significance in diverse migratory contexts.

B273/P0266

Cellular protrusions as wave propagations coupled with membrane curvatures

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Cell migration depends on the coordinated formation of actin-based protrusions, which can take diverse forms—lamellipodia, filopodia, macropinosomes—often thought to arise from distinct pathways. Using Dictyostelium as a model, we show that cortical signaling waves propagate with characteristic three-dimensional deformations matching protrusion geometry, supporting a continuum hypothesis in which diverse protrusions are unified by a common excitable signaling network. Guided by this framework, we identify class I myosin motors as key elements defining the refractory phase of the excitable network, linking biochemical activity with mechanical and geometrical feedback so that cell form and function are tightly coupled. High-

resolution SIM and LLSM imaging of protrusion transitions further supports the continuum model, and similar curvature–wave coupling is observed in neural crest lamellipodia. These findings establish curvature-coupled excitability as a conserved organizing principle for diverse protrusive behaviors.

B255/P0248

Design and characterization of a Rac-specific localization-based biosensor for real-time imaging of GTPase activity

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Rho family GTPases, including Rac, are critical regulators of actin cytoskeleton dynamics, controlling cell migration, adhesion, and proliferation. Rac cycles between active (GTP-bound) and inactive (GDP-bound) states, controlled by guanine nucleotide exchange factors (RhoGEFs) and GTPase-activating proteins (RhoGAPs). Dysregulation of this network contributes to cancer, inflammation, and other pathological conditions. Since aberrant Rac activity often results from altered regulation by GEFs and GAPs rather than changes in expression, measuring protein levels alone is insufficient. Understanding Rac signaling thus requires tools that detect its activity with high temporal and spatial resolution, as activation occurs on minute and micrometer scales. To address these challenges, advanced tools that enable real-time, high-resolution measurements of endogenous Rac activity are essential. Two types of Rho GTPase biosensors exist: FRET sensors and effector localization sensors. FRET sensors have been widely used to study Rho GTPase dynamics but they exhibit limitations: they report net GEF/GAP activity rather than localization of endogenous GTPases, have limited dynamic range, and occupy a broad spectral range, restricting multiplexing. In contrast, effector localization sensors, consisting of a small GTPase-binding domain fused to a fluorescent protein, enable direct visualization of endogenous active GTPases. However, they have seen limited adoption in mammalian studies, as they require a high level of expression, which can lead to higher background fluorescence from unbound probes and potential dominant negative effects. Critically, no Rac-specific localization sensors exist, since most Rac effectors (e.g., PAK) also bind Cdc42. Here, we describe the design and development of a new biosensor for active Rac using the Rac-binding domain of Plenty of SH3 domains (POSH), a previously characterized Rac-specific effector, as the sensory unit. Through iterative optimization combining pulldown assays and live-cell imaging, we tested multiple designs and fluorophore tags to assess performance and specificity relative to other Rho GTPases. The best-performing constructs reliably detected endogenous Rac activity under physiological conditions such as cell migration, showing recruitment to the leading edge of protrusions in correlation with protrusion velocity. A single-point mutation that disrupts Rac binding abolished recruitment, confirming specificity. In summary, this sensor will enable direct, high-resolution visualization of endogenous Rac activity in live cells, overcoming limitations of traditional FRET-based sensors and providing a powerful tool for studying Rac signaling dynamics in health and disease.

B261/P0254

Dynamic pathway switching and repressive interactions between FGF, Wnt, and Netrin-independent UNC-5/UNC-40 signaling regulate fates, proliferation, and migration of C. elegans mesodermal progenitors

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Cell migration is orchestrated by multiple families of secreted signaling proteins that act as attractive or repulsive cues. In many cases, the same pathways that control migration also regulate cell fate and proliferation, yet how cells integrate and prioritize multiple signals to execute precise behaviors at specific times remains unresolved. To address how different signaling pathway interactions lead to context-dependent outputs in vivo, we used Caenorhabditis elegans muscle progenitors called sex myoblasts (SMs) to investigate how signaling pathways coordinate migration, proliferation, and fate. During larval development, Fibroblast Growth Factor (EGL-17) acts as a chemoattractant for migrating SMs, which fail to complete their migration in FGF/egl-17 or FGFR/egl-15 mutants. Intriguingly, cell ablation experiments showed over two decades ago that SM migration defects in FGF mutants are mediated by an unknown repulsive cue that is produced by the same cells that express egl-17. Here, we identified this long-sought repulsive mechanism through cell-type specific depletion of tagged endogenous proteins. We found that in the absence of FGF signaling, the Wnt ligand MOM-2 repels migrating SMs by non-canonical Wnt signaling through the receptors Frizzled/LIN-17 and Ryk/LIN-18. The Netrin receptors UNC-5 and UNC-40 act in parallel with non-canonical Wnt signaling to prevent migration independent of Netrin/UNC-6. We also uncovered functions for canonical Wnt signaling in proliferation and cell fate, and for non-canonical Wnt signaling in early migration and in establishing mesodermal symmetry. Unlike gonad-dependent repulsion, these developmental roles are independent of FGF, highlighting how distinct Wnt modules independently govern multiple developmental processes in the same cell lineage. Combined depletions with RTK signal transduction components showed that Ras and ERK can act downstream of non-canonical Wnt and UNC-5/UNC-40 signaling during SM migration, demonstrating unexpected inter-pathway coupling. Finally, we developed a synthetic construct that enables functional dissection of canonical versus non-canonical Wnt signaling, providing insights into how pathway branching shapes distinct outcomes. Collectively, our findings uncover how a model cell lineage integrates multiple signals in a context-dependent manner to ensure robust developmental outcomes.

B275/P0268

Effect of DMP Plasticizer on the Cell Migration of HaCaT and NIH-3T3 Cell Lines

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Microplastics, an ubiquitous collection of pollutants, are prevalent in various consumer products and the environment. Though their ingestion and inhalation have been widely studied, the impact of microplastics on integumentary function and tissue regeneration remains less understood. Using dimethyl phthalate (DMP), a common plasticizer found in personal care products and medical devices, we examined its effects on HaCaT (keratinocyte) and NIH-3T3 (fibroblast) cell lines, which are critical to epidermal and dermal wound repair, respectively. Both cell types were cultured with and without DMP over duration of four weeks, followed by a scratch assay to model wound healing. Cell migration across the wound site was imaged every 12 hours, and upon closure, gene expression was analyzed using qPCR to assess markers of inflammation and migration. Results revealed that DMP-treated HaCaT cells demonstrated significantly slower migration compared to controls, accompanied by downregulation of genes associated with cell movement and inflammation, alongside an upregulation of NFKB. Conversely, DMP-treated 3T3 cells showed enhanced migration and upregulation of inflammatory and migratory genes, suggesting a hyperactive repair response. These findings implicate that microplastics may impair epidermal healing while simultaneously overstimulating dermal fibroblasts, potentially leading to imbalanced or dysfunctional tissue repair.

B260/P0253
Endoplasmic Reticulum - Plasma Membrane contact sites restrict RTK signaling in cell migration
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Directional cell migration is crucial for processes such as development, immune responses, and cancer metastasis. Cells respond to environmental cues by polarizing, forming membrane protrusions, and migrating. Receptor tyrosine kinases (RTKs) trigger this process by activating PI(3,4,5)P3 signaling and stimulating small GTPases like Cdc42 and Rac at the leading edge, leading to actin polymerization and directed migration. Simultaneously, signaling at the cell rear must be inhibited to maintain proper directionality, but the mechanism for this inhibition is not fully understood. Our lab recently showed that ER–PM contact site gradients (MAPPER gradients) spatially restrict RTK signaling by enabling ER-localized PTP1B to dephosphorylate RTKs at the rear. We hypothesize that ER–PM contact density thresholds regulate RTK output and directional migration. Using circular micro-patterns to guide single RPE-1 cells, live-cell imaging revealed that migration velocity correlated with PIP3 but inversely with MAPPER density. A ~20% local MAPPER reduction preceded protrusion by ~20 min, while PIP3 accumulation lagged velocity peaks by ~15 min. E-syt1 knockdown reduced MAPPER density and migration velocity, eliminated MAPPER–velocity correlation, and caused erratic migration, highlighting the importance of ER–PM contacts. To define phosphorylation inputs, we examined eight major EGFR sites (pY845, 974, 992, 1045, 1068, 1101, 1148, 1173). TIRF imaging showed pY992 and pY1068 were highly EGF-sensitive and strongly depleted by PTP1B inhibition, indicating their importance in MAPPER-modulated EGFR signaling. To directly test downstream functional effects, we generated stable RPE cell lines expressing single phosphorylation site Y to F mutants. Under siRNA knockdown of endogenous EGFR condition, the pY992F mutant exhibited the strongest loss of PIP3, identifying pY992 as a critical node linking EGFR to PI3K signaling in the context of MAPPER regulation. Together, these findings support a model in which ER–PM contact density thresholds gate EGFR phosphorylation and PI3K output, ensuring directional migration. Future studies will test whether targeted modulation of MAPPER in defined regions can reprogram protrusion sites and cell trajectory.

B241/P0234
Epiblast signals direct mesoderm migration during human gastrulation
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Collective migration of mesoderm cells is essential for gastrulation, producing progenitors of the heart, bone, muscle, and immune system. Yet how human mesoderm delaminates and migrates beneath the epiblast has remained unresolved due to limited post-implantation models. We leverage micropatterned human embryonic and iPSC-derived organoids, which offer precise control of epiblast size, geometry, and cell number, to dissect mesoderm dispersal at high spatiotemporal resolution. Live imaging of a mesoderm reporter (TBXT–mNeonGreen) shows that mesoderm cells form a dense niche and undergo EMT with loss of junctions; some then escape and invade the epiblast, mimicking ingression. 3D tracking reveals that cells positioned beneath the epiblast lead this migration, while others trail. Mesoderm also accelerates and increases radial velocity toward the colony center, reorganizing the tissue independently of epiblast neighbors, as confirmed with a SOX2–GFP epiblast reporter. Mechanistically, we identify a conserved directional cue. Mesoderm expresses the Apelin receptor APLNR (a G protein–coupled receptor), while adjacent epiblast provides its ligands (Apelin/Apela). APLNR expression increases over time, conferring migratory competence. Inducible APLNR

knockdown blocks dispersal and traps mesoderm within the niche. Altering epiblast geometry indicates that mesoderm establishes a self-generated signaling gradient that guides collective migration. This is the first demonstration in humans of a direct epiblast to mesoderm guidance cue mediated by APLNR, answering how mesoderm is directed toward the epiblast. These findings show that epiblast signals integrate with intrinsic mesoderm programs to coordinate collective migration and establish early axis patterning.

B262/P0255

Epithelial State Transitions and EFNA3 Metabolic Signaling Drive Collective Migration Without EMT

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Contrary to the long-standing EMT paradigm, we show that mammary epithelial cells migrate collectively while retaining epithelial identity. Snail1 directs motility through cytoskeletal regulation rather than EMT induction, revealing dynamic epithelial state transitions as the true basis of migration. We further identify EFNA3 as a basal cell–derived signal that enhances oxidative phosphorylation in luminal leader cells, fueling collective migration during development and breast cancer metastasis. These findings redefine the mechanisms of epithelial motility and highlight new therapeutic opportunities in cancer.

B244/P0237

Glioblastoma neurospheres contract prior to cell invasion, which depends on actomyosin and microtubule activity

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Glioblastoma multiforme (GBM) is one of the most aggressive brain tumors, with a 5-year survival rate of less than 7%. This poor prognosis is largely attributed to the highly invasive phenotype of GBM cells. These cells invade the surrounding healthy brain tissue, forming tumors with diffuse and ill-defined margins that are difficult to fully resect, leading to frequent relapse and poor survival outcomes. In this study, we investigated the invasive behavior of GBM cells using patient-derived neurospheres. When cultured in Matrigel, these neurospheres spontaneously invade the surrounding microenvironment, making them an effective model for studying GBM invasion. To better understand cell-microenvironment interactions during invasion, we performed three-dimensional traction force microscopy (3D TFM) on the invading GBM neurospheres. Interestingly, we observed a gradual increase in cellular contractility near the neurosphere surface prior to the onset of invasion, suggesting that a threshold level of traction force is required for cells to detach from the neurosphere and invade the Matrigel. This increasing contractility over time was driven by actin- and microtubule-rich protrusions extending from the neurospheres, highlighting the importance of actomyosin and microtubule activity in this process. Furthermore, invading cells displayed an elongated morphology with prominent actin-rich protrusions at the leading edge and microtubule structures along the cellular body, consistent with a mesenchymal migration mode. From 3D TFM analysis, we also observed localized contraction of the Matrigel at the tips of the protrusions, further emphasizing the role of actomyosin contractility during invasion. To test this dependency, we inhibited myosin activity and microtubule polymerization, which significantly reduced the cells' ability to invade the Matrigel. However, a degree of invasion persisted following myosin inhibition, indicating that some invasion mechanisms do not rely on cellular contractility. Moreover, inhibition of microtubule polymerization led to a loss of the elongated cell morphology, suggesting that

microtubules are critical for maintaining the elongated phenotype necessary for effective invasion. Together, we demonstrate the roles of myosin II and microtubules in GBM cell contractility and invasion.

B264/P0257

Heritability of behavioral heterogeneity in T cell migration

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Cell heterogeneity, such as the phenotypic cell-to-cell differences in behaviors like cell movement, is always present to some degree in a cell population even when the cells are genetically identical. In this work, we have explored the degree of heritability of heterogeneous migratory behavior and the associated transcriptional characteristics of the EL4 cell line, a proliferative migratory T-cell-like line derived from mouse (Mus musculus). During six rounds of the transwell assay with stromal cell-derived factor-1α (SDF-1α, CXCL12), we observed a gradual increase in the migration rate, reaching approximately 50% over successive rounds, and selectively collected a highly motile cell population that exhibited enhanced migration in response to various chemoattractants. The more migratory cells showed faster movement than the control group in both 2D and 3D conditions, with or without external cues. To understand the transcriptional properties of the highly migratory cells, we conducted bulk and single-cell RNA-seq, and identified differentially expressed genes (DEGs) in the more motile cells compared to the control group. Specifically, the dimensionality reduction analysis of scRNA-seq revealed that the transcriptional profiles promoting cell behavior originated from a behaviorally heterogeneous original population. Finally, by regulating the expression of a single identified DEG in original EL4 cells, we bidirectionally validated the relationship between enhanced migratory behavior and transcriptional characteristics. These findings indicate that intrinsic heterogeneity within a single cell line can be selected for heritable enhancements in migratory phenotype. This work enhances our understanding of the molecular basis for behavioral heterogeneity within a cell population.

B269/P0262

How Do New Cells Invade Into Old Monolayers? - Investigating The Mechanism Of Apical Intercalation

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Epithelial cell intercalation is a key process in embryonic development, wound healing, and tissue morphogenesis. Apical intercalation involves top-down insertion of incoming cells into a confluent epithelial monolayer through adhesion, tight junction (TJ) remodeling, and polarity changes (Pfannenstein, Dev Cell 2023). This study investigates investigates the rate-limiting role of tight junctions in apical intercalation into mammary epithelium. Cas9-mediated editing was used to generate Eph4 cells lacking the TJ polarity protein Pals1, required for barrier function and TJ assembly, with ZO1 endogenously tagged by the stable fluorophore mStayGold (mSG) to monitor TJ integrity. Loss of Pals1 disrupted TJ continuity. In an in vitro assay, fluorescently tagged cells were plated onto confluent monolayers and live imaged. Intercalation was accelerated nearly ten-fold in Pals1-KO monolayers, showing that intact TJs are a barrier to apical entry. Re-expression of Pals1 restored junctions and suppressed intercalation. Work in Drosophila suggests intercalation proceeds via a zippering mechanism mediated by homophilic adhesion along lateral membranes. To test this, E-cadherin was deleted in incoming Eph4 cells. On Pals1-KO monolayers, loss of E-cadherin strongly reduced intercalation, consistent with the need for zippering between E-cadherin molecules. Some E-cadherin–deficient cells transiently entered but were unstable and often extruded within hours. A corollary of the zipper model is that

active migration driven by actomyosin contractility may not be required. To test this, I am using a genetic tool based on CA-MYPT1, a regulatory subunit of Myosin Phosphatase, which inhibits contractility by dephosphorylating myosin light chain. Inducible expression will determine whether blocking contractility in incoming cells, the monolayer, or both prevents intercalation.These studies indicate that TJ integrity restricts apical intercalation, while E-cadherin adhesion promotes stable insertion. Ongoing experiments will clarify the contribution of actomyosin contractility. This work defines molecular requirements for late-stage intercalation and may have significance for cancer dissemination and organ development in tissues such as the mammary and salivary glands.

B265/P0258
How prototypical phagocytes detect and respond to gram-positive bacteria?
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Phagocytes rely on distinct receptor systems for chemotaxis and phagocytosis—GPCRs for detecting chemoattractants and PRRs for recognizing and engulfing bacteria. The social amoeba Dictyostelium discoideum, a model phagocyte, intriguingly lacks canonical PRRs yet efficiently detects and consumes diverse bacteria. Previously, we demonstrated that D. discoideum uses a GPCR, fAR1, to mediate both chemotaxis toward bacterial-secreted folate and phagocytosis of gram-negative bacteria via lipopolysaccharide (LPS) detection. In this study, we investigated how D. discoideum recognizes gram-positive bacteria. We identified lipoteichoic acid (LTA), a key component of gram-positive bacterial surfaces, as a trigger for ERK phosphorylation (ERK-P) via a GPCR-dependent pathway. Using G-protein null mutants, we found that Gα4 and Gβγ subunits are required for LTA-induced ERK-P. Notably, LTA failed to activate ERK-P in grlL and grlG null cells, suggesting that both GPCRs, GrlL and GrlG, are essential for LTA recognition. Live-cell imaging revealed that LTA triggers transient translocation of key signaling reporters—RBD-GFP (Ras), PH-GFP (PI3K), and LimE-GFP/RFP (F-actin). Structural predictions using AlphaFold3 suggest that GrlL and GrlG form a heterodimer that associates with Gα4 and Gβγ, enabling downstream signaling. Together, these findings uncover a novel mechanism wherein class C GPCRs function as pattern recognition receptors in D. discoideum, facilitating detection and response to gram-positive bacteria. This supports a broader paradigm in which GPCR-mediated signaling coordinates both chemotaxis and phagocytosis in ancestral phagocytes, representing an evolutionarily ancient recognition strategy.

B276/P0269
Investigating the role of ApoD in cranial neural crest cell migration
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Cranial neural crest cells are a transient, embryonic cell population that contributes to the craniofacial skeleton, cranial ganglia, and epidermis. Cranial neural crest (NC) undergo extensive and tightly controlled migration, and dysregulated NC migration often results in atypical craniofacial morphogenesis. Toward a better understanding of this regulation, we recently identified Apolipoprotein D (ApoD) as a key player in regulating craniofacial development. ApoD is a multifunctional lipocalin that binds lipid species and regulates inflammation, oxidative stress, proliferation, and cell migration in other contexts. Yet, how ApoD regulates cranial neural crest development remains unclear. We performed HCR-FISH experiments in developing chick embryos to determine the spatiotemporal pattern of ApoD expression and observed strong expression in

delaminating NC, with lower levels maintained throughout migration. To uncover the role of ApoD in neural crest cells, we transfected gastrulating embryos with control or ApoD-targeting morpholinos on contralateral sides and analyzed the resulting NC cell development at migratory stages. By measuring neural crest area, we observed reduced cranial NC migration after ApoD loss, suggesting that ApoD is required for cranial NC migration. Since this phenotype could also result from loss of specification, we counted NC numbers by Pax7 and Sox10 expression in transverse sections. From this analysis, we did not find a difference in the total number of NC cells after ApoD loss, which suggests that ApoD-dependent migratory behaviors are independent from NC specification. To further examine the role of ApoD in NC migration, we categorized the migratory patterns of NC cells in ApoD morphants and found that ApoD deficiency increased migratory streaming behaviors over the more sheet-like migration of contralateral controls. To explore more subtle migratory dynamics, we tracked individual cells during ex vivo migration of cranial NC explants and found that ApoD deficient NC cells at the leading edge of migratory collectives migrated more slowly than paired controls. The results from our in vivo and ex vivo analysis indicate that ApoD is a novel regulator of migration in cranial NC cells and suggests that cellular lipid management determines the mode of cell migration during craniofacial development.

B278/P1802

Matrix stress relaxation flips the regulatory effect of cofilin on glioblastoma 3D invasion

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Glioblastoma (GBM) is the most aggressive and prevalent primary intracranial tumor in adults, with over 10,000 patients diagnosed in the United States each year. Despite advances in surgery, chemotherapy, and radiotherapy, GBM patients typically only survive 12-18 months after diagnosis. This dismal prognosis is typically ascribed to the infiltrative nature of the tumor, which compromises essential brain structures, prevents complete resection, and promotes resistance and recurrence. GBM tumor cells spread by penetrating tightly confined spaces within the brain, including junctions between cells and regions surrounding white matter tracts and blood vessels, all of which are embedded within a three-dimensional (3D) extracellular matrix rich in hyaluronic acid (HA). Our laboratory recently showed that the actin-severing protein cofilin slows migration within elastic 3D HA matrices, with cofilin-1 (CFL) knockdown surprisingly speeding migration (Tran et al., MBoC 2025). However, brain tissue is viscoelastic (stress-relaxing), and it is unknown how viscous properties influence CFL regulation of 3D invasion. Here we address this question by investigating 3D GBM cell migration using HA matrices with tunable stress relaxation properties. We fabricated hydrogels with matched storage moduli but varied in stress-relaxing (SR) kinetics: fast-relaxing high molecular weight (80%, HMW H80) and slow-relaxing (20%, HMW H20) HA formulations. U87 GBM cells expressing either non-targeting (NT) or CFL KD constructs were embedded as spheroids in each matrix with or without integrin-ligating RGD peptides. In the fast-relaxing H80-RGD matrices, CFL KD reduced invasion compared to NT controls. By contrast, in slow-relaxing H20 matrices, NT and CFL KD cells invaded with similar speeds. These results suggest that stress relaxation reverses the effect of CFL on 3D invasion, with CFL activity speeding 3D invasion in fast-relaxing matrices. Based on our past finding that fast-relaxing matrices promote collective, mesenchymal invasion (Carvalho et al., Adv Mat2024), we propose that CFL promotes invasion by liberating actin subunits for incorporation into contractile bundles.

B250/P0243

Microfluidic Analysis of Geometric Confinement Effects on Nuclear Transit during Cell Migration

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In vivo, cell migration frequently occurs through physically confined environments, such as dense extracellular matrices or narrow intercellular junctions, where the geometry of confinement can significantly influence nuclear mechanics and cell motility. To systematically investigate the effects of confinement geometry on nuclear dynamics, we fabricated polydimethylsiloxane (PDMS)-based microfluidic channels with 20 µm in width and 30 µm in height. These channels were patterned with distinct geometrical features that produced minimum confinement widths of 2.5 µm, 3 µm, and 4 µm. Five geometric configurations were employed to impose confinement: (1) Opposing Half-Ovals: Two half-ovals with a major axis of 45 µm facing each other. (2) Circular Constrictions: Circles with diameters of 17.5 µm (2.5 µm confinement), 17 µm (3 µm), and 16 µm (4 µm). (3) Rectangles: Two rectangles with a 17.5 µm edge length. (4) Trapezoids: Two trapezoidal structures with a long edge length of 17.3 µm at a 60° angle with smaller lengths of 7.2 µm (2.5 µm confinement), 7.5 µm (3 µm), and 8.1 µm (4 µm). (5) Semicircle–Flat Wall Combinations: Semicircles with radii corresponding to confinement widths of 2.5 µm, 3 µm, and 4 µm (radii: 17.5 µm, 17 µm, and 16 µm, respectively). We used MDA-MB-231 breast cancer cells to assess nuclear dynamics during migration. Specifically, we measured nuclear velocity and transit time through the confined regions. The results indicate that minimum confinement width alone did not significantly influence nuclear transit time. However, nuclear exit velocities were significantly higher when cells exited 2.5 µm-wide confinements, particularly in semicircles and rectangles, compared to 4 µm-wide confinements. Among the geometries tested, confinement shape exerted a more pronounced effect on nuclear dynamics than confinement size. Semicircle and half-oval geometries were associated with longer nuclear transit times, whereas rectangle geometries facilitated faster nuclear exit velocities relative to semicircle, half-oval, and trapezoid shapes. These findings suggest that geometric features of confinement, particularly shape, play a critical role in modulating nuclear transit and exit behavior. Square-like geometries appear to promote more efficient nuclear passage, while curved structures may impose greater mechanical resistance. Ongoing work will further determine the mechanobiological interactions between confinement geometry and nuclear deformation.

B274/P0267

Microplastics-cell association and its impacts on cell proliferation and motility

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The accumulation of microplastics and nanoplastics (MNPs) in human tissues has raised growing concern about their impact on human health. Recent studies have confirmed significant MNP deposition in human organs, with higher accumulation reported in decedents with diagnosed dementia and evidence of a rising trend over the past decade. Whether and how MNPs affect fundamental tissue cell functions, however, remains a central but unresolved question. Although largely chemically inert, MNPs act as rigid foreign bodies once internalized, which are likely to interfere with cytoskeletal organization and remodeling. This is particularly relevant for nanoplastics (NPs) of ~100 nm, a size comparable to the mesh size of the actin network. At this critical length scale, NPs can alter actin network mechanics and dynamics, potentially impairing intracellular trafficking and thereby disrupting cell proliferation and migration. Using microfluidics-calibrated fluorescence microscopy, we quantify NP accumulation in vitro, matching cellular NP concentrations to levels reported in human tissues. NP

exposure at these levels broadly impair cell proliferation across epithelial, endothelial, fibroblast, and immune cells, with sensitivity varying by cell type. Using a combination of motility assays and a novel phenotype-profiling approach, we reveal that NP exposure reduces motility in T cells (including primary human CD4+ T cells) and fibroblasts, with more complex effects observed in macrophages. In addition, we show that NP–cell association and trans-epithelial transport involves not only classical endocytic regulators but also pathways related to ion and water transport. Notably, NP association and release are highly sensitive to physiological extracellular fluid environments, particularly viscosity. Together, these findings reveal the impact of NPs on tissue cell function and uncover novel regulatory mechanisms, establishing a quantitative framework for investigating NP–cell interactions under biologically relevant conditions.

B270/P0263

Mitochondrial dynamics are governed by cell-matrix interactions in primary human fibroblasts

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The rapid cytoskeleton remodeling in cells migrating across 2D surfaces necessitates the repositioning of mitochondria to sites of high ATP consumption such as the leading edge. Primary mesenchymal cells migrating through highly crosslinked 3D matrices require additional actomyosin contractility to pull the nucleus through small pores in the extracellular matrix. This contractile force is transmitted to the nucleus via the cytolinker plectin and vimentin intermediate filaments (vimIFs), enabling the nucleus to function as a piston that pressurizes the anterior cytoplasm while maintaining a low-pressure posterior. It is currently unclear how mitochondria are positioned in cells when this nuclear piston mode of migration is triggered. We examined mitochondrial distribution and dynamics in primary human dermal fibroblasts using the nuclear piston and found that mitochondria in the perinuclear region, where vimIFs are enriched, are more fragmented and less dynamic compared to mitochondria at the leading edge. Live-cell imaging revealed that mitochondria overlapping with vimIFs exhibit dramatically reduced motility in 2D. Knockdown of vimIFs decreased mitochondrial membrane potential and reduced mitochondrial size in 2D. Furthermore, inhibition of Rho-associated kinase (ROCK) abolished anterior mitochondrial accumulation specifically in CDMs, suggesting a matrix-specific requirement for actomyosin contractility in mitochondrial localization. Together, these findings demonstrate that the nuclear piston machinery, through vimIFs and actomyosin contractility, regulates mitochondrial localization and morphology in fibroblasts migrating through 3D matrices. We propose that the perinuclear, fragmented mitochondria in the cell's anterior generate the additional ATP required for fibroblasts to power the nuclear piston while migrating through crosslinked extracellular matrices.

B249/P0242

Model of collective cell migration with varying repolarization bias and intercellular interactions

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The coordinated migration of cell collectives through confined and heterogeneous environments underlies key physiological processes such as tissue repair, immune infiltration, and cancer invasion. We used computational modeling to better understand how small clusters of deformable cells navigate constricting microenvironments, and how their internal polarity cues interact with intercellular mechanical forces to maintain cohesion and directionality. In a two-dimensional bead–spring model, the membrane and cell cortex of deformable cells responds to stretching, bending, area conservation, and is subject to active protrusive and

contractile forces that drive locomotion along a polarized cell axis. Neighboring cells interact through a tunable adhesive force. We introduce a biased repolarization mechanism in which the polarity direction of each cell shifts relative to the net intercellular interaction force. A bias strength parameter measures the polarity direction's tendency to orient either toward or away from the net interaction force exerted due to neighboring cells. Simulations in obstacle-free environments reveal that the direction of repolarization bias affects the collective dynamics. When cells repolarize toward the net intercellular interaction force, the cluster covers larger spatial ranges over time. In contrast, repolarization away from the net interaction force results in reduced displacements and more confined motion. Ongoing work extends this framework to environments with fixed obstacles that mimic constricting extracellular matrices with varying cluster size, adhesion strength, and repolarization bias.

B266/P0259

Nerve Growth Factor and Oligopeptide-215 Accelerate Endothelial Wound Closure in Human Umbilical Vein Endothelial Cells Scratch Assays

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Endothelial monolayers preserve vascular barrier function and regulate thrombosis, leukocyte trafficking, and angiogenesis; loss of coverage after intervention or inflammation exposes a pro-thrombotic surface, making rapid re-endothelialization a clinical priority. Two-dimensional wound-healing (scratch) assays in human umbilical vein endothelial cells (HUVECs) model coordinated migration/proliferation during gap closure. We asked whether nerve growth factor (NGF), known for neurotrophic and endothelial roles, and the synthetic peptide Oligopeptide-215 (O-215; WKPep® Salaregen S), with anti-inflammatory and healing properties but undefined effects on endothelial repair, enhance closure kinetics and whether combined treatment merits testing. HUVECs were grown to 90% confluence, and a linear gap was introduced with a 10 µL pipette tip. Visualized on a Zeiss 200M microscope, monolayers of HUVECs received one of five conditions: (1) untreated control, (2) DMSO solvent control, (3) phosphate-buffered saline (PBS) solvent control, (4) O-215 (100 ppm in DMSO), or (5) 1× NGF (100 ng/mL in PBS). Phase-contrast images acquired every 30 min for 5 h were analyzed in ImageJ to quantify gap area versus time; rates were normalized by nuclear density using NucBlue™ Live ReadyProbes™ Reagent (Hoechst 33342) stain. The three control conditions exhibited comparable closure rates. Both NGF and O-215 increased wound-closure rate relative to controls. Notably, O-215 showed a delayed-onset profile: closure lagged behind controls during the first 90 minutes, exceeding the NGF-only condition thereafter. The confluence of the cells was controlled at 90 percent due to the aberration of scratch gaps with high-confluency samples. NGF and O-215 conditions each accelerated gap sealing relative to matched solvent controls, supporting both neurotrophic and peptide cues can directly potentiate re-endothelialization. The delayed-then-accelerated profile of O-215 suggests a mechanism distinct from NGF's rapid receptor signaling evident after 90 min. Because closure rates were normalized to live nuclear density, the dominant effect is likely on collective migration rather than simple increases in cell number. A combined O-215 and NGF condition is therefore a rational next step; we hypothesize supra-additive effects after the early lag and will evaluate synergy using dose–response matrices and Bliss/Loewe analyses. Although we mitigated confounding errors with matched solvent controls and normalized nuclear density, limitations include the use of a single endothelial subtype, one dose per agent, and scratch-assay artifacts (variable wound width, edge damage) suggests more conclusive understanding after extensive repeats of experiments.

B245/P0238

Neutrophil migration patterns change in response to elevated extracellular viscosity

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Highly viscous mucus is associated with diseases such as cystic fibrosis, IPF, and COVID-19. The affected airways are prone to incur epithelial wounds, which expose cells initially protected by the epithelial barriers to pathogens and viscous mucus. Those cells include mesenchymal cells such as fibroblasts and immune cells such as neutrophils. We previously found that mesenchymal cells with active ruffling lamellipodia can sense extracellular viscosity levels. At increased viscosity, cells with ruffles flatten, and focal adhesion turnover increases, counterintuitively resulting in faster migration. In this study, we investigated the effects of elevated mucus viscosity on neutrophils. We used simple healthy and diseased mucus-mimetics made by supplementing long-chain polymer methylcellulose in medium, resulting in a viscosity two orders higher than that of the control. We also used a synthetic mucin hydrogel to physiologically represent mucus in healthy and diseased airways. We used differentiated HL-60 (dHL-60) to model neutrophil behaviors. In diseased mucus-mimetics, dHL-60 increased their surface area by 2-fold, migrated 4 times faster with 3-fold higher directional persistence compared to healthy mucus-mimetics. Similar trends were observed using mucin hydrogels. We note that dHL-60 and neutrophils only exhibit such persistence in the presence of a chemo-gradient at low viscosity. Strikingly, unlike the case where chemo-gradient is present, each dHL-60 moved persistently in different directions under high viscous environments. We then inspected actin dynamics in dHL-60 expressing Ftractin-mStayGold. When subjected to diseased mucus-mimetic medium, each dHL-60 would not only form one, large, leading pseudopod, but also assemble multiple actin puncta of ~450 nm diameter near the leading edge. We also characterized the changes in the nucleus, and found 17% lower chromatin condensation in cells subjected to diseased mucus-mimetic. These results suggest that mucus viscosity may change actin dynamics and alter epigenetic profiles.

B247/P0240

Novel spatial arrangement of signaling modules driving mechanics of bleb-based migration.

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Cancer cell migration plays a crucial role in invasion and metastasis. During metastasis, tumor cells migrate through their surrounding microenvironment, navigating various tissue and extracellular matrix (ECM) geometries, including confined spaces, to spread to distant sites. In confined microenvironments with low adhesion, metastatic cells often adopt a bleb-based mode of migration that differs fundamentally from integrin-dependent mesenchymal motility. Using metastatic melanoma cells under non-adhesive confinement, we investigated the mechanisms that govern the formation and stability of the large, pressure-driven leader bleb that defines cell polarity in this context. High-resolution live imaging and molecular perturbations revealed that EGF signaling via PI3K is critical for maintaining a stable, polarized leader bleb. Surprisingly, biosensors showed a rear-to-front gradient of EGFR and PI3K activity, with downstream effectors PIP3 and Rac1-GTP enriched at the rear of the bleb, while PIP2 and RhoA-GTP localized to the front. This spatial organization is inverted compared to classical mesenchymal migration. Disruption of this gradient via optogenetic tools led to bleb collapse, highlighting its functional importance. We further identified that,

transmembrane picket protein CD44 and membrane-cortex linker proteins restrict EGFR mobility within a dense actin cortex at the bleb rear, stabilizing the signaling gradient. Combined modeling and experimental data support a feedback mechanism linking cortical architecture to signaling dynamics. Our findings uncover a novel polarity axis in bleb-based migration driven by localized signaling and cytoskeletal interactions. This work underscores how distinct spatial arrangements of conserved signaling modules on the membrane enable plasticity in migration strategies, allowing metastatic cells to adapt to biophysical constraints of the tumor microenvironment.

B258/P0251

Oscillatory dynamics enable cells to sustain migration under high hydraulic resistance

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Cell migration through dense tissues is essential for cancer invasion, immune surveillance, and morphogenesis, yet most mechanistic insight derives from low-resistance or two-dimensional systems. To examine how physical resistance alters migration, we tracked MDA-MB-231 cells moving through PDMS-based microfluidic channels of identical cross-section (3 μm × 10 μm) but variable length (200 μm and 1500 μm) to impose quantifiable differences in hydraulic resistance. Strikingly, cells in high-resistance (1500 μm) channels were significantly elongated and displayed robust oscillations in cell length and migration velocity. The standard deviation of cell length under high resistance nearly doubled that under low resistance (200 μm), and multiple oscillatory velocity patterns emerged, including persistent, near-zero, and burst-like behaviors. Despite increased resistance, mean migration velocity was maintained or slightly elevated, and oscillatory cells moved faster than non-oscillatory ones. Time-lapse fluorescence imaging further revealed that F-actin intensity and distribution fluctuated periodically, sometimes in synchrony with changes in cell length. High resistance also produced multi-timescale oscillations that were absent under low resistance. Together, these findings identify oscillatory adaptation as a previously unrecognized strategy that enables cells to sustain or enhance motility in mechanically restrictive environments. Rather than simply impeding migration, hydraulic resistance acts as a physical cue that induces dynamic rhythmic behavior. This migratory pattern reveals a new biophysical principle of how cells navigate through confined tissue spaces.

B254/P0247

Oxidation of INPP4B, the inositol polyphosphate 4-phosphatase, is critical for actin polymerization and migration of glioma cells

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Intracellular H2O2 production in response to a variety of extracellular stimuli in mammalian cells is required for signal transduction by oxidizing and inactivating protein tyrosine phosphatases. The effect of intracellular hydrogen peroxide (H2O2) molecules on phosphoinositide signaling, which is implicated in numerous cellular processes such as cell growth and proliferation is unclear. INPP4B, the inositol polyphosphate 4-phosphatase, was reported to be a tumor suppressor gene or an oncogene in a cell context-dependent manner in many cancers. How INPP4B enzyme activity is regulated by H2O2 and its physiological consequence for cancer cell migration has not been studied yet. In this study, we investigated whether INPP4B protein is oxidized and

inactivated by H2O2 accumulated during extracellular stimulation in PTEN-deficient U87MG glioma cells. Our results revealed that H2O2 produced by platelet-derived growth factor (PDGF) stimulation induced the inactivation of INPP4B by oxidizing the catalytic cysteine842 residue of this protein. H2O2-dependent oxidation of INPP4B results in the accumulation of PtdIns(3,4)P2 around the plasma membrane, thereby promoting the recruitment of lamellipodin and the Arp2/3 complex, which play critical roles for filamentous actin formation at the leading edge of lamellipodia and cell migration. A wound healing assay and live cell tracking experiment demonstrate that expression of INPP4B results in reduced cell migration, and inhibition of oxidized INPP4B by antioxidants reduces cell migration further. In addition to the cell study, an informatics analysis on brain tumor patients demonstrates that low expression of INPP4B is associated with poor prognosis of patients with glioblastoma or gliomas, suggesting that the INPP4B functions as a tumor suppressor in brain tumors. Inactivation of INPP4B by intracellular H2O2 molecules produced around the plasma membrane in cells activated with growth factors is critical for cancer cell migration.

B253/P0246

Parasitic flatworm locomotion: motility mechanism of the Schistosoma mansoni schistosomule

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To successfully infect a human host, the schistosomule stage of the parasitic flatworm Schistosoma must navigate through diverse host tissues. Schistosomules tunnel through skin, migrate into and through vasculature, and traverse narrow lung capillaries. Despite the substantial global health burden of schistosomiasis, which affects over 250 million people, the biophysical mechanisms enabling this remarkable tissue navigation remain poorly understood. Here, we investigate the fundamental locomotive strategy of Schistosoma mansoni schistosomules and characterize schistosomule motility at distinct developmental stages. We find that unconfined schistosomules exhibit rapid axial body contractions, and that contractions power productive movement when schistosomules are embedded in fibrin and collagen gels. We hypothesize that backward-pointing actin-rich spines on the schistosomule surface create asymmetric friction, converting bidirectional axial contractions into net forward displacement – similar to how earthworms use setae for locomotion at the macroscopic scale. To test this hypothesis, we seek to quantitatively understand the relationship between spine patterns, contraction gaits, substrate mechanics, and motility outcomes. Ongoing work examines how motility mechanics differ between skin- and lung-stage schistosomules and whether these adaptations are tuned to the distinct mechanical properties of these tissue environments. This research seeks to provide a biophysical framework for understanding schistosomule motility and to explore general principles of how parasites adaptively navigate host tissues.

B240/P0233

PI(4,5)P2 regulates Arl4D self-interaction to activate Arl4D-Pak1 function cue for cell migration

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Self-interaction of small GTPases is a crucial mechanism for regulating intracellular signaling dynamics. Among the ARF small GTPase family, GTP-binding is essential for self-association, though other regulatory mechanisms are less known. Our previous studies show that fibronectin promotes the cooperative recruitment of Arl4D and Pak1 to the plasma membrane and sustains Pak1 activation by facilitating Arl4D binding to the chaperone-like

protein HYPK, which stabilizes Arl4D at the plasma membrane. In this study, we demonstrate that phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] triggers Arl4D self-interaction, which is essential for Arl4D-Pak1 function in cell migration. Arl4D self-interaction occurs at the plasma membrane, not in the cytosol. We generated an Arl4D mutant that binds GTP and localizes to the membrane but lacks self-interaction, indicating that GTP-binding alone is not sufficient. The polybasic C-terminal region of Arl4D is critical for its PI(4,5)P_2-rich membrane localization and self-interaction. In addition, Arl4D, but not self-interaction defect Arl4D mutant, preferentially interacts with PI(4,5)P_2 containing liposomes. Fibronectin stimulation enhances Arl4D self-interaction via Pak1 phosphorylation, which is crucial for recruiting Pak1 to the plasma membrane and maintaining Arl4D stability. Our findings reveal a complex interplay of lipid signaling and Pak1 feedback that regulates Arl4D self-interaction, enabling effective signal transduction and directed cell migration in response to extracellular matrix cues.

B246/P0239
PIP5K-Ras bistability initiates plasma membrane symmetry breaking to regulate cell polarity and migration
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Symmetry breaking is a fundamental process that underlies key cellular behaviors such as cell polarity and migration. Yet, how a uniformly structured plasma membrane and cell cortex transition into an asymmetric state, particularly in the absence of cytoskeletal assemblies or receptor-mediated inputs, remains unclear. In this study, we integrate optogenetic manipulations, targeted genetic screening, single-molecule analyses, and computational modeling to show that a core biochemical circuit involving mutually inhibitory interactions between PIP5K and RasGTP is necessary and sufficient for symmetry breaking. The process is initiated by stochastic, localized Ras activation coupled with a decrease the association of PIP5K with the membrane. A resulting localized reduction in PI(4,5)P2 facilitates the recruitment of a RasGEF to that domain, amplifying RasGTP production through a positive feedback loop. Dissociated PIP5K relocates to other membrane regions, where it suppresses Ras activation. These events dynamically segregate the membrane into distinct active and inactive zones. Beyond symmetry breaking, this biochemical circuit guides the spatial organization of downstream signaling and cytoskeletal activities, and thereby, generates protrusions involved in motility and defines the overall axis of a migrating polarized cell.

B263/P0256
Quantifying Forces In Amoeboid Cancer Cell Migration
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During metastasis, epithelial cancer cells transform into mesenchymal cells, enhancing their migration abilities. In confined tumors, these cells often start to bleb, adopting amoeboid traits that enhance their ability to navigate through complex environments. Amoeboidal cancer cells have a worse prognosis, increased invasion, and drug resistance. Surprisingly, however, the mechanisms by which they migrate remain unclear. Unlike mesenchymal cells, amoeboid cancer cells do not rely on large focal adhesions or cyclic contractions, but instead create pressure-driven blebs that are believed to enable them to move across the substrate using friction. In 3D collagen gels, previous works have shown that cells establish gradients of proteins in large leader blebs, and that adhesive proteins like talin, vinculin, paxillin, PI3K, and beta1 integrins all accumulate at the base of transient blebs. When confined between two rigid, passivated surfaces, cells are able to migrate

efficiently in the direction of the leader bleb. While this phenotype has been observed in multiple cell types, the mechanics of this migration mode and the role of these protein localizations remain unclear. A variety of computational models have been proposed, but experimental validation remains elusive. To address this gap in knowledge, we have confined A375 cells, a cell lined derived from a malignant melanoma, between two soft elastic substrates to measure their mechanical interactions with the substrate. This approach allows us to measure both the amplitude and directionality of forces (e.g., pushing or pulling), and will allow us to validate the molecular mechanisms that explain how amoeboid cancer cells migrate in confinement. Our preliminary results indicate that amoeboid cancer cells require a gradient in the magnitude of confinement to migrate efficiently, and that they often push themselves into the substrate to create this force, but still exert localize contraction. These findings may suggest that leader bleb migration is likely a superposition of migration modes, and that the geometry of the confinement is a critical factor in determining a cell's ability to migrate. By better understanding the mechanisms of this adaptive migration mode, we hope to identify new ways to targeting amoeboid cancer cell migration.

B271/P0264

Ras-ERK signaling regulates collagen cleavage to govern the mode of normal human fibroblast 3D migration

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Cells sense and respond to the physical structure of the extracellular matrix (ECM) to migrate efficiently through 3D environments. When migratory fibroblast cells encounter covalently crosslinked 3D matrices, such as in cell-derived matrix (CDM), they activate actomyosin contractility in the anterior of the cell to pull the nucleus forward like a piston in a cylinder. This causes cytoplasmic pressure to increase in front of the nucleus while leading protrusions switch from actin-based lamellipodia to pressure-driven lobopodia. While it is unclear how cells sense covalently crosslinked matrices and activate the nuclear piston, matrix metalloproteinase (MMP) activity, including MMP14, inhibits piston activation and the formation of lobopodial protrusions. To determine how the physical environment and reduced MMP activity can lead to activation of the nuclear piston, we compared the phosphoproteomes of fibrosarcoma cells migrating in 3D collagen with the piston on versus off. Phospho-proteomic analysis revealed 131 phospho-proteins which either increased or decreased their phosphorylation in cells using the nuclear piston compared to piston-off cells. Interestingly, eight of these phosphoproteins were associated with Ras/ERK signaling, suggesting a role for this pathway in regulating piston activation in response to the physical environment. To test this hypothesis, we overexpressed Ras and constitutively active Ras in cells migrating through 3D matrices. We found that hyperactivating Ras inactivated the piston in cells moving through 3D CDM, while inhibiting either Ras or Erk activity activated the piston in cells migrating in 3D collagen. Importantly, we found that using an MMP inhibitor to activate the piston significantly reduced phospho-Erk, consistent with Erk regulating piston activation downstream of MMP activity. Surprisingly, ERK inhibited cells had a significantly lower amount of cleaved collagen, suggesting Erk can also act upstream of MMP activity, potentially by regulating MMP secretion. To determine if the ECM affects the secretory pathway, we compared MMP14 surface expression between cells migrating on glass or through a 3D matrix. We found that MMP14 expression is significantly decreased for cells migrating through CDM and this corresponded to a reduction in overall Golgi volume. Together, these results suggest that mechanical signals from crosslinked ECM govern Ras-ERK and MMP activity to regulate nuclear piston activity during 3D cell migration.

B243/P0236
Reelin Controls the Directional Orientation, Apical Localization and Length of Primary Cilia in Principal Neurons in the Cerebral Cortex
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The primary cilia of pyramidal neurons in inside-out laminated regions are recently found to orient predominantly toward the pial surface, reflecting a reverse movement during postnatal development. However, the mechanisms underlying the directional cilia orientation and reverse movement are unknown. Here we show that the primary cilia of pyramidal neurons are localized near the base of the apical dendrites and aligned on the nuclear side opposite to the axon initial segment. However, this pattern is not observed in atypical pyramidal neurons in the deep neocortex, excitatory neurons in the amygdala and other non-laminated brain regions, interneurons, or astrocytes, where their cilia are irregularly positioned around the nuclei and lack preferred orientation. In Reelin-deficient mice (reeler), the directional orientation and apical localization of cilia in late-born neocortical and CA1 neurons are partially impaired, because the initial impairments can be partially corrected during postnatal development, along with a realignment of apical-basal orientation. In contrast, loss of Reelin completely disrupts the directional orientation of primary cilia in early-born neocortical neurons and principal neurons in evolutionarily older cortical regions; consistently, their cilia and centrioles do not dominantly localize to the base of the apical dendrites. Additionally, Reelin deficiency increases the cilia length of principal neurons in the cerebral cortex after P14 when WT cilia stabilize, but not in interneurons, astrocytes, or excitatory neurons in non-laminated regions. Together, Reelin controls the directional orientation, intracellular localization, and length of primary cilia in principal neurons in the cerebral cortex. These results underscore primary cilia as a key apical component particularly prominent in late-born neurons.

B251/P0244
Regulation of Rap1 GTPase Signaling During Collective Epithelial Migration
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Collective cell migration is critical for embryonic development and tissue repair. However, cells can also use the same migration machinery for spreading cancer, resulting in metastasis. To understand the molecular mechanisms that drive collective cell migration, we investigate wound healing in epithelial tissue using the Drosophila embryonic epidermis. Wounds are generated by ablating 4-5 epidermal cells in stage 14 embryos using a high powered laser. The lesions heal rapidly without scarring or inflammation, forming a supracellular actomyosin cable connected by reinforced adherens junctions at the leading edge of the migrating cells. Our previous work has demonstrated a role for the small GTPase Rap1 in coordinating the cell adhesion and cytoskeletal rearrangements required for rapid wound healing. Rap1 GTPase is misregulated in many metastatic cancers, and the Rap1 signaling pathway plays a role in epithelial integrity. Thus, understanding the factors that promote Rap1 activity is critical for important questions in human health and disease. Rap1 is known to be regulated by specific GEFs - PDZ-GEF, Epac, and C3G - and a GAP - Rapgap1. In unwounded epidermis, endogenously tagged PDZ-GEF:GFP localizes to cell-cell boundaries. RNAi-mediated knockdown of PDZ-GEF, verified via Western blotting and loss of PDZ-GEF:GFP fluorescence, had no effects on the rate of embryonic wound closure or cytoskeletal polarization. PDZ-GEF shows no localization to the wound edge.

Together this suggests that other Rap1 GEFs and GAPs are playing a more important role in controlling Rap1 activity to drive migration. Future work will examine localization and RNAi-mediated knockdown of Epac, C3G, and Rapgap1 to better understand the regulation of Rap1 during collective cell migration.

B259/P0252

Role of Rap1 GTPase in coordinating migration of human epidermoid carcinoma cells

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Collective cell migration—the coordinated movement of cell groups—is a critical biological process fundamental to development, tissue regeneration, and disease progression, yet remains incompletely understood. This process relies on the cytoskeleton to generate mechanical forces and on cell adhesions to facilitate interactions with neighboring cells and the extracellular matrix. The small GTPase Rap1 functions as a molecular switch and is known to regulate cytoskeletal dynamics and adhesion remodeling, with dysregulation implicated in metastatic cancer. Our previous studies have established a pivotal role for Rap1 GTPase in initiating and coordinating cell migration during wound healing in Drosophila embryos. We hypothesize that Rap1 signaling is a conserved key driver of collective cell migration in human epithelial tissues. To test this hypothesis, we utilized A431 epidermoid carcinoma cells stably expressing fluorescently tagged E-cadherin. Cells were cultured as monolayers on Matrigel-coated glass-bottom dishes and treated with either DMSO (control) or the Rap1 inhibitor GGTI-298. Wounds were induced using a high-powered laser mounted on a confocal microscope, and after a 2-hour healing period, cells were fixed and stained for Rap1A or Rap1B and F-actin. Quantitative imaging and analysis were performed on migrating cells and cells in an unwounded monolayer. Rap1B was strongly localized to E-cadherin-mediated cell-cell adhesions in non-migrating cells. In migrating cells, Rap1B re-localized to lamellipodia protrusions at the leading edge. In general, GGTI298 treatment disrupted Rap1A intensity and Rap1B localization in non-migrating and migrating cells. Although A431 cells demonstrated wound closure over the 2-hour interval, wounds in the control group exhibited more extensive healing than those treated with GGTI-298. Furthermore, inhibitor-treated wounds had a rougher wound edge with some cells leaving the collective and migrating independently. E-cadherin localization was also reduced at cell-cell adhesions close to the wound. These results suggest that Rap1 activity is essential for coordinating cellular behavior and maintaining cohesion during migration. Future directions include comparative studies of Rap1A and Rap1B localization and effects of Rap1 disruption on migration rate, localization of additional cytoskeletal and adhesion markers, and modulation of cytoskeletal contractility. These studies will determine if Rap1A and Rap1B have independent functions during collective cell migration and establish their roles in cytoskeletal and adhesion remodeling.

B272/P0265

Rotational migration in human pancreatic ductal organoids depends on actin and myosin activity

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Rotational migration is one specific form of collective cell migration when epithelial cells are confined in a spherical geometry, such as in the epithelial acini. This tissue-level rotation motion is crucial for the morphogenesis of multiple epithelial systems. Here, we introduce a new primary human model for the study of rotational migration, pancreatic ductal organoids. Live imaging revealed the persistent rotation of the

organoids over time. By tracking the nuclei, the three-dimensional trajectory of the cellular movement was reconstructed, and the velocity of the rotation was quantified. The presence of focal adhesion clusters and prominent actin stress fibers were observed at the basal side of the organoids, suggesting the interactions between the cells and the surrounding extracellular matrix. Finally, our inhibition study showed the dependence of pancreatic ductal organoid rotational migration on myosin activity, actin polymerization, and actin branching. We believe this model will enable future studies with human primary cells, which are more faithful to normal epithelial cells.

B256/P0249

Talin as a novel regulator of gene expression

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Talin is a focal adhesion (FA) adaptor protein traditionally known as a regulator of integrin-mediated adhesion. Integrins are a heterodimeric class of transmembrane receptors mainly responsible for linking the extracellular matrix (ECM) to large cytoplasmic complexes intracellularly, with Talin at the core. These complexes ultimately connect to the actin cytoskeleton, regulating many responses to the extracellular environment including, cancer cell migration and metastasis. Recent studies show that a subpopulation of Talin localizes to the nucleus, where it may regulate gene expression. We hypothesize that this provides a novel layer of regulation by Talin and an unexplored mechanism for it to contribute to cancer progression. We propose that both intracellular cues, such as integrin activation, and extracellular stimuli, including matrix stiffness and chemotactic signals, influence Talin localization to nuclear vs. cytoplasmic compartments. To explore these dynamics, we investigate how altering the microenvironment impacts Talin localization in three contexts – 1) fibroblasts cultured on polyacrylamide hydrogels of varying stiffness, 2) Transforming Growth Factor β (TGF-β) induced epithelial-to-mesenchymal transition (EMT) in breast cancer cells, and 3) outside-in induced integrin activation with manganese chloride treatments in fibroblasts. In addition to quantifying co-localization via immunofluorescence, we are confirming results with western blotting of cell fractions and exploring the transcriptional regulation of Talin-dependent genes via RNA-seq. Together, this work highlights Talin's emerging nuclear role and aims to uncover how various contexts may regulate Talin localization, ultimately shaping cancer cell behavior and gene regulation.

B252/P0245

The Influence of Cell Cycle Progression on Migration Morphology and Persistence

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Cell motility is a fundamental property that underlies numerous physiological and pathological processes in vivo, including tissue development, immune responses, and cancer metastasis. However, most studies of cell migration have analyzed heterogeneous populations, without accounting for potential variability across different phases of the cell cycle. As a result, the extent to which cell motility is modulated by cell-cycle progression remains insufficiently explored. In this study, we investigate the relationship between cell-cycle phase and cell motility using MDA-MB-231 human breast cancer cells as a model system. To distinguish cell cycle phases, we employed the FUCCI system, which differentiates G1-phase cells from those in S/G2/M based

on phase-specific degradation of fluorescently tagged cell cycle regulators. Images were acquired at three-minute intervals during live-cell imaging. Cell motility was quantified using two primary parameters: path length (total distance traveled) and persistence (net displacement divided by total path length). Path length and persistence were calculated within 1-hour time-lapse windows to reduce trajectory overlap and temporal bias. Quantitative analysis revealed that persistence was significantly higher in cells in the G1 phase compared to those in the S/G2/M phases, indicating more sustained and directed migration during G1. In contrast, no significant differences in path length were observed between cell-cycle phases, suggesting that the overall migratory capacity is not dependent on cell-cycle stage. Cell morphology was assessed by computing the aspect ratio, defined as the ratio of cell length to width, with the length axis aligned to the direction of migration at each time point. Cells in the G1 phase exhibited higher aspect ratios than those in the S/G2/M phases, indicating a more elongated morphology during the G1 phase. Collectively, these findings demonstrate that cell-cycle phase influences both the directionality of migration and cellular morphology, likely through dynamic regulation of actin cytoskeleton architecture. Ongoing investigations will elucidate the molecular mechanisms by which cell-cycle progression governs cytoskeletal remodeling and migration behavior.

B277/P0270

Tissue geometry and mechanochemical feedback initiate rotational collective migration in Drosophila

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Collective migration of epithelial cells drives diverse tissue remodeling processes. In many cases, external asymmetries such as a free tissue edge can polarize epithelia for directed migration. In contrast, edge-free epithelia, such as cultured epithelial spheres, and those grown on circular micropatterns or other curved substrates, spontaneously break symmetry and undergo persistent rotational migration. The ability to self-organize for rotational migration appears to be a fundamental property of epithelia that adopt distinct geometries, but the mechanisms that initiate and orient epithelial rotation are unknown. To determine how symmetry is broken in these tissues, we study the rotational collective migration of follicular epithelial cells in the Drosophila egg chamber, an ovarian follicle. Follicle cells form a topologically-closed epithelium that surrounds a central cluster of germ cells. During early development, follicle cells migrate collectively along a basement membrane (BM) that encapsulates the egg chamber. This drives rotation of the entire egg chamber around its anterior-posterior (AP) axis within the BM that remains stationary. The onset of rotation depends on the atypical cadherin Fat2 which functions to align lamellipodial protrusions made by each cell across the tissue. How Fat2 mediates the initial symmetry breaking event that drives rotation onset is unclear. Additionally, the mechanisms that orient rotation around the AP axis remain unknown. By employing new methods for long-term live imaging and for delaying the onset of rotation, we reveal the dynamic behaviors of the follicle cells as rotation initiates and show that symmetry breaking is a self-organized process. Combining experiments and theoretical modeling, we identify a positive feedback loop in which Fat2 stabilizes and aligns individual cell protrusions, and the resulting tissue motion polarizes Fat2 to cells' trailing edges. The coupling between the mechanosensitive behavior of Fat2 and rigid-body dynamics of the egg chamber breaks chiral symmetry leading to sustained tissue rotations. We also identify mechanical constraints arising from cell-cell interactions and tissue geometry that may ensure rotation is oriented around the AP axis across developmental time. Our findings reveal a biophysical mechanism — combining Fat2-mediated velocity-polarity alignment, rigid-body dynamics, and tissue geometry — by which a closed epithelial tissue self-organizes into persistent, large-scale rotational migration in vivo, expanding current flocking theories.

B239/P0232

TRIM9 is novel regulator of adhesion and migration in melanoma

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Coordinated cell adhesion and cytoskeletal dynamics sculpt cell morphology and dictate cell motility across development and disease. Identification of cell type specific regulators of cell adhesion, cytoskeletal dynamics, and motility are critical for addressing specific disease states, such as melanoma metastasis. Our lab previously identified TRIM9, a brain enriched E3 ubiquitin ligase, as a potent regulator of growth cone dynamics, axon guidance, and morphogenesis in developing neurons. In neurons, TRIM9 negatively regulates actin dynamics via non-degradative ubiquitination of the actin polymerase VASP. TRIM9 expression has been noted in a subset of non-neuronal motile cells, including many cancer types (lung, melanoma, breast), In melanoma, TRIM9 was identified as a potential prognostic biomarker. However, the non-neuronal roles of TRIM9 are unknown. We data mined expression studies and found that TRIM9 is upregulated in a neural crest like subtype of melanoma, which exhibits invasive behavior. Here we investigate how TRIM9 deletion alters cellular behavior in two human melanoma cell lines (1205Lu and WMM266-4), both with high TRIM9 expression. We show that TRIM9 colocalizes with VASP at filopodia and lamellipodia tips, as well as at focal adhesions. Live cell imaging indicated TRIM9 visits focal adhesions. Loss of TRIM9 reduced filopodia number and length, and lamellipodia width. Loss of TRIM9 increased VASP localization to focal adhesions and increased focal adhesion number and area in the cell, suggesting that TRIM9 is a negative regulator of VASP at focal adhesions. Fluorescence recovery after photobleaching revealed that loss of TRIM9 increased VASP and zyxin turnover at focal adhesions. Preliminary data suggest that TRIM9 mono-ubiquitylates VASP in melanoma, as we previously found in neurons. Supporting that TRIM9 regulates VASP via ubiquitylation, a nonubiquitylatable VASP (VASPKR) exhibited rapid turnover at adhesions, even in the presence of TRIM9. Consistent with a phenotype of increased adhesion, we find that loss of TRIM9 resulted in increased cell contractility, as measured by collagen gel contraction assays, acute cell spreading after plating, and reduced protumoral cell blebbing on soft substrates. On several substrates (glass, PDL, Laminin, Fibronectin, and soft hydrogels) loss of TRIM9 increased the rate of cell migration, but reduced directional persistence. Together these data indicate that TRIM9 is a novel regulator of cell adhesion and cell motility present in some non-neuronal cancer types.

B268/P0261

Vimentin promotes collective cell migration through collagen networks via increased matrix remodeling and spheroid fluidity

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The intermediate filament protein vimentin, expressed in mesenchymal cells and different cancer cells, is functionally associated with cell migration through fibrous tissues. Vimentin increases cell polarization needed for migration, yet also acts as a cytoskeletal cage that hinders cells squeezing through small spaces. To determine how vimentin facilitates cell migration through the extracellular matrix and around neighboring cells in tissues, we examine the collective invasion of cell spheroids embedded in collagen networks. Unlike single-cell migration in collagen networks, the collective invasion of cells through the collagen network is

increased by vimentin for both mouse embryonic fibroblasts (MEF) and cocultures of MEF with MDA-MB-231 breast cancer cells. Using multiple experimental systems, we show that vimentin increases spheroid contractility and that vimentin-mediated collective cell expansion depends on matrix metalloproteinases (MMP), which degrade collagen networks. In addition, through advanced imaging and a computational 3D cell vertex model, we find that vimentin enhances the polarization of cells in spheroids embedded in collagen, indicating increased spheroid fluidity and active collagen contraction. Altogether, these results reveal new insights on vimentin's effects in enhancing migratory persistence in 3D matrix environments through MMP transportation and tissue fluidity.

B242/P0235
Wound-induced endogenous electric fields guide neutrophils to sites of injury in larval zebrafish epidermis
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Skin wound-healing requires precise spatio-temporal coordination among multiple cell types, including epidermal epithelial cells and immune cells such as neutrophils. Intact skin maintains a trans-epithelial potential (TEP) due to asymmetric ion transport. Wounding the epithelium disrupts the TEP and induces a lateral electric field (EF) with the cathodal pole at the site of injury. Isolated neutrophils and isolated basal epidermal cells have been shown to migrate toward the cathode of an applied electric field in tissue culture, and we hypothesize that the wound-induced EF may contribute to the directional migration of both cell types in wounded skin. To distinguish between directional migration guided by the wound-induced EF versus other wound-induced signals, including osmotic shock, we compared the speed and directionality of migration for these two cell types in the first 30 minutes following epidermal laceration in zebrafish (Danio rerio) larvae at 11-14 days post-fertilization under varying media conditions. In comparison to the rapid, dramatic, wound-directed migration of epidermal cells in normal hypotonic embryo media (E3), we find that wounding in isotonic media containing NaCl (eliminating both the osmotic shock and the EF) fails to induce any wound-directed epidermal cell migration at all, while wounding in isotonic media containing sorbitol (eliminating osmotic shock while preserving the EF) induces migration with normal directionality but reduced cell speed. In contrast, neutrophils migrate with increased speed (as compared to E3) after wounding in both isotonic NaCl and isotonic sorbitol. Most strikingly, rapidly migrating neutrophils in animals wounded in isotonic NaCl move in completely random directions, with no preference for migration toward the wound, while neutrophils in animals wounded in isotonic sorbitol have normal wound-directed movement. These results strongly suggest that both basal epidermal cells and neutrophils use the endogenous wound-induced EF as a directional signal, dominating their choice of migration direction in the critical minutes immediately following epidermal injury.

Cell Adhesion, Migration, and the Extracellular Environment - Cell-cell Adhesion

B196/P0191
Chromosomal instability induced by Mps1 inhibition as a driver of basal cell extrusion in the intestinal epithelia
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The intestinal epithelium is a highly dynamic structure that self-renews about every five days. Self-renewal is driven by the constant extrusion of apoptotic enterocytes from villar tips and generation of new enterocytes by

crypt stem cells. These extrusion events are uniformly apical and require the engagement of the signaling molecule S1P with its receptor S1PR2, as this controls the localization and activity of an actomyosin ring at the cell base that pushes the cell into the gut lumen. Importantly, enterocytes harboring oncogenic mutations that can lead to aneuploidy (e.g. truncated APC and KrasV12) are often extruded basally i.e. into the underlying tissue. Basal extrusion, which likely involves a failure of the actomyosin ring to migrate to the cell base, can have severe consequences if the aneuploid cell harbors oncogenic mutations, as it increases the chances that the cell will survive and migrate, leading to metastasis. Our long-term goal is to determine precisely how the actomyosin machinery is harnessed differentially to drive the apical extrusion of apoptotic cells and the basal extrusion of aneuploid cells. Towards that end we are using intestinal organoids, which retain the crypt-villus topology of the intestine in the form of a 3D, stem cell-driven tissue model amenable to structure: function analyses. In an effort to model oncogenic mutations that induce aneuploidy/basal extrusion, we treated organoids with an inhibitor of the spindle assembly checkpoint (SAC) kinase MPS1, as this inhibitor induces aneuploidy by allowing mitotic progression before kinetochore attachment is complete. As anticipated, organoids treated with 3 μM inhibitor start extruding cells basally into the Matrigel within ~12 hours. Interestingly, SIPR2 appears to be down regulated in these organoids, suggesting that chromosomal instability may promote basal extrusion by downregulating S1PR2, as this leads to actomyosin ring mis-localization. In addition, cells that extrude basally appear to generate less contractile force prior to extrusion, and unlike in apical extrusion, maintain adherence to the basement membrane prior to extrusion. In summary, our work shows a potential link between chromosomal instability, basal cell extrusion, and mechanisms of colon cancer metastasis.

B193/P0188

Immune infiltration into cell-packed tissues governed by divisions-induced connectivity disruption

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Immune cells dissemination and function relies on their ability to invade between juxtaposed tissues and to migrate within densely packed environments without preexisting paths. How do cells find the convenient spot to open up the path is unknown. Studying such invasive migration of macrophages in the Drosophila embryo we have found that the first macrophage penetrates the tissue barrier only when there is a cell that divides at the site of invasion and disassembles focal adhesions that attach a cell to its neighbors*. To investigate if this mechanism plays a role in cellular infiltration in other systems, we turned to co-cultures of vertebrate macrophages and organoids. We observe macrophages actively invading into normal murine pancreatic ductal organoids and other types of normal and cancerous epithelial organoids (lacrimal gland, airway, salivary gland). Close imaging shows that as in Drosophila an entering macrophage is always flanked by a dividing organoid cell, and that macrophages invade more vigorously in faster dividing organoids. Pharmacological inhibition of the cell cycle reduces the number of macrophages infiltrated into organoids. We observe division-specific reorganization of cell-cell junctions preceding division, alongside opening of extra intercellular space that correlates with the movement of the macrophage cell body. Our study demonstrates that cell division at a tissue edge is a key regulator of cell invasion into a confined tissue environment in many different systems with distinct architectures and can affect immune cell infiltration in normal and disease contexts. *Akhmanova, M. et al., Cell division in tissues enables macrophage infiltration. Science 376, 394–396. (2022).

B190/P0185
Investigating the mechanoaccumulation and function of the LIM domain proteins zyxin and LPP at epithelial tricellular junctions under mechanical challenge
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Epithelial cells form cohesive sheets that generate barrier function to restrict the entry of harmful substances and selectively regulate permeability. Epithelia must sense and respond to mechanical challenges in order to maintain their function. One way cells can respond to increased mechanical stress is by recruitment of LIM domain-containing proteins to strained actin filaments. Here, we investigate how two members of the LIM domain protein family, zyxin and LPP, help maintain epithelial integrity and barrier function at tricellular junctions (TCJs), sites that experience heightened mechanical strain compared to bicellular junctions. We find that zyxin and LPP are enriched at TCJs in the Xenopus embryonic epithelium under baseline tension and further mechanoaccumulate at TCJs when tension increases. To determine which domains contribute to the mechanoaccumulation of zyxin and LPP, LIM domain chimeras were generated. Additionally, conserved aromatic residues associated with LIM domain mechanosensitivity were mutated. We find that the chimeras and proteins with the conserved aromatic mutations mechanoaccumulate at TCJs to a lesser extent than the wildtype proteins. While our results demonstrate that zyxin and LPP's isolated LIM domains can mechanoaccumulate on their own, results from the chimeras and conserved aromatic mutations suggest that both the LIM domains and N-termini of the proteins contribute to the proteins' mechanoaccumulation. Since high levels of zyxin are associated with kidney disease, we are currently overexpressing zyxin and LPP to test their roles in regulating junctional integrity and barrier function in mammalian kidney epithelial cells (MDCK II). Together, these studies will advance our understanding of the dynamic interplay between LIM domain proteins and the actin cytoskeleton in maintaining junction stability under mechanical challenge.

B192/P0187
Measuring Intercellular Adhesion Strength Using a Custom-Built Atomic Force Microscopy System
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Intercellular adhesions play a crucial role in cellular assembly and maintaining tissue strength. This is particularly important for tissues such as skin, where keratinocytes in the epidermis interface together through specialized adhesion molecules to create strong and durable tissues. However, quantifying the adhesion energy between cells in both native and synthetic cell-cell adhesions remains technically challenging. To address this, we have been developing an experimental protocol for measuring intercellular adhesion energy using Atomic Force Microscopy (AFM) using a custom-built AFM system. By coating AFM cantilevers with either antibodies or ligands for intercellular adhesion molecules and holding the cantilever in contact at constant force, we can measure how adhesion strength changes during different mechanical loading conditions. This technique can be used to measure both the role of interaction force, contact time, and detachment rate for both native cell adhesion molecules and recently developed synthetic cell adhesion molecules (synCAMs).

B187/P0181

Mechanistic analysis of epithelial barrier dysfunction mitigation by zonulin inhibitor against inflammatory cytokines

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The intestinal epithelial barrier plays a critical role in maintaining gut homeostasis by regulating selective permeability and preventing the intrusion of harmful pathogens, toxins, and antigens. Dysfunction of this barrier, commonly referred to as increased intestinal permeability or "leaky gut", has been strongly linked to the development and exacerbation of inflammatory diseases, including inflammatory bowel disease (IBD), and other systemic inflammatory conditions. In inflammatory conditions, inflammatory cytokines and immune mediators disrupt epithelial tight junction integrity, allowing uncontrolled penetration of antigens and pathogens into subepithelial tissues. Zonulin is a physiological modulator of intestinal barrier function, controlling tight junction permeability between intestinal epithelial cells. Elevated zonulin levels disrupt barrier integrity, increasing intestinal permeability and causing the situation called "leaky gut". Larazotide acetate (AT-1001) is a synthetic peptide that antagonizes zonulin signaling by stabilizing tight junctions and reducing pathological increases in intestinal permeability. Therefore, targeting the zonulin pathway with larazotide acetate is a promising therapeutic approach for impaired intestinal barrier function. However, there are still many unknowns in zonulin signaling. In this study, our group analyzed the effects of larazotide acetate against inflammatory cytokines by using human colorectal adenocarcinoma cell line Caco-2 cells and human colon cancer cell line HT29 cells as human intestinal epithelial cell models. Co-culture model of Caco-2 and HT29 cells was also utilized for epithelial barrier function assay. Treatment with inflammatory cytokines including TNFα and HMGB1 decreased epithelial barrier function of cell culture model, and treatment with AT-1001 mitigated this decrease. Our results indicate that larazotide acetate might be a new candidate for treating inflammatory diseases including IBD in terms of epithelial barrier function.

B197/P0192

Membrane dynamics driven by the Arp2/3 complex regulate myofibers growth and myonuclear accretion during skeletal muscle postnatal development

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Skeletal muscle is a highly organized tissue, composed of large multinucleated cells called myofibers. During postnatal development, muscle growth is mainly driven by the addition of new myonuclei to the myofibers (myonuclear accretion) through the fusion of mononucleated progenitor cells (myoblasts). Cell-cell fusion in skeletal muscle has been extensively studied in myoblasts, but the mechanisms involved in myofibers fusion are largely unknown. The Arp2/3 complex is a seven-subunit complex that polymerizes branched actin networks, having a wide range of cellular functions. In skeletal muscle, the Arp2/3 complex was shown to be involved in the fusion of myoblasts during embryonic development, in the migration of myonuclei to the periphery of the myofibers, and in the transverse organization of T-tubules in triads. Our study aims elucidate how Arp2/3-mediated actin dynamics contribute to myofibers postnatal development in vivo. Using an inducible skeletal muscle-specific knockout mice model, we depleted Arpc4 (a core subunit of the Arp2/3 complex) in myofibers during the first postnatal week (Arpc4-/-). When compared to littermate controls,

Arpc4-/- mice presented smaller myofibers with less myonuclei. We found that myoblast fusion into Arpc4-depleted myofibers is severely inhibited both in vitro and in vivo, leading to an accumulation of proliferating myoblasts and a reduction in myonuclear accretion in Arpc4-/- mice. This impairment in myofibers postnatal growth directly affected muscle function in vivo, since P7 Arpc4-/- neonates presented gait abnormalities (asymmetric movement and increased foot angles) and signs of muscle weakness when performing the hindlimb suspension test. Moreover, we developed a co-culture system to study myoblast-myofiber interaction and fusion in vitro. Our results demonstrated that the Arp2/3 complex accumulates at the plasma membrane of differentiated myofibers, specifically in regions of contact with myoblasts. Additionally, myofibers generate membrane extensions enriched in the Arp2/3 complex when interacting with neighboring myoblasts before fusion. The formation of these membrane extensions depends on the activity of the Arp2/3 complex, as they were significantly reduced following Arpc4 depletion in myofibers. Overall, our findings unveil a new fundamental role of the Arp2/3 complex in mediating myofibers growth during skeletal muscle postnatal development.

B191/P0186

Neuroblast cell dynamics in C. elegans: regulation by the sole Toll homolog tol-1

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Chordate neurulation involves internal reorganization of neuronal precursors and epiboly of overlying epidermis. C. elegans embryos undergo similar morphogenetic movements at the end of gastrulation: (1) ventral cleft closure (CC) and (2) ventral epidermal enclosure (VE). CC involves formation of two rosettes with apical enrichment of HMP-1/α-catenin, as ventral neuroblasts (VNs) migrate to the midline and seal the open surface left behind by internalizing cells. During VE, epidermal cells migrate over VNs to meet at the midline. Actomyosin foci in epidermal cells and VNs facilitate VE. We sought to identify novel, conserved molecular components involved in these processes, and identified a single Toll homolog (tol-1) which had previously only been implicated in innate immunity. At 15°C, tol-1(nr2013) deletion homozygotes exhibit <10% survival, with lethality at multiple developmental stages due to morphogenetic defects. Zygotic tol-1(nr2013) mutants are defective in VN migration, rosette formation and adhesion during CC and/or VE. tol-1(nr2013) mutants display abnormal clustering of HMP-1 to vertices of VN cells in rosettes and non-muscle myosin II (NMY-2) is more diffuse in tol-1(nr2013) mutants. Endogenously tagged TOL-1 localizes to the surface of VNs at the end of CC and through VE. Our data suggest TOL-1 acts at the cell surface given (1) putative null alleles of C. elegans homologs of the Toll signaling pathway lack developmental defects; (2) there is no homolog of NF-κB in the C. elegans genome; (3) deletion of the toll-interleukin 1 receptor (TIR) domain of TOL-1, the intracellular signaling domain, is largely dispensable for embryonic development. LAT-1, homolog of vertebrate latrophilin and an adhesion GPCR, was recently shown to directly interact in vitro with TOL-1. lat-1 and tol-1 binding interaction mutants show similar CC and VE defects, and LAT-1 and TOL-1 are expressed in overlapping groups of midline VNs. We are currently investigating the mode of interaction between LAT-1 and TOL-1 during CC and/or VE, and tissue-specific requirements for TOL-1 through tissue-specific degron-mediated knockdown. We propose TOL-1 promotes rearrangement of VNs into rosettes during CC, via accumulation of NMY-2 to apical cortices. Our work identifies new roles for a Toll homolog during neuroblast migration, a fundamental morphogenetic process in metazoans.

B188/P0182
Tetraspanin 31 as a Potential Neuronal Adhesion Receptor for the beta2 Subunit of the Na^+, K^+-ATPase
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The β2 subunit of the Na^+, K^+-ATPase (β2/AMOG) is an adhesion molecule involved in neuron–astrocyte interaction. In transfected CHO cells, a homotypic interaction of the protein between different cells was demonstrated for its adhesive function. However, studies in adult rats and humans reveal the absence of β2/AMOG in most mature neurons, suggesting that it may interact with another molecule in neuron–astrocyte communication. Therefore, the neuronal receptor for β2/AMOG is under investigation. Tetraspanin 31 (TSPAN31) has been proposed as a potential candidate, as it fulfills key criteria, including plasma membrane localization. Furthermore, interactome studies have reported a possible interaction with β2/AMOG in transfected HEK cells. To study the possible interaction, molecular docking was performed between the ectodomain of β2/AMOG and the TSPAN31 dimer, followed by molecular dynamics (MD) simulations to evaluate complex stability. In the experimental assays, a pull-down technique was carried out using CHO cells transfected with TSPAN31 fused to turbo green fluorescent protein (tGFP), and MDCK cells transfected with β2/AMOG fused to a 6-Histidine tag (6His). The results were analyzed using Western blot (WB). In the in silico assays, we obtained a stable model of the TSPAN31–β2/AMOG interaction. In the experimental assays, the interaction between TSPAN31 and β2/AMOG was observed via WB.

B189/P0184
The Effects of High Keratin 19 Expression and Related Type I Keratin on Cell Adhesion and Migration
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Keratin 19 (K19) is a type I intermediate filament protein that plays a vital role in maintaining cell adhesion, cellular architecture, mechanical strength, and cell proliferation. K19 forms a mature 10 nm filament through heterodimerization with type II keratin 8 (K8). Keratins bind to desmosomes and hemidesmosomes, supporting proper cellular structure. K19 is highly expressed in various cancers, including breast cancer. Despite its importance in maintaining tissue resilience and integrity, the effects of high K19 expression, along with other closely related type I keratins such as K17, K16, and K14, on cell adhesion and migration are not well understood. Using MCF7 breast cancer cells as a model, we hypothesize that overexpression of K19 and its closely related type I keratins will enhance cell-to-cell adhesion and inhibit cell migration. Our adhesion, migration, and biochemical assays show that overexpressing K19 in both MCF7 parental (P) and K19 knockout (KO) cells improves cell-to-cell adhesion and shortens wound healing time. When K17, K16, and K14 are overexpressed in MCF7 P and KO cells, we observe a similar positive effect on cell adhesion and a decrease in wound healing time, like K19 overexpression. We also demonstrate that K17, K16, and K14 can interact with K8 and the desmosomal protein desmoplakin, as shown by co-immunoprecipitation. Additionally, K19 directly interacts with desmoplakin and plakophilin, causing a conformational change in the desmoplakin N-terminus. Our results indicate that K16 interacts with desmoplakin like K19, and to a lesser extent, K17 and K14. These findings reveal a new role for K19 in cell adhesion and highlight a complex interplay among type I keratins, which breast cancer may exploit to strengthen interactions between cancer cells and potentially facilitate collective cell migration. In conclusion, K19 plays a role in cancer, and it is a promising biomarker for breast cancer patients.

B194/P0189
The Role of Rap1 in Primary Pigment Cell Development in the Drosophila melanogaster eye
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Morphogenesis requires dramatic cell shape changes and cell movements, which are powered by the contractile force generated by actin and myosin linked to the plasma membrane at cell-cell and cell-matrix junctions, as well as through cell adhesion molecule mechanisms. Although many of the physical mechanisms have been determined, it is less clear how factors that promote cell fate ultimately lead to changes in cell shape. The Drosophila eye contains ~750 ommatidia in an exquisitely precise hexagonal arrangement that enables tight packing and acute vision. One morphogenetic event that occurs during pupal development involves a group of four cone cells that signal via the Notch pathway to adjacent cells on either side, forming primary pigment cells. The primary pigment cells then enwrap the cone cells, separating them from interommatidial cells. It is likely that actomyosin contractility and/or heterophilic cell adhesion are involved in the enwrapment process; however, the mechanisms underlying this process remain unclear. Rap1, a Ras-family small GTPase, regulates diverse functions that are highly context-dependent in various cell types, including proliferation, differentiation, cell survival, cell adhesion, polarity, and movement. To determine whether Rap1 has a role in primary pigment cell enwrapment, we employed the UAS-GAL4 system to drive the expression of dominant-negative Rap1 (Rap1N17) in primary pigment cells. What we found in our fixed samples were extra primary pigment cells in some ommatidia. Additionally, our time-lapse observations revealed that these extra cells originated from interommatidial cells. Time-lapse imaging shows that the primary pigment cells initially begin to enwrap the cone cells. However, later during morphogenesis, interommatidial cells will often invade the primary pigment cells to replace them. This resembles defects observed in Notch mutants, suggesting that Rap1 and Notch are both involved in this process. By combining live-cell imaging and genetic screening, we aim to elucidate the relationship between Notch and Rap1 during the specification of primary pigment cells through cell-cell interactions during ommatidia development.

B195/P0190
Vascular tortuosity causes curvature-dependent endothelial cytoskeletal and cell-cell adhesion disorganization and local loss of barrier function.
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Tortuous blood vessels are a hallmark of many vascular diseases and tumor progression. A common vascular defect associated with tortuous vessels, regardless of disease origin, is vascular permeability. Our previous studies showed that the endothelial cytoskeleton is sensitive to cell-scale curvature imposed on it by the local extracellular matrix or physical environment. Since the cytoskeleton is critical to barrier function, we hypothesized that vessel shape may contribute to altered permeability through mechano-regulation of the cytoskeleton. We used sacrificial ink-mediated printing of tortuous vessels of defined curvature under physiological flow to study the effects of local vessel shape on the endothelial cytoskeleton, cell adhesion, and local barrier function. We find that endothelial cytoskeletal organization and cell polarization are reduced in cells lining the inner curvature of tortuous vessels. In these regions, activated myosin 2 and migfilin, a tension-dependent cytoskeletal protein, both exhibit decreased cytoskeletal recruitment, suggesting lower tension at inner curvatures. In addition, cell junctions in these regions lose smooth, continuous morphology and form

focal adherens junctions that recruit a vinculin biosensor that shows decreased levels of tension. A novel assay allowing micron-scale localization of permeability sites reveals that inner curvature-associated changes in cytoskeletal and junction tension and organization are coincident with locally increased vascular permeability. We propose that endothelial cell shape and flow dynamics imposed by vessel curvature cause mechanosensitive alterations in the cytoskeleton that alter cell-cell adhesions to allow loc

Cell Adhesion, Migration, and the Extracellular Environment - Cell-matrix Adhesion

B203/P0198
Cellular Responses to Surface Curvature on Hydrogels
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The objective of this study was to investigate how cells respond to hydrogel surfaces with varying curvature. Cell–environment interactions are a central focus in biology and biomedical engineering. Surface composition and stiffness have been shown to significantly affect cell morphology, adhesion and growth. Two-dimensional surface geometry also influences cell growth and organization on rigid substrates. However, cell responses to curved hydrogel surfaces remain largely unexplored. To address this knowledge gap, we systematically examined cell morphology on flat, cylindrical, and spherical hydrogel substrates. Methods: Alginate was used as the supporting matrix to develop hydrogels for cell attachment and growth. Microparticles and microfibers were fabricated by extruding alginate solution into $CaCl_2$ using a custom-built device, while bulk alginate hydrogels were prepared in disk molds to provide flat two-dimensional surfaces. After curing, all alginate hydrogels were sequentially incubated in gelatin and genipin for modifying alginate surface with gelatin. Mesenchymal stem cells (MSCs) and 3T3 fibroblasts were cultured on flat hydrogels or traditional culture flasks as controls. For the study of cell growth on curved surfaces, cells were incubated overnight with alginate microparticles or microfibers to enable attachment, followed by washing to remove unattached cells. Cell morphology was examined by confocal microscopy at multiple time points, and images were analyzed to calculate elongation, spreading area, and aspect ratio. Results: Cells on cylindrical and spherical hydrogel surfaces exhibited markedly smaller spreading areas compared to those on flat hydrogels or flasks. On cylindrical substrates, cells elongated in a manner similar to those on rigid surfaces, with a preferential alignment along the z-axis of the cylinder but not the radial axis. In contrast, cells cultured on spherical particles and flat hydrogels appeared rounder, with a lower aspect ratio. These findings suggest that curvature strongly influences cell morphology. Convex geometries can either limit or promote spreading, while the anisotropic nature of cylinders guides elongation along the z-axis. Conclusion: This study demonstrates that hydrogel surface curvature significantly impacts cell morphology. Cylindrical geometries promote axis-directed elongation. These results highlight curvature as a critical design parameter in biomimetic hydrogels. Ongoing studies will evaluate gene and protein expression to link morphological changes with functional outcomes. Our findings provide valuable insight into how hydrogel curvature modulates cell behavior, offering implications for biomaterial design and the advancement of hydrogel-based cell therapies.

B213/P0208
Control of epithelial cell adhesion and ECM organization by mRNA localization in vivo

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mRNA targeting to specific subcellular destinations is widely observed in diverse mammalian cell types. Nevertheless, the physiological function of mRNA localization has remained almost entirely uncharacterized. Here, we identify the Net1 mRNA as prominently basally localized in a variety of mammalian epithelial tissues. We show that Net1 mRNA is targeted to the dermal-epidermal junction (DEJ) in stratified squamous epithelia. Strikingly, close examination of this region revealed that Net1 mRNA accumulates at uncharacterized protrusion-like structures that interdigitate with the basement membrane and connect to a mechanosensitive network of microfibrils. Injection of antisense oligonucleotides (ASOs) that prevent the localization of Net1 mRNA altered mouse epithelial organization including DEJ morphology, keratinocyte-matrix connections, and induction of premature keratinocyte differentiation. These defective aspects of keratinocyte physiology led to thinning of the epithelium and a disrupted tissue architecture. Mechanistically, Net1 mRNA localization controls the cortical accumulation of Net1 protein and its activity as a RhoA GTPase exchange factor (GEF). Altered RhoA activity is in turn sufficient to alter the ultrastructure of the DEJ by remodeling the microfibril matrix. This work provides a high-resolution in vivo view of mRNA targeting in a physiological context. It further demonstrates that altering the subcellular localization of an individual mRNA can alter mammalian epithelial tissue organization, thus revealing an unappreciated level of post-transcriptional regulation that controls tissue physiology.

B214/P0209

Investigating the role of PKA phosphorylation of tensin-3 in cell-matrix adhesions

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Cell adhesion to the extracellular matrix (ECM) is a fundamental aspect of multicellular life. Cell-matrix adhesions (CMAs) are multi-molecular structures responsible for mediating cell-ECM contact by coupling integrins to the actin cytoskeleton. This positioning allows CMAs to serve as mechano-sensing signaling hubs, transmitting bi-directional cues between the cell and ECM that regulate many diverse cellular processes. The cAMP-dependent protein kinase (PKA) is a ubiquitous Ser/Thr kinase also known to regulate a myriad of cellular processes, including cell migration. Despite the central role of both PKA and CMAs in cell migration, PKA had not previously been known to act within CMAs. Recently, our lab showed PKA to be a functional component of CMAs, prompting the hypothesis that PKA may phosphorylate components of the “adhesome”. In vitro screens found many candidate PKA substrates in CMAs, including previously characterized substrates (e.g. VASP, ezrin) as well as several novel targets. Among the most prominent of these was tensin-3 (Tns3), a core CMA component that directly couples integrins to actin and serves as a multi-molecular scaffold. Additionally, while Tns3 is present in tension-dependent focal adhesions (FAs), it is enriched in fibrillar adhesions (FbAs), a subset of CMAs that persist upon the reduction of cellular tension and participate in ECM remodeling. Tns3 also participates in many signal transduction pathways and is highly phosphorylated. While Tyr-phosphorylation of Tns3 is well-studied, less is known about its Ser/Thr phosphorylation. Here, we show that in silico and mass spectrometry analyses of Tns3 predict 8 PKA phosphorylation sites, most of which occur in the intrinsically disordered domain (IDD), a unique region whose function is ripe for characterization. Furthermore, we show validation of 2 of the 8 PKA phosphorylation sites on Tns3 in living cells, one of which resides in the Tns3 actin binding domain and the other directly upstream of a small helical motif that facilitates the Tns3 interaction with talin. Efforts are underway to characterize the Tns3 protein-protein interactions affected by PKA phosphorylation, to assess the impacts of such interactions on signaling through CMAs, and to generate cell lines in which endogenous Tns3 is replaced with Tns3 phospho-mutants for assessing CMA morphometrics and dynamics, ECM remodeling, durotaxis and 3D matrix invasion. The discovery of Tns3 as a

substrate for PKA reveals the need to understand the axis by which Tns3 and PKA, two molecules essential for the coordination of cell adhesion and migration, interact.

B205/P0200
Investigation of CNNM3 and PRL3 in MCF7 Breast Cancer Cells
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CNNM3 is a member of the CBS domain–containing CNNM family of Mg^{2+} transport mediators that modulate cellular Mg^{2+} homeostasis and are regulated by the phosphatases of regenerating liver (PRL1–3). PRL3 is frequently overexpressed in metastatic cancers, yet the mechanisms underlying its oncogenic activity remain unclear. A prevailing model posits that overexpressed PRL3 binds CNNMs to raise intracellular Mg^{2+}, thereby promoting proliferation and metastasis. CNNM3 is likewise overexpressed in breast cancer. To define CNNM3 function, we knocked out CNNM3 in MCF-7 breast cancer cells, where CNNM3 is more highly expressed than other CNNM isoforms. Using data-independent acquisition mass spectrometry (DIA-MS) in wild-type and CNNM3-KO cells, we mapped CNNM3-associated proteins and observed enrichment for cytoskeletal proteins. Consistent with these associations, CNNM3 localized to focal adhesions, and its loss altered cytoskeletal organization in MCF-7 cells. Together, our findings suggest that CNNM3 links Mg^{2+} homeostasis to adhesion–cytoskeletal pathways in breast cancer cells and may cooperate with PRL3 to drive malignant phenotypes.

B212/P0207
Light-induced phase separation of adhesion adapters controls the de novo assembly of integrin-mediated adhesion
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Integrin-adhesion complexes (IACs) form spontaneously in cells on extracellular matrix substrates, allowing them to sense matrix composition and transduce force. However, IACs often do not form uniformly across a cell, which begs the question: What is required to nucleate an adhesion, and what factors enable the stabilization of an IAC once it has formed? Many factors have been suggested to promote formation and the subsequent stabilization of IACs. It is difficult to explicitly test these factors in vivo as IACs undergo constant remodeling. Here, we employ optogenetics to explicitly test the ability of talin in different activity and phase states to nucleate and stabilize IACs in regions where none are present. We find that fusion of intrinsically disordered regions directly to talin enhances its adhesion nucleation potential and allows new adhesions to be produced in response to optogenetic talin clustering. Similarly, expression of factors previously shown to enhance biocondensation in vitro, such as paxillin, the paxillin N-terminus, or unfolding of talin, allows for adhesion nucleation and biocondensation of talin. We show that these biocondensates of talin can cluster and activate integrins even in the absence of extracellular matrix. By applying optogenetic activation to regions of the cell with or without ventral actomyosin, we demonstrate actomyosin engagement promotes the formation and stability of adhesions. These results are corroborated by theoretical modelling which shows that phase separation of talin is enhanced by differential clutch formation in the presence of actomyosin thus enabling peripheral adhesion formation and stability. This work establishes a model in which increased cooperativity of talin enables IAC nucleation through talin biocondensation, which clusters and activates integrins. In addition to the findings we generate multiple optogenetic tools that enable local nucleation or enhancement of IACs.

B209/P0204

MAP4-mediated Crosstalk between Mitochondria and Focal Adhesions governs Cell Migration

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Mitochondria are dynamic organelles essential for diverse cellular processes, including maintaining cellular ATP balance, redox homeostasis and ionic buffering. Traditionally viewed as static organelles with exclusive metabolic functions, emerging evidence highlights their ability to respond to diverse cellular cues, such as integrin-mediated cell adhesion to the surrounding extracellular matrix (ECM), by altering their architecture and function. Integrin-driven signaling is central to cellular responses to physicochemical signals from the ECM via mechanosensing. Although aspects of the ECM like stiffness, rigidity and composition seem to drive mitochondrial phenotypes, if and how cell-ECM adhesions themselves regulate mitochondria and whether mitochondria regulate adhesion dynamics remains unknown. We tracked focal adhesions (FAs) and observed mitochondrial recruitment proximal to areas of FA assembly and retraction from regions undergoing disassembly. Spatiotemporally controlling cell geometry using micropatterning enabled us to conduct precise quantitative analyses of mitochondria and FAs in cell protrusions vs. the cell body. This setup revealed tight reciprocal regulation where perturbing FAs altered mitochondrial localization to protrusions and proximal to FA. Furthermore, we observed spatially distinct pools of mitochondria localizing to protrusions, with FA-proximal mitochondria exhibiting a higher membrane potential, even though the cytoplasmic ATP levels were lower near adhesion-rich protrusions than in the FA-distal cell body. Further investigation identified a FA resident microtubule-associated protein MAP4 to negatively regulate mitochondrial localization to FAs in a phosphorylation-dependent manner. Conversely, depletion of mitochondrial transport proteins MIRO1 and MIRO2, restricting mitochondria to the cell body, decreased FAs and impaired cellular spreading. Therefore, we propose that mitochondria near FAs have functions beyond ATP production, supported by mildly elevated intra-mitochondrial Ca+2 concentration and ROS levels. We are currently investigating the significance of spatially controlled mitochondrial localization and heterogeneity in driving corresponding cellular phenotypes. Our study highlights a novel crosstalk between mitochondrial and cell-ECM adhesion with implications for migration, particularly relevant during cancer cell migration.

B200/P0195

Nematic fluctuation of FA elements, energized by active forces, mediates "patrolling" mechanosensing

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Cells not only respond to mechanical perturbation but also actively explore their physical environment, in order to adhere, migrate or just to stay and grow. Focal adhesions (FAs) are membrane-less organelles that connect the cytoskeleton to the extracellular matrix while transmitting force. Although originally described as “plaque-like”, the mechanotransduction within FAs are known to be discontinuous and dynamic. However, knowledge on how discrete FA sub-regions fulfill local mechanosensing remains scarce and can be interpreted from their motions. We utilized super-resolution live-cell imaging to capture the dynamics of FA proteins at the endogenous level and uncovered distinct movement patterns of FA elements from different proteins. Intriguingly, vinculin elements exhibited nematic fluctuations (diffusion-like motion along the long axis of FAs) in low-motility cells, and this behavior was mechanosensitive. Simultaneous super-resolution imaging of vinculin elements and DNA-based force probes revealed that this nematic diffusion correlates with force

transmitting integrins. Mathematical simulations suggest that this motion is driven by active fluctuating forces arising from the anti-parallel organization of myosin II within FAs and is independent of actin retrograde flow. Subsequent PREM and STED imaging provided supporting evidence for such structures at FAs. Together, our study identified a patrolling mechanosensing that is propelled by active forces to ensure effective sensing of local forces, especially in low-motility cells.

B210/P0205

PHACTR4 is a novel regulator of migration and matrix adhesion of intestinal epithelial cells.

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Intestinal epithelial cell (IEC) migration is critical for the establishment and maintenance of the intestinal barrier as well as for repairing this barrier following inflammatory injuries. IEC migration is regulated by a variety of signaling events that eventuate in the remodeling of the actin cytoskeleton to drive cell motility. PHACTR proteins serve as unique dual regulators of actin filament dynamics and protein phosphatse-1 (PP1) signaling. Their functions in different epithelia remain poorly understood. In this study we examined the roles of PHACTR4 in the regulation of intestinal epithelial cell migration. A bulk and single cell RNA sequencing analyses demonstrated high PHACTR4 expression in different IEC populations in normal human colonic and small intestinal mucosa. Importantly, immunofluorescence analysis observed decreased PHACTR4 expression in the inflamed intestinal mucosa of inflammatory bowel diseases patients. shRNA-mediated knockdown of PHACTR4 in HCA7 and Caco-2 human IEC lines revealed paradoxical effects, such as increased collective cell migration during wound healing, and attenuated individual cell migration according to time-lapse imaging. The accelerated collective cell migration of PHACTR4-depleted cells was accompanied by increased IEC proliferation and was mitigated by inhibition of cell proliferation with mitomycin. Individually migrated PHACTR-depleted HCA7 cells displayed decreased average speed, average accumulated distance and diminished directionality of cell migration. These motility defects were accompanied by the increased adhesion of PHACTR4-depleted IEC to the extracellular matrix. Furthermore, immunofluorescence labeling and confocal microscopy demonstrated increased cell spreading after PHACTR4 knockdown. The enhanced attachment and spreading of PHACTR4 deficient cells were due to accelerated assembly of focal adhesions, increased focal adhesion signaling and establishment of more prominent actin filament bundles at the spreading cell edges. In summary, our results highlight PHACTR4 as a novel positive regulator of IEC migration that controls assembly of the actin cytoskeleton and matrix adhesion complexes in migrating cells. Supported by Leona M. and Harry B. Helmsley Charitable Trust grant to A.I.I.

B201/P0196

Photoactivatable proximity labeling reveals local protein synthesis and metabolic regulation by ECM stiffness

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Cellular metabolism, a central biochemical process influencing numerous cellular processes, has intimately link with mechano-transduction. Uncovering the mechano-metabolomic crosstalk will provide promising new perspective in understanding cell physiology and disease. Focal adhesions (FAs) are mechano-transduction hubs, that regulates cell motility and fate. Recent studies have highlighted the role of ECM rigidity in lipid and glucose metabolisms via FA signaling. It has also been identified that protein synthesis can take place at FAs.

However, the molecular components, functional roles, and regulatory mechanisms of this mechano-metabolic interplay of proteins are still poorly characterized. Furthermore, current research predominantly focuses on intracellular mechanosensitive responses, whereas knowledge of the secretory phenotype and intercellular communication driven by mechanical stimuli to influence metabolism is limited. Proximity labeling methods use enzymes to catalyze the covalent binding of small molecules to biomolecules near the target protein, offering an antibody-free approach that can detect weak or transient interactions. To overcome the limitations of background labeling and improve temporal resolution, optogenetic approaches have been employed to control labeling enzyme activity by tuning exposure time and region. Total internal reflection fluorescence (TIRF) excites fluorophores in a thin layer (~100 nm) at the interface between the substates and cell ventral surface, reducing background fluorescence in the cytosol and improving axial resolution. Thus, TIRF microscopy is widely used for FAs imaging. However, commercial objective-based TIRF microscopy has a limited field of view (~2.5×10^{-5} cm^2), which is insufficient for large-scale proteomics sample collection if combined with optogenetic control of proximity labeling. Here, by combining an optimized optogenetic proximity labeling (PA-ultra)-mass spectrometry with customized pTIRF excitation, we reveal the interactome of FAs and look for transient interactions that may potentially unveil new roles of FA-mediated mechano-transduction. Our study uncovers a new mechano-metabolic axis linking extracellular matrix (ECM) stiffness to selenium (Se) homeostasis. We identify the dynamic interaction of FAs with SerRS which is critical for SELENOP synthesis. FA-localized SELENOP mRNA and newly-synthesized SELENOP have been revealed by biochemical and imaging assays. Functionally, increased ECM stiffness enhances FA assembly and upregulates intracellular SELENOP level.

B202/P0197

Protein Glycosylation Pathway Mutants of Pseudomonas aeruginosa Show Distinct Phenotypes in Biofilm Formation

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Pseudomonas aeruginosa is an opportunistic human pathogen commonly acquired in hospitals by vulnerable individuals like those with cystic fibrosis or burn wounds. The increasing prevalence of antibiotic resistance in P. aeruginosa, as well as its ability to form strong biofilms makes it challenging to treat these infections. However, while P. aeruginosa is a model organism for studying biofilm development, structure, and function, the roles of protein glycosylation in this process have not yet been elucidated. Therefore, we have analyzed biofilm formation in knockout mutants of two glycosylation pathway enzymes, FgtA and TfpW, that have previously been described to modify flagella and pili, respectively. Together with their corresponding wild types, PA14 and Pa5196, we performed 96-well plate adhesion assays with crystal violet staining to quantitatively assess their ability to form biofilms. Interestingly, after 1 h, 4 h, and 24 h, decreased biofilm formation in comparison to the wild type was observed for the fgtA deletion strain, but not the tfpW mutant. In addition, Confocal Laser Scanning Microscopy (CLSM) was used to obtain three-dimensional images of the biofilms produced over 24 h. In line with the 96-well plate assay results, CLSM images showed thinner biofilms with fewer characteristic mushroom-shaped cell clusters for the fgtA mutant than for their wild type PA14. Furthermore, even though no quantitative changes were observed for the tfpW mutant, their biofilm structure showed larger clusters of cells than for the wild type PA5196. These findings indicate not only that protein glycosylation plays critical roles in mature biofilm formation of P. aeruginosa, but also that mutants of different glycosylation pathways show distinct biofilm phenotypes. These insights may contribute to potential strategies for combating biofilm-associated P. aeruginosa infections, such as by targeting specific glycosylation pathways.

B208/P0203
Serotonin as a target modulator of cell migration in breast cancer cells
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Cell migration requires a delicate balance of cellular adhesion to the extracellular matrix (ECM). Focal adhesion complexes are responsible for this balance. In the context of breast cancer, dysregulation of such adhesions contributes to disease progression and metastasis. Recently, the serotonergic signaling network has become a target of interest in breast cancer progression. Increases in serotonin result in increased cell survival, invasion, and proliferation in breast cancer cells, yet the underlying mechanisms of its tumor-promoting properties are poorly understood. Serotonin plays a key role in maintaining mammary epithelium homeostasis evidenced by the expression of both the rate-limiting enzyme in its biosynthesis, tryptophan hydroxylase, and various serotonin binding sites such as serotonin receptors and the serotonin transporter. Peripherally produced in enterochromaffin cells, serotonin is stored in the cytoplasm of platelets. Upon activation, platelets aggregate, secreting serotonin in the process. Among other factors, serotonin has been suggested to influence platelet aggregation through its transamidation to Rho GTPases, a process known as serotonylation. Rho GTPases are known to modulate focal adhesion dynamics, which ultimately influence cell migration. As abnormal cell migration is the fundamental issue of breast cancer progression and metastasis, we aim to investigate the role that serotonin plays in cell migration. In order to address this question, we have treated breast epithelial cell lines with serotonin and transforming growth factor beta (tgfB). Our initial characterization utilizes immunofluorescence staining to visualize and further quantify changes to the focal adhesion complexes of both cancerous and noncancerous breast epithelial cells as a result of serotonin treatment. Given that tgfB treatment induces metastatic-like characteristics in normal breast epithelial cells, we expect to see similarities in the characterization of focal adhesion complexes between both treatments.

B206/P0201
Spatiotemporal coordination between actin retrograde flow and stress fibers drive focal adhesion life cycles
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Cell migration is essential for many physiological functions such as embryonic growth and immune defense. While the physical mechanisms of cell migration are not well understood, the answers to these fundamental questions are believed to be key to understanding diseases such as cancer metastasis. To migrate, many cells rely on focal adhesions (FAs), integrin-based transmembrane linkages that connect the cell to the extracellular matrix (ECM) and exert actomyosin-based traction. FAs are dynamic subcellular attachments that form de novo, engage with actin retrograde flow, mature in size by engaging with stress fibers, and eventually, disassemble to facilitate migration. Despite their importance, the underlying mechanisms and associated principles that underlie the complete life cycles of FAs are unclear. Combining theoretical modeling with live-cell imaging, we establish that actin retrograde flow and stress fibers coordinate their mechanical actions in time and space to drive the assembly and disassembly of FAs through processes dependent on the catch-slip bond behavior of FAs. Our work identifies a natural mechanism by which the life cycles of FAs are coupled with cell edge protrusion and retraction, offering novel insight into the fundamental mechanisms of cell migration.

B204/P0199

SPECTr biosensors that report the stretch-mediated opening of specific talin domains

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Talin is a large, mechanosensitive adapter protein that plays an essential role in integrin adhesions by serving as the primary link between the actin cytoskeleton and integrins. The rod domain of talin contains 13 helical bundles (R-domains) that function as force-dependent switches that unfold stochastically in response to tension. R-domain stretching alters their specificity for downstream proteins allowing cells to 'feel' the extracellular environment by changing adhesion composition and signaling. However, it has not been possible to observe the timing and location of talin stretching in live cells. Here, we have developed a series of biosensors that detect the conformation of specific domains in real time, capable of single-molecule imaging in living cells, based on a new approach called SPECTr (Small Peptide Exposure for Conformation Tracking). Short peptides are embedded within each domain so that colocalization with a separate 'binder protein' indicates R-domain unfolding. Using SPECTr-Talin, we correlate cell and adhesion behavior with talin stretching at sub adhesion resolution. Our work shows that talin undergoes specific and localized conformational changes in migrating cells and provides insight into the complexity of talin mechanotransduction.

B211/P0206

Tensin3 drives Ras signaling by recruiting GRB2 to modulate mechanosensing

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The extracellular matrix (ECM) and cell adhesive interactions play essential roles in the pressure-sensing function. This study aims to identify the mechanistic basis of Tensin3 (TNS3), a focal adhesion and mechanosensing protein, in driving tissue contractility and stiffness in primary human trabecular meshwork (HTM) endothelial cells. The TM cells play a crucial role in regulating intraocular pressure (IOP). TM cells were utilized as a cell biology model system, and changes in eye pressure in mice served as the physiological readout to study the role of TNS3. To assess the functional impact of TNS3 - A) On IOP in vivo, adenoviral TNS3 (AdTNS3) and control vector (AdMT) were intravitreally delivered in C57BL/6 mouse eyes, and IOP was monitored longitudinally. AdTNS3-injected eyes exhibited a significant and sustained IOP elevation (*$p \leq 0.05$, n=14 eyes) persisting through day 40, suggesting the role of TNS3 in IOP homeostasis. B) On ECM remodeling, we performed deoxycholate fractionation assays in primary HTM cells. This revealed a marked increase in insoluble fibronectin deposition upon TNS3 overexpression (n=3, *$p \leq 0.05$). C) Actin-based contractile changes were evaluated through gain and loss-of-function experiments. TNS3 overexpression resulted in a pronounced increase in actin stress fiber formation, while TNS3 silencing via siRNA led to a reduction in these fibers, as demonstrated by immunofluorescence analysis. Filamentous actin/Globular actin (F/G) fractionation analysis showed an increase in F/G actin ratio in AdTNS3-transduced HTM cells (n=3, *$p \leq 0.05$). In parallel, TNS3 overexpression increased the phosphorylation of myosin light chain, indicating elevated cellular contractility (n=3, *$p \leq 0.0003$). D) To delineate downstream signaling pathways, we performed an unbiased proteomic profiling of TNS3-overexpressing HTM cells (n=4). This analysis revealed upregulation of the focal adhesion adaptor protein GRB2 ($p=0.004$), which is involved in Ras activation, and downregulation of the Ras inactivator RASA1 ($p=0.04$), suggesting sustained Ras signaling. E) Immunoprecipitation with GRB2 in HTM cells confirmed

its interaction with TNS3, suggesting that TNS3 orchestrates a novel GRB2-Ras signaling axis that links focal adhesion signaling to cytoskeletal contractility. Together, these findings establish TNS3 as a central regulator of TM mechanobiology, acting through dual mechanisms of ECM stiffening and cytoskeletal remodeling. This study reveals previously unreported and novel roles for TNS3 in actin polymerization and Ras signaling, expanding our understanding of its function and identifying it as a potential therapeutic target for diseases driven by abnormal mechanotransduction.

B198/P0193

TMEM184A activation by heparin modulates integrin recycling in vascular cells

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In response to heparin treatment, the heparin receptor, transmembrane protein 184A (TMEM184A), internalizes and alters cell signaling to result in decreased proliferation and decreased inflammatory signaling in vascular smooth muscle cells (VSMC) and endothelial cells (EC). TMEM184A activation of the heparin-induced signaling pathway through eNOS is visualized at the basal surface. In addition to binding heparin, TMEM184A interacts with the heparin sulfate proteoglycan, syndecan 4. TMEM184A and syndecan 4 work synergistically in regenerating angiogenesis in zebrafish caudal fins. The presence of TMEM184A also facilitates increased VE-cadherin stability in endothelial cells. In our current work, we examined whether TMEM184A is involved in recycling integrins in vascular cells. We have employed short term heparin treatment to activate signaling and TMEM184A internalization and observe concurrent movement of integrin α5 and TMEM184A toward the perinuclear regions of ECs and VSMCs. These molecules become more co-localized with a trans-Golgi network marker – TGN48 suggesting that heparin treatment facilitates recycling of integrin α5 through classical recycling pathways. Similar movement of integrin αV does not occur in response to heparin treatment. Transient knockdown of TMEM184A in ECs alters basal distribution of integrin α5 and decreases relocation responses to heparin. After stable TMEM184A knockdown in VSMCs, heparin has little impact on integrin α5 movement, and levels of integrin are lower than in wild type cells. Together, our data support a role for TMEM184A in recycling of integrin α5. Funding source: HL54269 to LJLK.

B207/P0202

Unraveling the mechanisms of talin1 mediated PKA anchoring within focal adhesions

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Cell adhesion to the extracellular matrix (ECM) is fundamentally important for a vast array of cellular functions. Focal adhesions (FAs) are highly dynamic, multi-molecular complexes that couple ECM-bound integrins to the actin cytoskeleton. In addition to their structural and adhesive function, FAs are also centers of bidirectional signal transduction from and to the ECM. The cAMP-dependent Protein Kinase (PKA) is a ubiquitous serine/threonine kinase that is responsible for regulating many physiological and cellular processes throughout the cell, including, but not limited to, cell migration. Subcellular localization of PKA is achieved through binding to AKAPs (A-kinase anchoring proteins), which scaffold PKA substrates and regulators in addition to the enzyme itself. Our lab recently discovered PKA binds to talin1, the archetypal FA protein, however the nature and role of this interaction remain to be fully understood. Talin1 is a large, mechanically complex protein that directly couples integrins to F-actin. Talin1 unfolds certain domains in response to tension from actomyosin

contractility, exposing “cryptic” binding sites for other FA proteins, such as vinculin. Recently, our lab identified PKA as a new mechanically-gated talin1 interacting protein, establishing talin1 as the first mechanically gated AKAP and a new mechanotransduction signaling niche for PKA. Recent data show that PKA also binds to talin2, suggesting a conserved role for talin-PKA binding. Delineation of the role of talin-PKA interaction is an important but complex next step. Previous work in the lab generated a double point mutant within helix41 of the talin1 R9 domain (A1806D/V1807D; AV/DD) that disrupts the hydrophobic face of the amphipathic helix while maintaining helicity. While this mutant helped confirm the region in talin1 involved in PKA binding and resulted in the loss of active PKA within FAs, its use in cellular assays is limited by its propensity to alter R9 conformation, leading to unfolding and constitutive activation of talin1. To find mutants within talin1 that reduce PKA binding while maintaining the structure and autoinhibitory function of R9, we made a series of mutations in the linker region between R9-10 through helix42 (R10) and found several that block PKA binding. Efforts are underway to characterize these mutants in in vitro binding assays with the talin1 head domain and direct structural analyses. Once established, talin1 mutants will be expressed in a cell system that allows elimination of endogenous talin2, insertion of WT and AKAP-mutant talin1, and knockdown of endogenous talin1. Once in hand, this system will allow us to rigorously test the effects of the loss of talin1-mediated PKA anchoring on talin biology and, in turn, cell adhesion and migration.

Cell Adhesion, Migration, and the Extracellular Environment - Extracellular matrix (ECM)

B225/P0220

A spectrin cytoskeletal network patterns transient extracellular matrix proteins to shape permanent acellular ridges in the adult cuticle of C. elegans

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The apical extracellular matrix (aECM) lines, shapes and protects exposed surfaces in animals and can organize to form elaborate, acellular three-dimensional structures. How animals pattern these large structures during development is poorly understood. The C. elegans alae, cuticle ridges that run along the sides of the worm in three long ‘racing’ stripes, are an example of one such extracellular structure. We are studying how the cytoskeleton patterns de novo formation of the adult alae. Previous research found that four longitudinal actin filament bundles (AFBs) form at the lateral (seam) epidermis and pattern assembly of a transient pre-cuticle aECM, required for alae formation. Additionally, ultrastructural data revealed a potential role of pre-cuticle delamination in alae morphogenesis, where four small horizontal areas of delamination demarcate the valleys between alae, while remaining points of matrix adhesion mark developing alae ridges. One hypothesis generated by this observation is that longitudinal AFBs exert mechanical forces on the matrix to trigger pre-cuticle delamination. We tested several cytoskeletal proteins with actin binding and plasma membrane binding domains, making them ideal candidates to aid in the assembly of seam AFBs and/or facilitate AFB-mediated mechanical forces on the matrix. Super-resolution imaging shows that the spectraplakin VAB-10 and intermediate filament bundles are arranged in a single line at the middle of the seam epidermis offset from AFBs, while the apical bH-spectrin SMA-1 is found superimposed on the AFBs. sma-1 loss specifically disrupts organization of the two medial-most seam AFBs that flank the site where the central alae ridge forms. Correspondingly, sma-1 loss also disrupts continuity of the pre-cuticle and cuticle matrix specifically in this central ridge region. sma-1 mutants appear to have increased cortical actin tension within the seam, as suggested by increased localization of a mechanosensitive LIM-domain protein, TES-1. Ultrastructural studies show that sma-1 mutants have longer areas of matrix delamination, likely because of increased cortical actin

tension. Overall, our data support the hypothesis that mechanical forces relayed by cytoskeletal structures play a role in the formation of the C. elegans adult alae.

B231/P0226

A tale of two sides: Coordination between the cortical cytoskeleton and extracellular basement membrane in tissue elasticity

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Tissues such as lung and vasculature must withstand repeated cycles of expansion and recoil for sustained function. This mechanical robustness relies on the cortical cytoskeleton and extracellular basement membrane (BM), which are connected by cell surface receptors and adaptor proteins. As a tissue expands, the enveloping BM stretches and stores energy like a taut trampoline mat. When the tissue relaxes, the BM releases energy in concert with actin-driven contractility to restore tissue shape. Despite its importance, how the BM and cytoskeletal networks coordinate to support tissue elasticity remains unclear, largely due to the lack of experimental models that allow real-time visualization and perturbation of these networks during rapid, repetitive tissue stretch and recoil events. To overcome this, we have developed C. elegans ovulation as an in vivo model to study how BM and tissue elasticity is established and maintained. During ovulation, the spermathecal tissue and its enveloping BM stretch ~1.7-fold and then recoil, accommodating oocyte entry, fertilization, and zygote exit. Our recent study demonstrated that the spermathecal BM network is reinforced by the BM crosslinker fibulin-1, which interconnects with the core BM component type IV collagen. Interestingly, fibulin-1 loss not only resulted in the accumulation of mechanical damage in the type IV collagen network during cyclic stretching, but also impaired actin organization leading to unbundling, ultimately disrupting elastic tissue recoil. These observations highlight the critical interdependence between the BM and actin cytoskeleton in supporting tissue elasticity. The spermathecal cell cortex also contains parallel arrays of microtubules uniquely enriched with α-tubulin isotype 4 (TBA-4). Depletion of TBA-4 disrupted tissue elasticity, leading to permanently elongated tissues. We discovered that tissue elasticity depends specifically on TBA-4 as loss of other α-tubulin isotypes present in the spermathecal tissue did not impair elastic tissue recoil. Notably, TBA-4 depletion reduced fibulin-1 levels in the spermathecal BM, raising the possibility that TBA-4-enriched microtubules support BM and tissue elasticity by mediating fibulin-1 transport to the BM. Together, this study demonstrates that the BM and cortical cytoskeletal networks coordinately promote cyclic tissue expansion and recoil.

B215/P0210

Adipocyte-derived soluble factors alter the transcriptional landscape of muscle cells

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Skeletal muscle tissue is composed not only of muscle cells but also of intramuscular adipocytes. Our previous research demonstrated that adipocytes release distinctive secretory factors during their differentiation (FEBS Open Bio. 2016; 6: 816-826), inspiring us to investigate how these soluble factors govern gene expression and cellular functions in muscle cells. However, the precise mechanisms by which these coexisting adipocytes influence muscle cells remain elusive. In this study, we explored the transcriptional changes in muscle cells under two distinct co-culture conditions: myoblasts partnered with differentiated adipocytes and myotubes

alongside preadipocytes. Notably, our microarray analyses revealed that gene ontology terms related to cellular adhesion, extracellular matrix (ECM) organization, and metabolic processes were significantly enriched in muscle cells subjected to both co-culture conditions. Furthermore, we examined how adipocyte co-culture influences myogenic differentiation and the expression of genes characteristic of muscle fiber types. While co-culture with differentiated adipocytes did not yield significant changes in myogenic regulatory factor expression in myoblasts, myotubes co-cultured with preadipocytes exhibited a pronounced upregulation of Myh2 and Myh4. These findings suggest that soluble factors derived from adipocytes profoundly reshape the transcriptional landscape of muscle cells, particularly by modulating genes involved in ECM remodeling and metabolic regulation. Such intercellular communication plays a crucial role in driving the structural and metabolic adaptations of skeletal muscle tissue in vivo, underscoring the intricate interplay between adipocytes and muscle cells.

B224/P0219

Analysing the Effect of Temperature on Collagen Turnover Dynamics in Zebrafish Notochord

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Temperature is a critical environmental factor that influences embryonic development. In zebrafish, high temperature accelerates the developmental rate and causes morphological defects such as bent body axes. The notochord, a central structural element of the axis, is encased in a collagen-rich sheath that provides mechanical stability during elongation. However, collagen has a relatively long production time due to its large size and extensive post-translational processing. The collagen sheath undergoes continuous remodeling during development. Therefore, understanding how collagen turnover responds to temperature stress is crucial for correlating environmental perturbations to tissue-level defects. To investigate this, I worked particularly on collagen IX, which is a non-fibrillar collagen. I constructed CRISPR knock-in Col9a2 lines by inserting the Dendra2 reporter sequence downstream of the signal peptide coding region within exon 1. I photoconverted green Dendra to red at 24 hours post-fertilization (hpf) embryos to track collagen turnover in zebrafish notochord under different temperature conditions (24°C, 28.5°C, 32°C, 34°C, 35°C), and performed readout at later stages (48hpf and 72hpf) under fluorescence microscopy. So, after photoconversion, the red signal is the collagen that already existed, and any new green signal is newly synthesized collagen that was added later. I analyzed images using computational workflows such as R and Python, calculated the turnover rate by measuring the collagen production and degradation rates, and developed a theoretical model. By combining experimental approach with theoretical modeling, my results revealed that collagen turnover initially increases up to 32°C. Beyond this threshold, turnover declines sharply. This is likely because enzymes required for collagen synthesis, processing, and degradation fail to function efficiently above optimum temperature. For embryos raised at all temperature conditions, collagen production is higher in the anterior notochord than in the tail, and the degradation occurs rapidly over time along the axis, indicating the non-uniform collagen dynamics across the body axis. These results suggest both temperature-dependent differences and regional differences in collagen turnover. Theoretical modeling incorporating synthesis and degradation constants closely matched the experimental fluorescence decay curve, validating the framework for predicting turnover rates. The findings of this study highlight that collagen turnover is dynamic and highly sensitive to temperature and, more broadly, how large structural proteins may act as a key bottleneck in maintaining developmental robustness under environmental stress.

B227/P0222

Cell confinement aftereffects include heritable loss of chromosomes and a persistently dysregulated division program

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Cell confinement and mechanical stress are common in solid tumors, but any impact on genetic changes remains unclear. Using chromosome reporters (ChReporters) that reveal heritable losses of chromosomes in live cells, we discover that ChReporter losses are surprisingly equivalent when cells are subjected to mild confinement (6-10µm) or severe confinement (2µm). Severe confinement causes interphase nuclear rupture in most cells, whereas mild confinement suffices to perturb mitotic spindles, prolong pro/metaphase, and eventually induce micronuclei. Indeed, micronuclei that form through chromosome mis-segregation in mitosis do not emerge during mild confinement but do emerge following release – indicating an aftereffect of the confined state. Live cell imaging post-confinement revealed that mitotic cells and their daughters also tend to arrest or die. Delayed induction of micronuclei and ChReporter loss are phenocopied by transient disruption of microtubules which prolongs pro/metaphase. Inhibition of cell cycle entry using a clinically deployed CDK4/6-inhibitor further shows ChReporter loss requires mitosis while inhibitor washout increases micronuclei and ChReporter loss. Consistent with a memory of spindle confinement, single cell and bulk RNA-sequencing demonstrate enrichment of chromosome segregation factors long after confinement.

B223/P0218

Cell type-specific recruitment of Zona Pellucida domain proteins in the apical extracellular matrix

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The interior of biological tubes is coated with an apical extracellular matrix (aECM), which contributes to their development and function. The composition of the aECM is specific to individual organs and even particular cell types. Zona Pellucida domain (ZPD) proteins are commonly found in aECMs across organisms, yet how they assemble into the correct matrix compartments is not well understood. The C. elegans vulva is an ideal system to connect aECM assembly to the biology of individual cell types. The vulva is an epithelial tube composed of only 22 cells divided into 7 morphologically distinct cell types (vulA/B1/B2/C/D/E/F), yet it produces a highly compartmentalized and dynamic aECM. Using live imaging of translational and transcriptional reporters, we compared the protein localization and gene expression of three ZPD proteins. LET-653, NOAH-1, and CUTL-18 are similar proteins with PAN domains preceding their ZP domains, yet localize to the matrix on different cell surfaces in the vulva. We found that these three ZPD protein genes are all broadly transcribed in the vulva, suggesting that their cell type-specific matrix localization is post-transcriptionally controlled. We identified a transcription factor, EGL-38/Pax, that is required for proper vulE/F matrix assembly. We propose that individual vulval cell types express membrane-associated “organizers” that recruit specific ZPD proteins, and that expression of the vulE/F organizer depends on EGL-38. To better understand the biological distinctions between vulval cell types and identify candidate matrix organizers, we performed single-nucleus RNA sequencing of the vulva in wild type and egl-38 mutants. Gene set enrichment analysis highlights significant differences in the expression of previously unstudied aECM components between vulva cell types. This suggests producing a distinct aECM is a key aspect of vulva cell identity. We have identified candidate matrix organizers with egl-38-dependent expression in vulE/F cells and are now testing their functional roles in

ZPD protein recruitment. Overall, these datasets will support continued studies of the mechanisms driving ZPD compartmentalization.

B221/P0216
Dissecting How ECM Properties Guide Basement Membrane Assembly in 3D Epithelial Culture
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Epithelial tissue structure and function depend on interactions with the basement membrane (BM), a specialized extracellular matrix (ECM) present at the basal surface. The BM is composed of four core components, laminin, collagen IV, perlecan, and nidogen, which are assembled into a thin sheet-like matrix that acts as an essential physical and molecular scaffold. Cells physically engage with the ECM and, in a reciprocal manner, actively modify it, remodeling its composition and structure for their developmental and functional needs. In tissue culture, epithelial cells can secrete BM components, yet the ability to assemble a continuous and functional BM emerges only in 3D hydrogel environments enriched in ECM molecules. This observation highlights the critical role of the physical and biochemical properties of the surrounding matrix in guiding BM formation. Leveraging 3D epithelial culture of MDCK cells presents a tunable, simplified system to interrogate how matrix properties guide and regulate BM secretion and organization. Here we are using a variety of 3D ECM hydrogels to investigate how the ECM properties guide BM secretion and assembly.

B228/P0223
Emerin-LINC modulation of mechanotransduction during cancer progression
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Breast cancer metastasis accounts for most of the breast-cancer-related deaths. During metastasis, cancerous tumor cells spread through the body by moving into and out of nearby blood vessels and squeezing through very small gaps in the endothelium. Nuclei in cancer cells are often smaller and more malleable, allowing for easier metastasis. Our lab showed invasive breast cancer cell lines and patient samples have less emerin expression, misshapen nuclei, and higher metastasis rates than non-cancerous controls. We hypothesize that reduced emerin expression serves as a key driver of breast cancer metastatic transformation by compromising the structural integrity of the nucleus. However, the mechanism by which emerin modulates nuclear stiffness is not well understood. Increasing stiffness of the tumor microenvironment (TME) was shown to increase nuclear softening. Here, we show emerin protein expression decreases upon extracellular matrix (ECM) stiffening. Thus, we predict emerin plays an important role in responding to changes in the TME by altering nuclear structure. We posit emerin acts as a mechanosensor to modulate structural and/or transcriptional outcomes by responding to extracellular stimuli through the linker of nucleoskeleton and cytoskeleton (LINC) complex. The end result of this mechanotransduction is nuclear softening to enable metastasis. Since, emerin binds to LINC through the SUN1 domain, we postulate the interaction between emerin and LINC is critical for enabling metastatic transformation. We used emerin and LINC mutants to perturb LINC interaction(s) with emerin and evaluated changes in TNBC cell nuclear size and shape, volume, invasion, and migration upon substrate stiffening. LINC disruption contributes to decreased nuclear size and increased migration in breast cancer cells, suggesting the LINC complex is intimately tied to maintaining nuclear architecture. Our analysis provides key insight into how emerin responds to substrate stiffening in metastatic progression as well as the

underlying mechanism of how emerin works with the LINC complex in response to changes in the tumor microenvironment.

B219/P0214
Etiology-specific chromosome 8q24 amplification associated with human intrahepatic cholangio- and hepatocellular carcinomas predicts MYC/ZEB1/MIZ1 transcriptional regulation and poor prognosis
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Genomic instability, in particular chromosome 8q24 amplification, is associated with late stage hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (iCCA) and the epithelial mesenchymal transition (EMT). Limited HCC patient studies suggest that 8q24 amplification may also be etiology-dependent. On this amplified region, is the known oncogene c-myc. MYC overexpression activates targets that promote cell proliferation and activates ZEB1 that drives EMT. Overexpressed MYC also represses tumor suppressors that antagonize EMT, including MIZ1. The connection between c-myc amplification and the corresponding changes in ZEB1 or MIZ1 have been reported, but not all three together. Also of note, is that these relationships have not been characterized in the context of HCC or iCCA across grades or with respect to etiologies. We confirmed 8q24 amplification using FISH and established the temporal expression patterns of MYC, ZEB1 and MIZ1 in human HCC and iCCA patient resections using immunohistochemistry and retrospectively compared them to patient histories. We found that focal c-myc 8q24 amplification occurs only in grade III/IV HCC and grade III iCCA which correlates with increased MYC and ZEB1 expression. In contrast, MIZ1 cytosolic expression was lost and only nuclear expression retained. We also observed, that of the high grade cancer patients that exhibited amplified c-myc, high MYC and ZEB1 expression and only nuclear MIZ1 expression were all associated with metastasis and poorer prognosis. Additionally, although majority of the cohort had a history of viral hepatitis, only those that also had a history of alcohol consumption exhibited this transcriptional regulation. To directly test MYC/ZEB1/MIZ1 transcriptional regulation, we examined EMT in human HCC-derived Hep3B or iCCA-derived HUCCT1 cells with amplified c-myc. Analysis revealed that alterations in MYC expression led to the predicted changes in ZEB1 and MIZ1 expression/distribution and in EMT further confirming MYC/ZEB1/MIZ1 transcriptional regulation drives EMT. Additionally, factors used to stabilize MIZ1 and decrease ZEB1 expression promoted an epithelial phenotype with a loss in EMT. Based on expression profiles of these markers, the clinician will be better able to grade a patient's HCC or iCCA, better predict its drug resistance and better decide what treatment options are available. As we continue to perform the basic science to define EMT more precisely, we will uncover novel therapeutic strategies for the treatment of alcohol-induced HCC and iCCA.

B218/P0213
MAL2 and rab17 selectively redistribute invadopodia proteins to laterally-induced protrusions in hepatocellular carcinoma: mutational analysis
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From our work and others, MAL2 (myelin and lymphocyte protein 2) and the small GTPase, rab17, have been identified as hepatocellular carcinoma (HCC) tumor suppressors. Little is known how their roles in hepatic polarized protein sorting/trafficking translate to their functions in nonpolarized cancer cells. We found MAL2/rab17 expression led to lateral actin-/cholesterol-dependent protrusion formation with reciprocal loss in

invadopodia, correlating with anti-oncogenic phenotypes. MAL2/rab17 selectively promoted redistribution of actin-associated invadopodia proteins (Mena, VASP, talin, α5β1 integrin) and invadopodia initiator (Tks5) to protrusion tips, correlating with decreased matrix degradation. Mutational analysis revealed protrusion formation and invadopodia protein redistribution required a putative MAL2 EVH1 recognition motif (in actin binding proteins) and GTP-bound rab17. Expanding our mutational analysis, we generated MAL2 serine mutations (S17A, S17D) in a predicted phosphorylation site adjacent to the EVH1 domain required for MAL2 anti-oncogenic properties and sumoylation-deficient rab17 (K41R, K68R) since mono-sumoylated rab17 is required for roles in polarized protein sorting. These were expressed in hepatoma-derived Clone9 cells (lack endogenous MAL2/rab17) and phenotypes were analyzed. In S17A-expressing cells, actin-based protrusions formed, but in S17D-expressing (phospho-mimetic) cells, the opposite occurred indicating ser-17 phosphorylation inhibits protrusion formation. Redistribution of invadopodia markers (Mena, VASP) was prevented in cells expressing S17D. In H2O2-treated cells (decreases sumoylation), rab17-induced protrusions were lost, consistent with our findings that sumo-deficient rab17 prevented protrusion formation and redistribution of invadopodia proteins. Our results indicate MAL2/rab17 expression alone or together promoted actin-protrusion formation at the expense of invadopodia formation, correlating with anti-oncogenic phenotypes. Rab17 sumoylation is required for anti-oncogenic properties whereas MAL2 phosphorylation at ser-17 prevents protrusion formation leaving invadopodia intact. MAL2 is not a direct target, but strategies to promote re-expression may be a viable approach. If MAL2 phosphorylation is involved in its anti-tumorigenic properties, kinases that phosphorylate MAL2 would be actionable targets. Rab17 expression is selectively repressed by ERK2, suggesting ERK2 inhibition may promote rab17 re-expression. Continued examination of rab17/MAL2 in polarized vs. nonpolarized context will provide mechanistic insight to understanding HCC progression and/or suppression while better defining mechanisms by which novel actionable targets function.

B217/P0212

Sensory extracellular matrix function and composition in C. elegans ciliated neurons

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The ability to sense and respond to our environment underlies all of our basic functions. Many of our senses are mediated by ciliated sensory neurons that are coated in extracellular matrix (ECM). Little is known about how this “sensory ECM” impacts a neuron’s ability to detect environmental cues. Furthermore, we lack an understanding of the composition of sensory ECM. C. elegans male-specific ciliated neurons provide an excellent system in which to study the function and composition of sensory ECM. A genetic screen for regulators of ciliary sensory channel localization revealed a role for mec-9. mec-9 encodes an ECM protein containing conserved Kunitz and EGF-like domains and is required for mechanosensation by non-ciliated touch neurons. We found that the putative neomorphic allele mec-9(ok2853) interferes with ciliated sensory neuron function (male mating) and causes ciliary localization defects of the TRP channel protein PKD-2. mec-9 null alleles do not cause mating defects or PKD-2 phenotypes. Ciliated neurons shed extracellular vesicles (EVs), which play roles in proteostasis and cell-cell communication. One population of ciliary EVs is released outside the animal while a second population is released within sensory organs. We explored how mec-9(ok2853) impacts ciliary EV release using a combination of transmission electron and super-resolution microscopy. mec-9(ok2853) mutants display accumulations of EVs in sensory organs. We observed no significant change in the number of environmentally released EVs from the neurons of the male tail. To determine the cell type supplying MEC-9 to the ECM surrounding ciliated sensory neurons, we examined a transgenic mec-9::GFP transcriptional reporter. We found that MEC-9 is not produced by ciliated sensory neurons themselves, but

instead supplied cell-non-autonomously by companion neurons. Taken together, our results support a model in which changes to the ECM interfere with sensation by causing mislocalization of ciliary sensory channels and EV accumulation. Current work is focused on identifying sensory ECM components using available fluorescent ECM reporters and candidates from the transcriptomes of sensory neurons and glia. Our work indicates that the sensory ECM has a unique composition compared to other tissues and neuron types, suggesting that sensory organs have specialized ECM "recipes" to support their unique functions.

B222/P0217
Shaping organs by patterning basement membrane turnover
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Organ formation requires extensive remodeling of the basement membrane (BM), a dense extracellular matrix (ECM) layer that encapsulates epithelial tissues. Yet, how BM turnover is spatially and temporally regulated during organ development remains poorly understood. To address this, we generated a Col4a2-HaloTag knock-in mouse model and developed pulse-chase labeling assays to systemically track BM turnover both in vivo and in ex vivo organ explants. Col4a2 encodes the α2 chain of type IV collagen, a key structural BM component whose loss causes embryonic lethality. The Col4a2-HaloTag mice were homozygous viable and fertile, with the fusion protein correctly localizing to BMs across embryonic and adult tissues, confirming that the tagged protein retains normal function. Using two-color pulse-chase labeling in Col4a2-HaloTag embryonic salivary glands—an established ex vivo model of branching morphogenesis —we mapped BM turnover across an entire developing organ. These assays revealed striking spatial heterogeneity: turnover was slow near the primary duct but markedly faster around the end buds. Notably, turnover decreased at nascent clefts, suggesting that local BM stabilization supports cleft formation and branching morphogenesis. Preliminary data also indicate that BM turnover dynamics changes across developmental stages. To identify molecular regulators of this spatial patterning, we are profiling the expression of ECM-remodeling enzymes and their inhibitors using expansion-assisted iterative fluorescence in situ hybridization (EASI-FISH). Early results show that Timp3 transcripts are highly enriched in mesenchymal regions in the vicinity of BM domains with low turnover rates, suggesting that transcriptional regulation of ECM remodeling activities modulates local BM stability. Together, these findings reveal that patterned BM turnover is a key mechanism guiding organ shape formation.

B216/P0211
Substrate Stiffness Regulates Tight Junction Formation and Epithelial-to-Mesenchymal Transition in Renal Epithelial Cells
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[Objective] Renal fibrosis, the convergent endpoint of chronic kidney disease (CKD), affects cell morphology and function. In vivo, the progression of renal fibrosis is correlated with a concomitant increase in tissue stiffness. However, conventional in vitro experiments are often conducted on glass or plastic devices, which are orders of magnitude stiffer than renal tissues. A growing body of evidence indicates that extracellular matrix (ECM) stiffness is a critical regulator of fundamental cellular processes including proliferation, morphology, and adhesion. However, the mechanism by which ECM stiffness specifically modulates the renal cell function and contributes to kidney diseases remains poorly understood. We used a polyacrylamide (PAA) gel-based system

to investigate how substrate stiffness influences renal epithelial cell morphology and function, and whether the in vitro observations can mimic fibrotic kidneys in vivo. [Methods] To simulate the physiological stiffness of the kidney in vitro, HEK293A cells were cultured on either PAA gels with normal kidney stiffness (0.9 kPa), fibrotic kidney stiffness (5 kPa), or glass. To generate fibrotic kidneys in vivo, we employed an ischemia-reperfusion injury model. We then examined the morphology and function of the cells and tissues. [Results] HEK293A cells on 0.9 kPa gels formed a monolayer without apparent intercellular spaces, which was accompanied by continuous co-localization of ZO-1, a tight junction (TJ) marker, and cortical F-actin. In contrast, cells on 5 kPa gels and glass left intercellular gaps when forming a monolayer, with discontinuous ZO-1 localization. A significantly higher level of permeation by 4 kDa fluorescein-dextran through a monolayer was observed on 5 kPa gels than on 0.9 kPa gels. Furthermore, cells on 5 kPa gels and glass showed increased protein expression levels of the epithelial-to-mesenchymal transition (EMT) marker α-SMA and phosphorylation of p38 MAPK relative to cells on 0.9 kPa gels. Pharmacological inhibition of p38 MAPK partially restored disrupted ZO-1 localization and reduced the α-SMA expression in cells on stiff substrates. In vivo, fibrotic kidneys exhibited elevated α-SMA expression levels, activated p38 MAPK, and reduced ZO-1 accumulation in the proximal tubules, which was consistent with the in vitro findings. [Conclusion] ECM stiffness is a critical regulator of TJ formation and EMT-related molecular profiles in renal epithelial cells. Our PAA gel-based system provides an effective in vitro model that closely simulates the in vivo mechanical environment, offering a more physiological tool for studying kidney diseases.

B229/P0224

The Absent in Melanoma 2 (AIM2) inflammasome is required for lamellipodia formation and fibroblast migration

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Fibroblast migration is a vital component of wound healing, where fibroblasts move to the wound site and deposit matrix proteins such as collagen and fibronectin into the wound matrix. Primary human fibroblasts exhibit migratory plasticity when moving through different 3D environments. Specifically, the F-actin binding protein tropomyosin 1.6 (Tpm 1.6) helps to activate the actomyosin machinery responsible for pulling the nucleus forward through the narrow openings in covalently crosslinked 3D ECMs, switching cells from low-pressure lamellipodia to high-pressure lobopodia-based migration. To determine how Tpm 1.6 controls migratory plasticity, we first identified Tpm 1.6 interacting proteins in human dermal fibroblasts. Our preliminary findings revealed an interaction between Tpm 1.6 and absent in melanoma 2 (AIM2), a cytosolic DNA sensor and an inflammasome-forming protein involved in wound healing and fibrosis. To investigate the potential role of AIM2 in migratory plasticity, we generated AIM2-deficient mouse embryonic fibroblasts (MEFs). Live cell imaging revealed that AIM2-deficient MEFs crawled across 2D surfaces much slower than their wild-type counterparts. Morphological and cytoskeletal analysis of AIM2-KO MEFs revealed disrupted lamellipodia at the leading edge, suggesting a role for AIM2 in actin remodeling and cell migration. In human dermal fibroblasts migrating on 2D surfaces, a small amount of AIM2 was enriched at the cell's leading edge, with the bulk of the protein targeted to the nuclear envelope and the perinuclear region, with occasionally filamentous appearance in the cytoplasm. In contrast, AIM2 became more uniformly distributed within the cytoplasm of primary human fibroblasts in both 3D collagen and cell-derived matrix (CDM), suggesting that AIM2 localization can be highly responsive to the physical environment. Finally, we transiently knocked down AIM2 in primary human fibroblasts and compared their 2D migration to control cells. Immunofluorescence staining with the lamellipodia marker cortactin revealed a significant reduction in lamellipodia formation in AIM2-deficient cells. Collectively, these findings show that the localization of AIM2 is responsive to the cell's

physical matrix environment and has an unexpected role governing lamellipodia formation and fibroblast migration.

B230/P0225
The healthy human breast extracellular matrix: a morphological foundation for biomimetic organoid development
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The extracellular matrix (ECM) of the human breast is a dynamic environment that orchestrates epithelial growth, differentiation, and morphogenesis, yet its structural features in healthy tissue remain scarcely defined. Our broader goal is to establish a physiologically faithful mammary organoid model by grounding its design in the native ECM. As a first step, we performed a morphological analysis of healthy breast tissue using histological staining to characterize collagen fiber distribution, stromal composition, and epithelial organization across the menstrual cycle in both the extralobular and lobular compartments of the tissue. Breast tissue samples were obtained from healthy women aged 20–59 years undergoing reduction mammoplasty (KULeuven S68111). Donors were grouped according to their menstrual phase: follicular (F) and luteal (L). Samples were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned, stained with Masson's Trichrome (F: n=5 and L: n=4) Hematoxylin-Eosin (F: n=5 and L: n=4), and Alcian Blue (F: n=1 and L: n=2), then scanned and analyzed with ImageJ. Masson's Trichrome revealed a visually higher proportion of extralobular fibers in the follicular phase compared to the luteal phase (F: 74.97 ± 10.61% ; L: 47.46 ± 32.43%), while adipocytes appeared more abundant in the luteal phase (F: 49.37 ± 34.19% ; L: 21.64 ± 12.23%), though neither with statistical significance. The lobular compartment (F: 8.75 ± 7.35% ; L: 8.10 ± 4.81%), epithelial content (F: 3.39 ± 2.92% ; L: 2.92 ± 3.26%), and intralobular fibers (F: 35.45 ± 13.87% ; L: 42.55 ± 4.22%) remained stable across phases. Hematoxylin-Eosin confirmed comparable epithelial thickness (F: 12.31 ± 2.52 µm ; L: 14.21 ± 5.01 µm), acini lumen area (F: 8578 ± 16509 µm² ; L: 381 ± 389 µm²), and peripheral lobular stromal thickness (F: 34.23 ± 12.70 µm ; L: 22.96 ± 7.57 µm). These observations indicate that epithelial and lobular architecture is largely conserved across menstrual phases. Exploratory Alcian Blue staining suggested compartment-specific glycosaminoglycan (GAG) patterns, with extralobular GAGs more abundant in the follicular phase than in the luteal (F: 68.5% ; L: 20.9 ± 6.3%), while intralobular GAGs were comparable (F: 39.1% ; L: 42.3 ± 9.6%). Given the small sample size, these results remain preliminary and descriptive. In conclusion, our preliminary analysis indicates that epithelial and lobular architecture appear conserved across menstrual phases, while stromal compartments may undergo phase-dependent fluctuations in fibers and adipocytes. GAG distribution showed compartment-specific differences, highlighting the importance of histological staining as a foundation for biomimetic organoid model development.

B220/P0215
Understanding how structural components shape cellular and tissue dynamics in live mice
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Cellular and tissue dynamics are strongly influenced by their structural matrix. In the vasculature, endothelial cells secrete Collagen IV, the predominant component of the basement membrane (BM), providing the

foundation for their architectural support. Previous work in our lab has identified the behaviors by which endothelial cells mature and maintain skin vasculature in vivo (Kam, 2023), but how endothelial cells interact with their secreted BM to shape vascular architecture remains unclear. This gap in knowledge is due to limitations in tracking BM's remodeling in its native context over time. To overcome this challenge, in collaboration with the Fujiwara lab, we developed an approach to simultaneously track endogenous components of BM (i.e. GFP-tagged Collagen IV alpha 2 chain) and endothelial cells during skin vasculature maturation in live mice. Preliminary data demonstrate that as endothelial cells transition from migratory to stationary, Collagen IV deposition increases. Despite this trend, Collagen IV turnover by endothelial cells declines as the vasculature matures as shown by Fluorescence Recovery After Photobleaching (FRAP) approaches. Lastly, Collagen IV requires interaction with endothelial cells as upon physiological or induced cell migration, remnant Collagen IV sleeves are unexpectedly degraded. Ongoing approaches explore the matrix metalloproteinases responsible for the enzymatic turnover in the early stages of vasculature maturation as well as delving into integrin mediated anchorage of endothelial cells to Collagen IV. By further investigating the spatiotemporal regulation of the BM organization as it supports endothelial cells in developing neonatal vasculature as well as the adaptive mechanisms that stabilize the adult plexus, our work advances the understanding of how structural matrix components shape tissue architecture and support cellular behaviors.

B226/P0221

Unveiling the Consistency: Evaluating EHS-ECMs in Stem Cell and Tumoroid Cultures Amidst Protein Composition Variability

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Engelbreth-Holm Swarm mouse sarcoma-derived extracellular matrices (EHS-ECM), commonly known as Geltrex™Flex or Matrigel®, are extensively utilized in stem cell research and tumor modeling due to their ability to provide essential biological cues that promote cell attachment, growth, and function. Despite their widespread use, EHS-ECMs are often cited as a potential source of variability due to their complex composition. This study compares the function of various commercial EHS-ECMs in multiple applications and characterizes their protein composition to describe any differences. First, we compared the growth and maintenance of human pluripotent stem cells, which are commonly cultured on EHS-ECMs. Cell growth was equivalent over multiple passages and cell colonies displayed a similar morphology. Flow cytometry with dual staining for the pluripotency markers OCT4 and NANOG showed a high expression (≥97%) across matrices. Further analysis by qRT-PCR confirmed high self-renewal gene expression and low expression of germ layer differentiation gene profiles, indicating that cells remained undifferentiated when cultured on the different matrices. Next, we explored primary tumor-derived organoids which are dependent on ECM composition to maintain morphology, growth and gene expression of interest. Human lung and colorectal patient-derived tumoroid lines were grown in 3D using embedding and suspension culture techniques. In each cell model we observed similar morphology and growth between matrices . Additionally, transcriptomic and genomic alteration analyses showed a high Pearson's correlation between tumoroid lines grown in the different EHS-ECMs (≥95%) after multiple passages, indicating that the matrices maintained the cell model phenotype of interest. Lastly, we characterized the composition of multiple lots of each EHS-ECM by mass spectrometry (MS). MS revealed that the primary constituent proteins—laminin, nidogen, and heparan sulfate proteoglycans—were present at similar concentrations among EHS-ECMs, and they collectively accounted for over 95% of the total protein content in the matrix. Variability between lots and vendors was primarily seen in the remaining 5% protein content which comprised of a diverse array of low abundance proteins (>5,000), including growth factors. Our findings indicate that the variability of the numerous but low abundance proteins was insufficient to drive significant differences in cell culture performance. Further characterization of

these differentially expressed low abundance proteins using gene ontology analysis is underway to elucidate their impact at the cellular level.

Cell Adhesion, Migration, and the Extracellular Environment - Extracellular vesicles and exosomes

B236/P0231
Analyzing Disease Biomarkers in Extracellular Vesicles Using Simple Western™ Automated Western Blot Platform
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Extracellular vesicles (EVs) play a vital role in intercellular communication and disease processes, providing a novel source for biomarker discovery. Understanding EV protein expression can provide insights into disease mechanisms and potential therapeutic targets. However, analyzing protein expression from EV samples is challenging due to the limited sample material and low protein expression levels. This makes antibody validation on EV samples critical to successful EV characterization. Simple Western™ automated western blotting systems are specialized to analyze protein expression in low sample volume and complex sample types, making them ideal for EV research. Development and validation of antibodies on EV samples for Simple Western will streamline and accelerate interrogation of EV-associated disease biomarkers. To improve the study of EV biomarkers, first we sought to validate antibodies on known EV samples isolated from various cell lines using the Simple Western platform. EVs were isolated using established guidelines. We implemented a rigorous antibody-validation criteria including multiple antibody concentrations, stringent signal-to-noise ratios, minimum and maximum peak height, and baseline, to ensure reproducible success. Markers tested in this initial study included canonical EV markers like CD9, CD81, Alix, Calnexin, and TSG101. Once antibody validation criteria was established on EV samples, we sought to identify and validate antibodies for critical disease biomarkers including cancer (e.g. CD73, EpCAM, Galectin-1), kidney disease (A2M, ANXA2, Galectin-3, UMOD), neurological disease (ANXA11, Annexin V, EphB2, Flotillin-1), and infectious disease (ANXA1, Cyclophilin A, THBS-1). Our study shows the importance of antibody validation on EV samples and a rigorous validation criteria for successful Simple Western assays. Antibody validation on Simple Western will further enable high throughput screening of EV-associated proteins for potential diagnostic application in cancer, kidney, neurological, and infectious diseases.

B233/P0228
Lens injury leads to vimentin release into the wound microenvironment through type III unconventional protein secretion to navigate the repair response
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Extracellular vimentin (eVim) function has been linked to promoting wound healing and fibrosis in response to lens injury. However, it is not known how eVim becomes secreted into the extracellular environment in response to injury. Protein secretion can occur through conventional secretion, whereby proteins with an N-terminal signal peptide enter the ER and transport to the Golgi to be exported out of the plasma membrane or unconventional protein secretion (UPS), which involves secretion of proteins that lack a signal peptide and

bypass the Golgi/ER. Vimentin lacks an N-terminal signal peptide, and depending on context and cell type can involve ER/Golgi function and/or UPS. Here, we investigated which mechanism of secretion, ER/Golgi and/or UPS, vimentin is released through, for its extracellular function in response to lens wounding. For these studies, a clinically relevant ex-vivo chick lens wound healing model was used which mimics cataract surgery to create wounding. To determine the vimentin-specific secretion mechanism, lenses were preincubated with vehicle, conventional ER and Golgi secretion inhibitors (Monensin, Brefeldin A) or inhibitors specific to type III UPS vesicular secretion (NH4Cl, Rapamycin) prior to surgery and post-surgery in culture. Media was collected day 1 post injury, concentrated using Amicon ultra centrifugal filters, and analyzed by western blot for eVim expression and normalized using ponceau red staining. Blocking the conventional ER/Golgi secretion pathway did not show any major change in eVim release compared to control. In contrast, inhibiting Type III UPS pathways, with NH4Cl or Rapamycin treatment, led to a reduction in vimentin secretion. Our findings begin to map a critical regulation step leading to eVim function in the extracellular environment in response to lens injury, revealing that eVim becomes secreted through type III UPS for its function. Targeting vimentin secretion may be a potential attractive therapeutic target for modulating eVim function in response to lens wounding.

B237/P0981

Leveraging blebbisomes, a new type of large extracellular vesicle, for therapeutic delivery

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Extracellular vesicles (EVs) are emerging as powerful tools for therapeutic delivery due to their intrinsic biocompatibility and ability to shuttle bioactive cargo between cells. However, the conventional EVs utilized for this are limited by their small size, inefficient cytosolic delivery, and unpredictable uptake across target cell types. We have recently identified a novel class of exceptionally large EVs—termed blebbisomes—that range from 5–20 µm in diameter and are generated through dynamic membrane retraction events during cellular migration. Blebbisomes are not detached cellular blebs, but rather enclosed vesicles that themselves exhibit blebbing and autonomous motility, retain key organelles including functional mitochondria, and engage in vesicle exchange. Our preliminary studies show that blebbisomes can enter cancer and endothelial cells, internalize extracellular cargo, and be transfected with plasmid DNA, suggesting they may serve as scalable, versatile platforms for cargo delivery. These findings establish blebbisomes as a novel, large EV population with unique origins, molecular composition, and robust uptake capabilities. Their size, composition, and internalization pathways highlight their potential as vehicles for therapeutic delivery of nucleic acids, proteins, and/or small molecules.

B234/P0229

Mucins form spontaneous self-packaging birefringent spheres in the C. elegans spermatheca

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The heterogenous collection of biological material that comprises cells and tissues can be well-mixed, or organized into polymers, sheets, and three-dimensional objects including polygonal crystals, and spheres. We visualized the popular model animal C. elegans using polychromatic polarization microscopy, in which the polarizer and analyzer of conventional polarization microscopy are accompanied by a special spectral state generator and an achromatic quarter wave plate, respectively. We explored the anatomy of C. elegans for

materials with a degree of local order high enough to afford them with birefringence. In addition to well-characterized muscles and intestinal granules with birefringence, we serendipitously discovered novel spherical birefringent structures in the spermatheca. Unlike the parallel or antiparallel arrangement of the linear particles that comprise muscle and most crystals, the objects comprising the novel birefringent material have a radial in three dimensions, or “spherulitic” arrangement. The globules, which ranged in size from undetectable to 4 microns in diameter, appear as a duplicated color wheel, exhibiting all colors of the visible spectrum per 180 degrees, such that blue is at 90 and 270 degrees, for example. Subjectively speaking, they are mesmerizingly beautiful. The appearance of these objects depended on development of the spermatheca but not on sperm, and was more frequent in hermaphrodites mated to males. The birefringent material is robust to protease treatment and highly enriched for mucins. The novel material was mechanically stable, elastically flattening and returning to spherical upon compression and relaxation. Interestingly, the center of mucin globules lost birefringence upon compression, while the perimeter, which adopted straight edges, remained birefringent, resembling beveled glass. Due to the globules’ chemical makeup and anatomical location, we hypothesize that they comprise a lubricant that aids passage of the fertilized oocyte through the valve that connects the spermatheca to the uterus, and passage of sperm back from the uterus to the spermatheca. Future work will define the apoprotein of the mucin and genetic control of glycosylation, the effects of loss of these genes’ function on reproduction, and the ultrastructure of the globules.

B232/P0227

Role of stress-dependent reduction of Murine Leukemia Virus-Gag in HT22 cells

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Exosomes contain nucleic acids (such as microRNAs) and proteins, and they are crucial mediators of intercellular communication by transferring these biomolecules to recipient cells. In the central nervous system, exosomes have been implicated in the propagation of pathological factors associated with neurodegenerative diseases. Oxidative stress, meanwhile, is a well-established phenomenon known to accelerate neurodegenerative disease; however, the effects of oxidative stress on the molecular cargo of neuron-derived exosomes remain unclear. This study aimed to investigate how oxidative stress influences protein encapsulated within exosomes secreted from HT22 cells, a murine hippocampal neuronal cell line. Following hydrogen peroxide (H2O2) treatment of HT22 cells, we isolated exosomes using ultracentrifugation. Two-dimensional gel electrophoresis and MALDI-TOFMS analyses identified factors whose expression levels were altered, among which a decrease in murine leukemia virus Gag (MLV-Gag), an endogenous retrovirus, was observed. Subsequent assessment of intracellular MLV-Gag expression demonstrated a reduction in protein levels concomitant with an upregulation of gene expression in response to H2O2 treatment. We investigate the functional impact of MLV-Gag reduction on HT22 cells, Knockdown experiments using small interfering RNA (siRNA) were conducted. These experiments revealed a decrease in HT22 cell viability, and flow cytometric analysis further demonstrated that the reduction of MLV-Gag expression triggered cell death. Overall, we demonstrate that oxidative stress decreases the levels of MLV-Gag in HT22 cell-derived exosomes and provide evidence that this reduction induces cell death. These findings suggest that endogenous retroviral elements may contribute to oxidative stress-induced neuronal cell death. Further elucidation of the physiological functions of exosomal MLV-Gag may contribute to the initiation and progression of neurodegenerative disease.

B235/P0230
Unconventional secretion of angiogenic sonic-hedgehog-containing extra-large exosomes (SHH-XLEVs) is stimulated via the PI3K-Rab18-GDP pathway
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Extra-large exosomes (XLEVs), with diameters >600 nm, play essential roles in special types of intercellular communications. However, information on the molecular mechanism underlying their roles and on their relevance remains scarce. Here we show that PI3K–Rab18-GDP signaling facilitates the secretion of XLEVs from human mesenchymal stem cells (hMSCs) and fibroblasts. These XLEVs contain a large amount of sonic hedgehog (SHH) protein and promote angiogenesis. Rab18 serves as the master facilitator of this secretion only in its GDP-bound form, which can be enriched by the Rab inhibitor CID1067700 or by the PI3K agonist SF1670. Rab18-GDP accumulates in the cell center and develops SHH-XLEV precursors from perinuclear endosomes. Rab18-GDP recruits heat shock protein 90α (Hsp90α) and neutral sphingomyelinase 2 (nSMase2) and facilitates vertical secretion of SHH-XLEVs with an Hsp90α corona from the cell center. This secretory machinery may play an important role in developmental and regenerative PI3K-mediated angiogenesis.

Cell Aging and Regeneration - Cell and tissue regeneration

B160/P1286
A novel strategy for epigenetic and metabolic reprogramming of aged human mesenchymal stem cells
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Adverse cellular changes in DNA, mitochondria, and protein processing occur with cellular aging. Epigenetic and metabolic reprogramming are two strategies to slow/reverse these processes. Apoptosis Signal-Regulating Kinase 1 (ASK1) is a MAP kinase whose inhibition has been shown to slow degenerative diseases. Activation of Sirtuin 6 (SIRT6), a NAD-dependent histone deacetylase epigenetically enhances longevity. Given these respective effects, this study explores the use of an ASK1 inhibitor (Selonsertib) and a Sirtuin 6 activator (MDL-800) together to regeneratively reprogram aged human bone marrow-derived mesenchymal stem cells (hBM-MSCs). Spheroidal micromasses made from hBM-MSCs of younger (27 ± 6yo) and older (79 ± 5yo) donors of both sexes (n=4-5 per group,17 total) were used. Transforming growth factor-beta 3 (TGFβ3) treatment was added to induce differentiation and treatment groups included TGFβ3 alone or in combination with Selonsertib, MDL-800, or both (dual treatment). Controls included no TGFβ3. Every 2 days, fresh media with treatments were added until day 10 when masses were collected for RNA/protein isolation and histology. Proteomics by Sequential Window Acquisition of all Theoretical Mass Spectra was used. Analysis using principal components, differential expression and Gene Set Enrichment (GSEA) analyses with UNI-PROT was performed. Significant downregulation of gene sets associated with ECM and ATP synthesis with upregulated DNA damage were observed in older untreated hBM-MSC micromasses. Confirmation of chondrogenesis with the addition of TGFβ3 treatment was indicated by increased enrichment of gene sets such as protein translation, ECM and decreased mitochondrial activity in both age groups. This result was confirmed using histology and alcian blue staining (proteoglycan) of the micromasses. Comparison of the effects of TGFβ3 treatment of micromasses from aged donors to younger donors showed significant downregulation in mitochondrial processes, NAD, Lipid/Fatty-acid metabolism and peroxisomes. Upregulated gene sets included

cell signaling, and endoplasmic reticulum/golgi processes. When the dual treatment was administered with the TGFβ3, these gene sets reverted to similar levels as those observed in the younger donors. In addition, dual treatment significantly upregulated NAMPT (Nicotinamide phosphoribosyltransferase) 3.5 fold in older hBM-MSCs. Functionally, ECM staining of donor micromasses for denatured collagen (using binding to the Gly-X-Y motif) showed increased staining with age that decreased with the dual treatment. Together these results demonstrate the dual treatment of Selonsertib and MDL-800 administered to older hBM-MSCs restores cellular and molecular processes to those of younger hBM-MSCs.

B162/P1288

Assessment of the environmental factors affecting DNA and chromatin integrity and stability, on leukocytes

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Introduction: Different environmental effects can affect the cellular chromatin composition leading to DNA fragmentation. Moreover, environmental effects can induce netosis in granulocytes and cause DNA double strand breakage in macrophages. Aims: We aimed to investigate the effect of the laboratory conditions encountered during processing of blood samples on DNA stability, netosis markers, DNA double strand break and cellular nucleic acid content. Materials and methods: K3EDTA blood samples (n=6) were collected. In addition to the temperature (4°C, 24°C) and light (dark, sunlight) we also examined the time since blood collection (0,2,4,6h). Mononuclear (M), granulocyte (G) cells, plasma fractions were separated. Comet assay, telomere length determination and DNA methylation of LINE-1 was assessed on cells. Nanopore and exome sequencing was used to analyse differentially methylated regions and mutations. CfDNA concentration was measured. DNA double break repair protein (gamma H2AX (gH2AX)), netosis markers (citrullinated H3 (H3Cit) and myeloperoxidase (MPO)) were labelled with antibodies and measured with flow cytometry. The cellular nucleic acid content were measured using acridine orange (AO) and flow cytometry. Results: After 2, 6 hours of incubation of M cells exposed to daylight at RT, more than 200%, significant DNA breaks were observed compared to control. The gH2AX positive cells where higher (30/100000 counted cell) in cells that were exposed compared to control (17/100000 counted cell). The change was significant (p=0.0111). Shortening telomere length was measured in both cell types in proportion to time. Global LINE-1 methylation was moderately affected by the above effects. CfDNA concentration increased (~10%). More H3Cit positive cells were measured (3835/100000 counted cell) in samples that were exposed to sunlight for 2 hours compared to control (2743/100000 counted cell). The number of positive cells decreased at the 4 hour time point. Moreover, no notable difference were observed in nucleic acid content in cells that were exposed to sunlight compared the ones stored in the dark. Exome sequencing showed difference in the mutational pattern of samples that were stored at different conditions compared to control. Conclusion: All in all, conditions before blood processing must be considered, especially the elapsed time and higher temperature.

B161/P1287

Building a spatiotemporal framework for Wnt-regulated cell and tissue dynamics during early limb blastema formation in limb regeneration in axolotl salamander

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The growing incidence of appendage amputations makes it increasingly important to study tissue regeneration mechanisms in a vertebrate model organisms that can regrow appendages, as this knowledge could inform clinical treatments in humans. Limb regrowth in axolotls depends on the coordination of individual cellular dynamics such as proliferation, migration, apoptosis, and dedifferentiation, amongst various other behaviors, to form a cone-shaped accumulation (blastema). Several signaling pathways have been identified as playing a crucial role during limb regeneration, and of these, we focus on the Wnt program, since its inhibition halts blastema accumulation, and thus regeneration. Yet precisely which cellular dynamics are regulated by the Wnt pathway have yet to be pinpointed. Moreover, despite the importance of cell behaviors during regeneration, the spatiotemporal cellular contributions to blastema formation and, ultimately, limb outgrowth, have yet to be detailed. To address this gap in information, we aim to construct a dataset capturing cell and tissue dynamics during this key stage of regeneration using novel histological methods, atomic force microscopy, and computational models of a regenerating limb in both control and Wnt-inhibited cases. Data on cell positional information and dynamic rates (such as mitotic division rates and angle distributions) inform a biophysical model of the regenerating limb made up of the mesenchyme tissue surrounded by a deformable, elastic epithelial layer. Preliminary model simulations informed us that we need to assume softening of the distal epithelium for the model to recreate a cone-shaped blastema of similar size and shape to what we observe in our control groups. To investigate how the Wnt signaling pathway contributes to tissue and cell behavior, we leverage a Porcupine enzyme inhibitor, C59, to inhibit the secretion of all Wnt signaling ligands, and by extension, inhibit limb regeneration. Preliminary results revealed significant differences in both cell and tissue morphometrics in the blastema in the Wnt-inhibited treatment group compared to the control treatment groups. This combined experimental-theoretical approach will provide a framework for understanding how Wnt signaling influences the formation of the early accumulation blastema at multiple levels of organization and how key cellular behaviors contribute to its formation.

B157/P1283

ERK Activates the Head Regeneration Gene Regulatory Network in Hydra vulgaris

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In highly regenerative animals, patterning pathways are deployed to rebuild lost body parts. Although the injury response activates similar transcription factors (TFs) and signaling pathways across animals, recovery from injury varies from scarring to complete restoration. Because the gene regulatory networks (GRNs) that trigger regeneration are largely unsolved, we do not yet fully understand how patterning pathways are activated by injury. Constructing the Hydra vulgaris regenerative GRN will serve as a point of comparison to animals with a variety of regenerative abilities, revealing what conserved components are responsible for scarless, robust healing. Hydra vulgaris is a freshwater cnidarian polyp that is capable of whole-body regeneration. Its main body axis is maintained through the head organizer that is formed by Wnt signaling. I am testing the model that a bisection injury in Hydra vulgaris activates ERK, which then activates Wnt transcription during injury and regeneration to form the head organizer. Regeneration assays reveal that ERK is required for rapid head regeneration. p-ERK levels rapidly rise after injury, then fall over the course of 12 hours. Further, RNA-seq and fluorescence in situ data supports the model that injury activates ERK signaling, which in turn activates the transcription of injury response, Wnt, and other patterning pathway genes to repattern the Hydra head. Additional perturbation experiments in a related Hydra species, Hydra oligactis, which is capable

of head regeneration but has significantly delayed Wnt activation after injury, will reveal the dynamics and degree of conservation of the ERK-induced head regeneration network.

B158/P1284
Induction of partial reprogramming to senescent cells using Xenopus laevis egg extracts
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Aging is a complex process that causes morphological alterations and functional decline in humans, often associated with the development of chronic diseases. While aging has been traditionally regarded as an irreversible process, research with iPSC reprogramming factors has shown a promising approach to rejuvenate aging-related phenotypes. This rejuvenation is believed to occur by restoring the epigenetic information that is lost during aging. This process, known as partial reprogramming, holds great promise for treating age-related diseases. Nuclear reprogramming of somatic cells, including cells derived from aged animals, can be efficiently achieved by oocyte/egg factors. In this research, we tried to induce partial reprogramming in cells from aged mice by egg factors. We then focused on a Xenopus egg cell-free extracts system that can induce a part of the reprogramming events in mammalian cells without genetic modification. We first established tail tip fibroblasts (TTFs) from aged and young mice. Aged TTFs showed lower cell proliferative rates than those from young individuals, as well as accumulation of DNA damage in nuclei and decreased expression of lamin B1, which are characteristic of cellular senescence. Next, we attempted to introduce Xenopus egg factors to TTFs for inducing partial reprogramming. The cell membranes of TTFs were reversibly permeabilized by non-ionic detergent, digitonin, and we optimized this reversible permeabilization process by the propidium iodide incorporation assay. These experiments identified a condition in which, TTFs were permeabilized for uptake of egg extract factors and can be re-culture after the extract treatment. Immunostaining analyses showed that oocyte/egg-specific factors such as linker histone H1.8 and lamin LⅢ were incorporated into the nuclei of mouse aged cells during incubation in egg extract. In addition, when the extract-treated aged cells were re-cultured, an increase in the number of replicating cells and a change in cell proliferative ability were observed as compared to the control. These results indicate that the egg extract treatment can induce the incorporation of oocyte/egg-specific proteins involved in nuclear reprogramming to aged cells, and that the properties of aged cells change after re-culture, reminiscent of partial reprogramming.

B163/P1289
Orphan nuclear receptor gene regulates cardiomyocyte maturation-dedifferentiation-proliferation transitions in zebrafish heart regeneration
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Myocardial infarction is the leading cause of death worldwide. It inflicts perpetual damage to adult mammalian hearts and elevates the risk of pathological cardiac remodeling triggering heart failure. Although a narrow regenerative window exists in neonatal hearts, adult mammalian hearts are unable to repair the damaged tissue owing to the post-mitotic nature of their cardiomyocytes (CMs). Contrarily, upon ventricular amputation, mature CMs of adult zebrafish heart can dedifferentiate to an immature state and proliferate from 3 days post amputation (dpa). Thus, zebrafish heart regeneration studies have helped identify major molecular players that

can be targeted to stimulate regenerative response in mammalian hearts. Using single nucleus RNA-sequencing of uninjured and injured 3 dpa hearts, our lab recently showed heterogeneous CM states associated with regeneration which regulate stress response, sarcomere remodeling, contraction, and proliferation. Functionally, these CMs can be grouped into four subtypes – mature, dedifferentiated, proliferating, and injured CMs. The immediate early-response orphan nuclear receptor gene nr4a3 is upregulated in dedifferentiated CMs of the regenerating heart. Additionally, our lab recently showed that the gene cited4a is essential to maintain mature CMs for heart contraction as regeneration proceeds. Maturation-deficient cited4a mutant hearts downregulate nr4a3 post-injury. Hence, we hypothesize that nr4a3 regulates CM dedifferentiation program in regeneration, downstream of cited4a. Using in situ hybridization, we showed that nr4a3 upregulation in dedifferentiated CMs of regenerating hearts is an injury-induced response. To further characterize its role, we generated nr4a3 loss-of-function mutants using CRISPR/Cas9 mutagenesis. Transcriptomic analyses revealed dysregulated metabolism and sarcomere disruption in mutant hearts post injury. Electron microscopy showed prevalence of fragmented mitochondria and misaligned myofibrils with irregular banding pattern in mutant hearts as opposed to wildtype (WT) hearts at 3dpa. Mutant hearts exhibit a pro-regenerative phenotype with increased proliferation compared to WT hearts. These findings position nr4a3 as a critical regulator that prevents excess dedifferentiated CMs from uncontrolled proliferation, pushing them towards a re-maturation fate to support heart function during regeneration. Loss of nr4a3 disrupts this regulation, predisposing mutant CMs towards increased proliferation but ultimately can cause metabolic defects and impair heart function. Thus, nr4a3 serves as an essential checkpoint that finetunes the balance between dedifferentiation-proliferation for regeneration vs maturation for maintaining heart function.

B166/P1292

Partial reprogramming by Yamanaka factors in post-mitotic cells extends lifespan in C. elegans.

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Previous studies with progeric mice demonstrated that cyclic induction of the Yamanaka factors, a set of transcription factors that reprogram differentiated cells into pluripotent stem cells, can extend the lifespan of the animals; however, the effects on non-progeric models have been less-extensively studied. Further, it is not known whether the lifespan extension provoked by partial reprogramming with Yamanaka factors requires cell division. To examine the effect of partial reprogramming in post-mitotic cells of non-progeric animals, we assessed whether expression of the C. elegans orthologues of Yamanaka factors alter aging and longevity when expressed after somatic cell divisions have been completed. We performed lifespan assays of animals at regular intervals across multiple cyclic induction regimens that varied in duration and frequency. Under optimal conditions, we observed a 50% increase in median lifespan of partially reprogrammed C. elegans ubiquitously expressing Yamanaka factors compared to control animals. These results suggest that partial reprogramming of post-mitotic, differentiated somatic cells can slow aging and increase lifespan in C. elegans. The Insulin/IGF-1 Signaling (IIS) pathway influences metabolism, development, and longevity and its inhibition results in extended longevity across metazoan phylogeny, including in C. elegans. To test whether Yamanaka-factor-induced lifespan extension acts through a mechanism that is dependent on IIS signaling, we investigated the requirement for DAF-16/FoxO, the transcription factor that mediates IIS, on lifespan extension in these animals. We found that a daf-16(-) mutation did not reduce lifespan extension resulting from partial reprogramming, suggesting that this effect acts independently from the IIS pathway. Our findings suggest that Yamanaka factor-induced lifespan extension does not require cell division in somatic cells and acts through an IIS-independent mechanism. Further studies will address the mechanisms through which Yamanaka factors act to slow aging in non-dividing cells.

B159/P1285
Studying the Effects of Vitamin B9 (Folic Acid) on Planarian Regeneration
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Tissue and cellular maintenance are essential characteristics of living organisms. Regeneration is the process of an organism replacing damaged and/or lost cells and tissues. The planarian flatworm is well-studied for its remarkable capability of whole-body tissue-renewal. We are interested in testing if folic acid, an essential B-Vitamin, has a role in regeneration. Folic acid has known roles in DNA synthesis, amino acid metabolism, and methylation, each of which may also play a role in regeneration. We are therefore interested in studying if exposure to folic acid, and other B-vitamins, affects the rate of regeneration in the planaria worm, Girardia dorotocephala. To analyze regeneration, we are using a scoring system to monitor features of regeneration including formation of eyespots, pigmentation, auricle development and blastema measurements. In addition, as the worm is regenerating its head, we will also be measuring behavioral features such as light avoidance and motility rates. We are in the beginning stages of this research project and hope to gather input from fellow peers as we start these experiments.

B165/P1291
The Ghost Acoel as a transparent model system for regeneration and stem cell dynamics
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The ability to regenerate missing body parts varies widely across animals. Traditional genetic model organisms have limited regenerative capabilities. In contrast, many animal phyla are capable of whole-body regeneration. This process is thought to require pluripotent adult stem cells, or collectively pluripotent adult cell populations, which allow animals such as planarians, cnidarians, and acoelomorphs to regenerate their entire bodies from a fragment. Despite increasing knowledge of the mechanisms that initiate regeneration and pattern regrowing tissues, we know relatively little about the cell-biological processes involved in whole-body regeneration, and current model organisms impose formidable technical barriers. To understand the cellular mechanisms that underlie whole-body regeneration, it will be essential to develop model systems that are amenable to modern cell biology approaches including genome editing and live imaging. Acoelomorph worms are particularly promising models based on the ability to obtain manipulable embryos under lab conditions and their phylogenetic position as basally branching bilaterians. However, current acoel models are not amenable to live imaging after early larval stages due to tissue opacity, pigment, and autofluorescence. To surmount this barrier, we are developing the Ghost Acoel (Amphiscolops sp.) as a new model for regenerative biology. Ghost acoels are capable of rapid, whole-body regeneration and are transparent throughout their life, making high-resolution live imaging possible even in deep tissues of adult animals. Ghost Acoels lay embryos consistently under lab conditions and reach reproductive maturity at 1.5-2 months. We have reared an inbred lab population of Ghost Acoels for five generations and sequenced the genome of a clonally established lab strain. We are seeking to develop methods for transgenesis and genome editing to establish a transparent bilaterian model for whole body regeneration that is amenable to modern cell and developmental biology tools. Additionally, comparing regenerative mechanisms between Ghost Acoels, other acoelomorphs, and additional phyla will provide insights into conserved and unique features of adult stem cell regulation and whole-body regeneration.

B164/P1290
The Prostaglandin F2a Analog Bimatoprost Activates Hair Follicle Progenitor Cells Through PTGFR-MAPK Signaling
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Bimatoprost, a synthetic analog of prostaglandin F2α (PGF2α), is clinically approved for the treatment of eyelash hypotrichosis due to its capacity to stimulate hair growth, however the underlying mechanisms remain poorly defined. Here, we investigate the cellular targets and molecular pathways through which bimatoprost activates hair follicle growth. Using immunostaining and fluorescence-activated cell sorting for the proliferation marker KI67 in treated and untreated mouse dorsal skin, we identify the secondary hair germ cell population of the hair follicle as the primary target of bimatoprost. To understand the mechanism of action on these cells, we first examined the expression pattern of the PGF2α receptor PTGFR through immunofluorescence staining, demonstrating its presence in the bulge and secondary hair germ structures and their derivatives during telogen. Using targeted lentiviral-mediated knockdown of either PTGFR or its Gq subunit, we determined that these effects are largely mediated through PTGFR-dependent MAPK activation as assessed through western blot analysis of phosphorylated ERK levels relative to total ERK. Together, our findings reveal that the PGF2α analog bimatoprost activates and promotes hair growth by targeting hair progenitor cells through a PTGFR-MAPK signaling axis. These findings allow for a mechanistic foundation for the future targeting of hair follicle progenitor cells in the treatment of alopecic hair disorders.

Cell Aging and Regeneration - Mechanisms of cellular aging and senescence

B131/P1257
Cardiac myosins and their role in sustaining oocyte integrity and developmental competence across their lifespan
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Time is one of the most powerful forces acting on biological systems, yet some proteins persist for months, even years, in cells that remain functionally dormant. This phenomenon defies the common understanding that proteins are short-lived and constantly renewed which raises fundamental questions about how molecular stability contributes to cell integrity. In oocytes, which remain arrested for extended periods, such long-lived proteins may be crucial for maintaining genomic and cytoplasmic integrity across the reproductive lifespan. Recent pulse-chase proteomic analyses from our lab in aging mice identified myosin heavy chain proteins typically restricted to cardiac muscle, Myh6 and Myh7, as exceptionally long-lived in oocytes. Their unexpected presence, in the absence of active transcription and with no previously defined role in the oocyte, presents a biological paradox. This research seeks to elucidate how these long-lived cardiac myosins influence age-related changes in oocyte chromosomal architecture and regulatory dynamics. The pharmacological inhibition of cardiac myosins during in vitro maturation results in a failure to form polar bodies, suggesting a critical and previously unrecognized role in oocyte meiosis. By uncovering the contribution of these long-lived myosins to oocyte development, this work may open new avenues to enhance oocyte longevity and restore reproductive potential with aging.

B128/P1254
Cell-size-driven senescence remodels the epigenome and nuclear biophysics
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Abnormal increases in cell size have been recognized as a driver for senescence and cell cycle exit. Recent studies have linked its mechanism with cytoplasm dilution, osmotic/replication-stress-induced p21 wave, impaired DNA damage response, and genome-dilution-driven proteome remodeling. However, how cell-size changes remodel chromatin and the nuclear biophysical environmental remains unclear. Here we arrest cells in G0/G1 with a CDK4/6 inhibitor and quantify size-dependent epigenetic and biophysical environmental changes in the nucleus. As nuclear size increases, LaminB progressively redistributes from the nuclear envelope to intra-nuclear puncta, with puncta number scaling with nuclear size. This reorganization correlates with an increase in facultative heterochromatin marked by H3K27me3, consistent with LaminB-heterochromatin interactions, and with impaired nuclear envelope integrity observed during replicative senescence. Moreover, combined epigenetic changes and cell size increases during cell senescence can be detected in nuclear biophysical environmental changes using a microrheology probe, nuclear genetically encoded multimeric nanoparticles (nucGEM). Together, our findings indicate that epigenetic remodeling is intrinsically linked to size-induced senescence and is reflected in measurable changes in the nuclear biophysical environment. nucGEM thus provides a sensitive, non-invasive readout of senescence progression.

B138/P1264
Cholesterol affects memory through impacting endothelial health.
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Alzheimer’s disease is the most common form of dementia. Prominent risk factors are high plasma cholesterol levels, however, the underlying molecular mechanisms are less understood. Our lab is interested in the cholesteryl ester transfer protein (CETP), which shuttles cholesteryl esters and triglycerides between plasma lipoprotein particles. Lower CETP activity is epidemiologically associated with longevity and good cognitive performance. We previously found that pharmacological CETP inhibition preserved memory in an AD mouse-model of amyloidosis. Mechanistically, we found that endothelial function underlies the beneficial effects of CETP inhibition. To dissect which CETP activity is detrimental or beneficial for endothelial health, we generated CETP mutants that were already described to be impaired for the shuttling of either the cholesteryl esters or triglycerides. Mutant CETPs were expressed in wild type mice using adeno-associated viruses. Cognitive performance was assessed using the novel object recognition and Y-maze tests. Blood lipoprotein profiles were determined using lipid quantification kits, and purification of high-density lipoproteins (HDLs). Functionality of HDL particles was tested based on their capacity to reduce reactive oxygen species and inflammation. We found that particularly the ability of CETP to transfer cholesteryl esters is detrimental for the protective effects of HDL. Thus, these CETP mutants are useful to further dissect the molecular mechanisms underlying the beneficial effects of pharmacological CETP inhibition on memory.

B145/P1271
Contribution of serum factors to age-associated changes in circadian rhythms
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Introduction: Circadian rhythms are internal timekeepers that regulate various physiological and behavioral processes. With age, circadian rhythms are dampened and advanced, leading to fragmented sleep and earlier activity schedules. These changes not only reduce the quality of life but also increase susceptibility to cognitive decline, chronic diseases, and weakened immune systems. At the molecular level, circadian rhythms are maintained by a transcriptional-translational feedback loop of four core clock genes: CLOCK, BMAL, CRY, and PER, which also regulate thousands of clock-controlled genes across peripheral tissues. Rhythmic gene expression of core clock genes in the periphery remains robust with age. However, circadian gene expression of clock-controlled genes in peripheral tissues dampens or phase-shifts with age, paralleling the phenotypes observed in the elderly. Our previous work showed that human age-dependent blood serum alters rhythmicity of clock-controlled gene expression in cultured fibroblasts, but the responsible serum factors are yet to be identified. The goal of this project is identifying specific serum substances that regulate age-related circadian defects. Method: Blood serum was collected from young (22 week-old) and old (88 week-old) mice to treat mouse 3T3 fibroblast cells. Cells were harvested every 4 hours over two days and RNA was extracted for qPCR analysis of specific clock-controlled genes. To test restorative effects, old serum was supplemented with serum factors enriched in young blood. Results: We established a mouse model that recapitulates age-related circadian decline observed in humans. Using this system, we identified young blood-enriched serum factors that can supplement old serum to restore rhythmic expression of certain clock-controlled genes in peripheral cells. Conclusion: Our results demonstrate that aging induces circadian-relevant changes in mouse serum, as in human serum, supporting their use as a suitable model for investigating the molecular mechanisms and physiological effects of aging serum factors on the circadian clock. Supplementing old serum with some young serum factors restored the rhythmic expression of specific clock-controlled genes, while having no significant effect on the core clock gene BMAL expression. These findings lay the foundation for developing therapeutic strategies to mitigate age-related circadian decline.

B140/P1266
Co-treatment of cardiomyocytes with palmitic acid and particulate matter accelerates senescence through ROS production and mitochondrial dysfunction.
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Cardiovascular disease (CVD) is the leading cause of death worldwide. Unhealthy diet and air pollution are two of the most important environmental factors we are exposed to daily. Epidemiological studies have shown that both increase the risk of premature cardiac aging and are closely associated with CVD. Cardiomyocyte aging can lead to a variety of pathological and physiological conditions. However, the molecular and specific mechanisms by which an unhealthy diet and air pollution jointly affect cardiomyocyte senescence remain unclear. Understanding the molecular pathways and associated mechanisms by which unhealthy diet and air pollution induce cardiomyocyte aging is crucial for developing new therapies for the early prevention and reduction of cardiovascular disease. Cardiomyocytes were co-treated with palmitic acid (PA, 150 μM) and particulate matter (PM, 50 μg/mL) for 24 hours. Western blot analysis showed that combined treatment with PA and PM significantly increased the expression of aging-related proteins p21, p53, p16, and p27, and

decreased pRB expression compared to the control group. The PA+PM treatment group showed strong β-galactosidase and γH2A.X (red) staining. ELISA assays revealed significantly higher levels of IL-6 (senescence-associated secretory phenotype, SASP) in the PA+PM treatment group compared with the control group. PA+PM treatment significantly increased ROS production and the number of cells with low ΔΨm. Combined PA and PM treatment significantly shortened mitochondrial length. Western blot analysis revealed significantly higher expression levels of ROS-related proteins (HIF-1α) and mitochondrial fission-related proteins (Drp1, FIS1, and MFF) in PA- and PM-treated cardiomyocytes compared with the control group, while expression levels of mitochondrial fusion-related proteins (MFN1 and MFN2) remained unchanged. The mitochondrial-targeting antioxidant MitoQ and the mitochondrial fission inhibitor Mdivi-1 significantly mitigated PA- and PM-induced cardiomyocyte senescence. Research findings on cardiac aging will have unique implications for clinical medicine and social well-being in the new era.

B144/P1270

Determining the Effect of Astrocytic Senescence on Developing Neurons

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Developing neurons require cues from their surrounding environment to produce mature dendrites and axons. These cues are often given from the predominant glial cell in the CNS, astrocytes. Astrocytes have multiple support functions for neurons in the CNS, including the support of neurotrophic development but astrocytes are also susceptible to stresses associated with aging. We developed a model of “aged” primary astrocytes that have a significant increase in p21 and p16 and a significant decrease in LMNB1. These “aged” astrocytes display molecular changes consistent with senescence. Additionally, these markers have also been found in the brains of patients with Alzheimer’s Disease. Our immediate goals are to further characterize senescence in both primary and iPSC-derived astrocytes and to quantify the impact that activation of the senescence program has on astrocyte function as it relates to neuronal development, function, and survival.

B137/P1263

DNA Damage Responses in hiPSC-derived Neurons as Drivers of Senescence

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Neurons exhibiting senescence-like features have been identified in both aged and diseased brains. These senescent neurons are postulated to be key contributors to dysfunction in neurodegeneration, but the upstream mechanisms that drive post-mitotic cells like neurons to senescence remain unclear. One candidate driver is the DNA damage response (DDR) normally coordinated by p53 signaling. To study this gap, we have developed a robust iNeuron system derived by human induced pluripotent stem cells (hiPSCs). This approach produces long-lived synchronized cultures. Using this novel system, we will define how (1) DNA damage activates p53 signaling and senescence-like phenotypes; (2) define how pathogenic tau exacerbate p53-dependent DDR; and (3) perform transcriptomic and secretomic profiling to uncover how DDR and tau converge on p53 signaling to drive neuronal dysfunction. Our preliminary data show that transcriptional stress induced by a topoisomerase II inhibitor produces DNA damage in neurons. Unexpectedly, short-term stress triggers aberrant cell cycle progression— a phenomenon not previously reported in long-term human neuronal models— while prolonged stress drives senescent-like features. This suggest that cell-cycle re-entry may

represent a unique DDR mechanism in iNeurons. We hypothesize that transcriptional stress activates a neuron-specific DDR that promotes cell-cycle re-entry and senescence-like phenotypes, and that tau oligomers amplify these defects by impairing p53-mediated repair. These studies will fill a major gap in our understanding of how neuronal DDR disruption drive senescence and dysfunction, offering mechanistic insight relevant to neurodegeneration.

B146/P1272
Establishing a System to Detect Calcium Flux in iPSC-Derived Neuronal Cultures
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Although it is still somewhat enigmatic, neuronal senescence is thought to be a major contributor to neurodegenerative diseases. In proliferatively competent cells, senescence is a state of persistent cell cycle stalling associated with elevated levels of p16. Elevated senescence markers, like p16, are found in brains afflicted with Alzheimer's disease; this is further exacerbated by DNA damage and metabolic stress. However, the neuronal senescent state and its relationship to persistent cell cycle stalling is unclear. Using a tractable induced-neuron (iNeuron) system, created by transducing an inducible Ngn2/Ascl1 vector into the KOLF2.1J iPSC cell line, we created neuron-like cells which express neuronal markers βIII-tubulin, MAP2, NeuN, and Synapsin-1, exhibit calcium signaling and form intricate neurite networks. We utilize this highly regulated system to evaluate neuronal senescence and the consequences of senescence on functional aspects of human neurons. Calcium flux, an indicator of excitatory neuronal activity, decreases in patients with neurodegenerative disease and often results in dysregulated calcium signaling. Therefore, we sought to establish a system to define features of neuronal senescence utilizing the calcium-sensitive fluorescent sensors GCaMP6 and Fluo-4-AM. Comparing senescent and non-senescent iNeurons stimulated with glutamate, we found that DNA damage may result in dysregulated calcium signaling, which further characterizes the neuronal senescent state.

B133/P1259
Expression of Atg8a in Somatic Follicle Cells Suppresses Age-Associated Decline in the Drosophila Ovary
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Aging is defined by a progressive deterioration of tissue and organ functionality during adulthood. The different organ systems in the body do not age at equal rates and the rate of aging can vary dramatically across individuals of the same species who have different "ageotypes." However, across species from invertebrates to mammals, the female reproductive system has consistently been found to be one of the first organs to lose functionality with age. Recent studies have highlighted the importance of ovarian somatic cells in reproductive aging, yet the molecular mechanism of aging in these cells are not fully understood. In the Drosophila ovary, somatic follicle stem cells (FSCs) maintain and establish a supportive environment for successful oogenesis. FSCs can divide to produce prefollicle cells that differentiate into either polar, stalk, or main body follicle cells. Despite the importance of the follicle lineage, little is known about how these cells contribute to age-related defects and the loss of reproductive capacity. We conducted a phenotypic analysis of aged ovaries and found aberrations in the typically well-organized layer of follicle cells that encapsulates germ cell cysts. These defects in the follicular epithelium are coupled with an extended S phase and increased oxidative stress, indicative of a

need for additional time to repair DNA damage caused by oxidation. As germ cell cysts migrate through the germarium, they undergo a quality control checkpoint, in which nearly 50% of germ cell cysts undergo complete degradation. We have identified a loss of this quality control mechanism in aged ovaries, contributing to dysregulation of the aging follicular epithelium. Overexpression of Atg8a, a key autophagy machinery gene homologous to mammalian LC3, specifically in follicle cells rescues age-related decline in the follicular epithelium. Single-cell RNA sequencing of young and old ovaries has corroborated our in vivo findings. Additionally, we have found that there are approximately 14x more genes that are differentially expressed in aging follicle cells, relative to aging germ cells, highlighting the value of investigating the role of age-related changes in somatic cells. Taken together, we have developed a model in which aging leads to an accrual of oxidative stress that triggers a longer S-phase and a loss of a germ cell quality control, which causes follicle epithelium dysregulation and a decline in fertility. Our current studies are using genetics, cell biological approaches, and genomics to further this model and identify genes associated with extending reproductive longevity.

B135/P1261

Investigating the Role of the MICOS Complex in Mitochondrial Integrity and Aging in Drosophila melanogaster

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Mitochondrial cristae support oxidative phosphorylation and ATP production, and their organization depends on the mitochondrial contact site and cristae organizing system (MICOS). While MICOS is essential for maintaining cristae architecture, its role in mitochondrial stability across the lifespan remains unclear. We hypothesize that MICOS dysfunction drives age-related decline through mitochondrial defects. While previous studies have linked MICOS dysfunction to mitochondrial defects in mammalian cells, our work leverages Drosophila melanogaster as a genetically tractable and rapidly aging model to dissect tissue-specific impacts of MICOS loss. This approach enables precise CRISPR-mediated knockouts in muscle and brain tissue and allows us to connect mitochondrial defects to organismal aging on a shorter timescale. To investigate this, we generated muscle-specific CRISPR-Cas9 knockouts of mitofilin (a core MICOS component) and Opa1(a mitochondrial fusion gene) under the Mef2 promoter. Functional decline was measured through locomotion (negative geotaxis) assays, while immunostaining of brain and muscle tissue with the oxidative DNA damage marker 8-OHdG assessed reactive oxygen species (ROS) accumulation. Preliminary findings indicate reduced neuromuscular function and elevated oxidative stress in MICOS deficient flies, suggesting that mitofilin and Opa1 are critical for maintaining mitochondrial integrity with age. Ongoing experiments include lifespan analysis and senescence-associated β-galactosidase staining (SA-β-Gal) to further define how MICOS dysfunction links mitochondrial defects to other hallmarks of aging such as cellular senescence. Defining these mechanisms may inform strategies to slow or prevent age-related disease.

B132/P1258

IRE1 orchestrates ER-Phagy by regulating a novel fusion module

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It is well-established that cellular stress responses, including the unfolded protein response (UPR), are attenuated with age. The UPR kinase/endoribonuclease IRE1 is essential for maintaining endoplasmic

reticulum (ER) homeostasis, but its role in regulating organelle-specific autophagy (ER-phagy) under homeostatic conditions is poorly understood. We discovered that constitutive, stress-independent IRE1 signaling is critical for the completion of ER-phagy. Our preliminary data from senescent cells and IRE1-KO epithelial cells reveal a stalled ER-lysosome fusion step. We identified a novel player in this process, a protein not previously implicated in autophagy, whose expression is dependent on the IRE1-XBP1s axis. We further demonstrate that overexpression of XBP1s or this new protein is sufficient to rescue the ER-phagy defect in IRE1-deficient cells. These findings challenge the prevailing paradigm that IRE1's autophagy-regulating function is reserved solely for conditions of ER stress. We propose that the age-related decline in basal IRE1 activity impairs this crucial fusion step, leading to dysfunctional ER-phagy and proteostatic failure in aging epithelia. Our work identifies a novel mechanism and a tractable therapeutic node, the IRE1-XBP1s axis, which could be targeted to restore cellular quality control and combat age-related diseases.

B130/P1256

Mapping Oxidative Damage on Telomere DNA from Oxford Nanopore Generated DNA Sequence

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Telomeres are long protective DNA repeats that cap each end of the chromosome with a repeat sequence to protect them from damage, but they are highly vulnerable to damage from oxidative stress. The repeat is a hotspot for oxidative stress because it contains many guanine residues, which, when oxidized, form the highly mutagenic 8-oxo-2'-deoxyguanosine (8-oxo-dG). Telomeric 8-oxo-dG also causes cell apoptosis and senescence. Our objective was to develop tools to map 8-oxo-dG on Nanopore derived telomere sequences. First, a training set was developed by amplifying a telomere repeat from a plasmids using primers containing 8 oxo-dG adducts at each of the three dG in the telomere repeat. Each PCR product was sequenced using Oxford nanopore r10.4.1 flow cells, "super-accurately" basecalled in Dorado software, and aligned to the plasmid. The resulting datasets were used with Remora create a training model that can recognize 8-oxo-dG. The Remora model was then tested with novel datasets and Integrative Genomics Viewer (IGV) was used to score the model's ability to detect 8-oxo-dG. Alignment parameters and quality filtering in Dorado, quality filtering in SAMTools, and AI training parameters in Remora were systematically varied to optimize the models (~200 trials total). The best models detected 8-oxo-dG at novel sites with ~1% false positives. Our results show that neural networks can be trained to read 8-oxo-dG on telomeres at the single-nucleotide level, and our pipeline can be used to map other adducts in telomere sequences.

B142/P1268

NS309 Restores Membrane Potential and Autophagy to Delay Post-Ovulatory Aging in Porcine Oocytes

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Post-ovulatory aging (POA) of oocytes leads to a progressive decline in oocyte quality, resulting in reduced fertilization capacity, impaired embryonic development, and increased risk of abnormal offspring. A key driver of this deterioration is the disruption of calcium (Ca^{2+}) homeostasis, mitochondrial dysfunction, membrane depolarization, and defective autophagy. NS309, a selective activator of small- and intermediate-conductance

calcium-activated potassium channels (SK/IK), has shown potential in regulating membrane potential and restoring cellular function, yet its role in oocyte aging remains unexplored. This study investigates whether NS309 can rescue porcine oocytes from POA-induced defects by restoring membrane potential, improving Ca^{2+} regulation within the endoplasmic reticulum and mitochondria, and alleviating oxidative stress and autophagy impairment. Using in vitro matured porcine oocytes, we assessed mitochondrial activity, ER distribution, Ca^{2+} dynamics, and autophagy-related markers through immunofluorescence, Western blotting, and functional assays. We further examined the protective role of NS309 against glibenclamide-induced KATP channel inhibition, a condition that exacerbates membrane depolarization and aging phenotypes. We anticipate that NS309 treatment will reduce oxidative stress, restore mitochondrial and ER function, promote protective autophagy, and stabilize membrane potential in aged oocytes. These findings will provide new mechanistic insights into the regulation of oocyte quality and may establish potassium channel modulation as a novel strategy to prevent age-related infertility.

B129/P1255

Reactivating RNA metabolism alters stress granule dynamics in senescence

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Nuclear prion-like RNA binding proteins (RBPs) such as FUS, TDP43, and hnRNPA1 often mislocalize and form cytoplasmic solid pathological aggregates in Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTLD). They also tend to get enriched in stress granules (SGs) upon mislocalization and form aberrant SGs in neurodegenerative conditions. Interestingly, only 5-10% of the familial cases of ALS and FTLD show mutations in these prion-like RBPs, suggesting the contribution of additional cellular factors. Aging has become the primary risk factor, driving many cellular and molecular changes that increase the onset and vulnerability to neurodegenerative diseases. It has been known that aging is also associated with a decline in proteostasis, RNA metabolism and organellar function. However, there are few insights about the RBPs mis-localization, aggregation and their relation to SGs in aging. Our quantitative live fluorescence microscopy experiments elucidate the presence of persistent SGs across different cellular and organismal aging models. These persistent SGs were enriched for its scaffold RBP, G3BP1 and other ALS-related prion-like RBPs. There is also a reduction in the total RNA and polyadenylated (polyA) RNA in the aged Drosophila brain and in cellular aging models. We found that the global decrease in RNA metabolism significantly increased the RBP to RNA ratio in the SGs of aged cells. We substantiate that an increased RBP to RNA ratio would be an underlying cause for the delayed clearance of SGs in aging, leading to its persistence. Interestingly, reactivating RNA metabolism through nucleoside addition significantly increased the total RNA levels in aged cells. This increased RNA levels consequently altered the SGs' RBP to RNA ratio and thereby increased the dissolution of SGs in aging. These results on reactivating RNA metabolism through nucleoside supplementation in aging highlight the role of RNA in SG clearance and would widen its implications in preventing the aggregation of prion-like RBPs and other altered RNA-RBP condensates in age-related diseases.

B136/P1262

Size-Based Flow Cytometry Sorting for Distinguishing Quiescent and Non-Quiescent Yeast Subpopulations

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Baker's yeast (Saccharomyces cerevisiae) is a powerful model for studying replicative aging, defined by the number of daughter cells produced before senescence, and chronological aging, reflecting the survival of non-dividing, stationary-phase cells. Within yeast populations, quiescent and non-quiescent (mother, and daughter) cells exhibit distinct physiological states that reveal conserved mechanisms of cellular maintenance, stress adaptation, and longevity relevant to human aging. Vacuolar function is central to these processes, as it regulates ion and pH balance, mediates the clearance of damaged proteins and organelles and supports lipid and amino acid homeostasis. Preservation of vacuolar integrity enhances stress resistance and lifespan across all three subpopulations. Conventional centrifugation-based methods for separating quiescent and non-quiescent cells are time-consuming, reagent-intensive, and can disrupt yeast morphology and vacuolar integrity. To address these limitations, we developed a flow cytometry–based sorting strategy that discriminates yeast cells by size and complexity into quiescent, daughter, and mother populations. This approach enables rapid, reproducible, and morphology-preserving isolation of subpopulations. It provides a scalable platform for molecular, metabolic, and phenotypic analyses of yeast aging and offers a broadly applicable framework for exploring conserved pathways of cellular aging in eukaryotic systems.

B139/P1265

Tenofovir disoproxil fumarate in the induction of premature senescence in primary human fibroblasts

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Human Immunodeficiency Virus (HIV) primarily targets and kills CD4+ T lymphocytes and can lead to Acquired Immunodeficiency Syndrome (AIDS). While there is still no cure, antiretroviral therapy (ART) has turned HIV into a manageable chronic disease by blocking viral replication at several stages of its life cycle. However, prolonged ART usage has been associated with premature aging phenotypes, including neurocognitive decline, renal and bone toxicity, and increased fracture risk. The objective of this study is to investigate the role of tenofovir disoproxil fumarate (TDF), a nucleoside reverse transcriptase inhibitor (NRTI), in inducing cellular senescence, an in vitro model of aging. Primary human lung fibroblasts (IMR-90 cells) were employed as a model system to assess the effect of TDF on senescence induction. We found that fibroblasts tolerated high levels (100 μM) of TDF in terms of viability based on MTT assays, but ceased proliferation within ~10 days of treatment in culture. Following 100 uM TDF treatment, cells had a drastically reduced population doubling level (PDL) compared with cells treated with a low dose (50 μM) of TDF and untreated cells. To determine if high-dose treated TDF cells entered senescence, we assessed beta-galactosidase levels and found ~70% of cells were beta-gal positive and had elevated expression of senescence-associated genes (Cdkn2a, Cxcl10, and Cxcl8). Given that reactive oxygen species (ROS) can promote senescence, we assessed ROS levels in high-dose TDF treated cells and found increased oxidative stress compared to control cells. Together, these results indicate that TDF promotes premature senescence in primary human fibroblasts through oxidative stress, offering potential insight into the accelerated aging phenotype observed in individuals receiving long-term TDF therapy.

B126/P1252

The Biology of Longevity: From Energy Metabolism in the Brain to Reproductive Aging

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Cellular longevity requires sustained energy production and the preservation of organelle integrity over extended lifespans. Our objective was to uncover mechanisms by which mitochondria and their protein constituents contribute to the longevity of both post-mitotic brain cells and reproductive systems. Using in vivo stable isotope pulse-chase mass spectrometry, mitochondrial isolation, and functional assays, we interrogated the persistence and turnover of mitochondrial proteins across diverse tissues. We found that select inner membrane and cristae organizing proteins exhibit exceptional stability in the mammalian brain, persisting for months in neurons where long-lived proteins support synaptic activity and metabolic resilience. In parallel, studies of reproductive tissues revealed that oocytes harbor extraordinarily stable mitochondrial structures and proteins that endure throughout the female reproductive lifespan, enabling developmental competence despite chronological aging. Complementary conceptual frameworks from comparative metabolism further highlight that mitochondrial adaptations underlie species-specific differences in reproductive span and brain function. Collectively, these findings establish that longevity in neurons and oocytes is supported by conserved principles of mitochondrial protein persistence and metabolic economy, while also exposing unique vulnerabilities in aging. We conclude that the biology of longevity cannot be understood without integrating mitochondrial proteostasis across cell types that rarely, if ever, regenerate. This work defines mitochondria not only as engines of energy metabolism but also as guardians of lifespan in cells central to cognition and reproduction.

B143/P1269

The Cytoprotective Effects of Sulforaphane on DNA Damage and Senescence in IMR-90 Cells

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Sulforaphane is a natural compound concentrated in cruciferous vegetables and known for its potent antioxidant, anti-inflammatory, and anticancer properties. Sulforaphane activates the redox-sensitive nuclear erythroid 2-related factor 2 (NRF2) pathway, which plays an essential role in DNA damage repair, cell cycle regulation, and antioxidant defense. Senescence is a permanent state of cell cycle arrest activated in response to various stressors, such as DNA damage and reactive oxygen species (ROS) accumulation. Though cells enter senescence as a protective mechanism, an accumulation of senescent cells over time has been shown to lead to age-related decline and disease, including cancer. We sought to investigate the impact of sulforaphane on DNA damage and onset of cellular senescence in the primary human fibroblast cell line, IMR-90. Etoposide is a chemotherapeutic agent that inhibits topoisomerase II, which leads to DNA damage and, ultimately, senescence in IMR-90 cells. We assessed the onset of etoposide-induced senescence in the presence of sulforaphane. Treatment with sulforaphane led to a significant upregulation in Nqo1 and Gclc expression, both genes in the Nrf2 pathway that function in DNA repair. Pre-treatment with sulforaphane resulted in decreased expression of senescence-associated genes Ptchd4, Mmp12, Cxcl10, and Purpl, as well as lower levels of senescence-associated beta galactosidase activity following etoposide treatment. The results of this study suggest that sulforaphane inhibits or delays the onset of etoposide-induced senescence in IMR90 cells. We speculate that sulforaphane protects against DNA damage induced by etoposide, thus reducing cellular senescence; ongoing research testing the direct effects of sulforaphane on DNA damage are currently ongoing.

B141/P1267

Tight Junctional Disruption between Salivary Gland Ductal Cells Might Cause Saliva Hyposecretion in Aged Mice

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Saliva hyposecretion causes “dry mouth”, leading to impaired mastication and swallowing. A recent meta-analysis reported that one-third of individuals aged 60 years or older experience reduced saliva secretion. Thus, addressing saliva hyposecretion in the elderly is expected to help extend healthy life expectancy. Among age-related changes, alterations in tight junctions—crucial epithelial barriers separating the external environment from the internal body—are particularly noteworthy. Previous studies have reported age-associated decreases in tight junction expression and abnormalities in localization in epithelial cells of the intestine, skin, and kidney. However, evidence for age-related changes in the salivary gland tight junctions remains limited. Non-muscle myosin II regulates the expression and localization of tight junctions. We previously demonstrated that NMIIC, an isoform of non-muscle myosin II, localizes around the tight junctions of salivary gland ductal cells, and that NMIIC knockout mice exhibited reduced expression of tight junction proteins and decreased salivary secretion. These findings suggest that tight junctions are indispensable for sustaining salivary secretion and that NMIIC may play a regulatory role. This background led us to hypothesize that salivary hyposecretion in the elderly results from impaired tight junction function in salivary glands and that therapeutic activation of NMIIC could be a viable strategy. Here, we used aged male mice (74-week-old), which we previously reported to have reduced saliva secretion, and young male mice (8-week-old) as controls. Western blotting revealed reduced expression of ZO-1, a tight junction protein, in parotid gland of aged mice. Immunofluorescence staining further demonstrated disrupted ZO-1 localization in parotid ducts of aged mice. In summary, although preliminary, these results suggest that the tight junctional disruption between salivary gland ductal cells may underlie saliva hyposecretion and NMIIC could represent a therapeutic target for treating age-related dry mouth.

B134/P1260

Uncovering the specificity of gene expression programs in cellular quiescence and senescence

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Most cells in the human body are not actively dividing and instead exist in states of cell cycle arrest, including quiescence and senescence. To understand the cell biological properties of these distinct physiological states, it is essential to explore the molecular basis governing their functional specificity. Quiescence is a state of reversible cell cycle arrest in which cells exit the cell cycle and yet retain the capacity to divide. In contrast, senescence is defined by an irreversible exit from the cell cycle and is coupled with the induction of an inflammatory secretory program. The physiological functions of quiescent and senescent cells are central to human health and aging, but our understanding of these cell states remains incomplete. Quiescent and senescent cells display substantial differences in their properties with respect to proliferative potential, cellular organization, and other behaviours. To uncover the molecular mechanisms underlying these differences, we analyzed key aspects of gene expression to comprehensively define factors that confer specificity to these cell states. At the global level, quiescent and senescent cells display significant global differences in chromatin organization and transcription and translation activity. Using targeted sequencing and proteomics approaches, we identified gene-specific differences in transcription start site usage, mRNA splicing, translation initiation,

and protein decay. We will describe specific proteins and variants that make important functional contributions to these distinct states. Our work reveals that the differential decoding of genes at multiple levels of regulation contribute to the specificity of quiescence and senescence as physiological states of cell cycle arrest.

Cell Aging and Regeneration - Wound healing and tissue repair

B152/P1278
A novel cellular response to nuclear membrane damage: ectopic cell-cell fusion.
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Nuclear membrane damage is often induced upon mechanical stress. While the most commonly studied consequences of nuclear membrane rupture include genomic instability and various cGAS/STING-dependent responses, we here report a novel consequence: cell-cell fusion. After tissue injury, we observe that nuclear membrane damage promotes ectopic epithelial cell fusion, and we propose a mechanism connecting the two processes through DNA repair and actin protrusion. Nuclear membrane rupture was induced in Drosophila notal epithelium wounded by laser ablation. Near the tissue wound, cells fused frequently to form multinucleated syncytia, as we and others have previously reported. Interestingly, wound-induced fusion occurred exclusively in cells that had nuclear membrane damage, and we demonstrated that these cells also suffer elevated DNA damage. Using loss-of-function genetic analysis, we found that a gene inducing DNA damage upon nuclear envelope rupture was also required for cell fusion upon tissue injury. How do nuclear membrane damage and the subsequent DNA damage promote ectopic cell fusion after wounding? We performed a candidate screen of DNA repair proteins and identified multiple hits that also mediated wound-induced fusion. These proteins all participate in the same actin-dependent DNA repair pathway, from the initial lesion recognition to the final activation of actin polymerization for lesion translocation. Some of the proteins we found form a complex whose role promoting actin polymerization has been suggested in previous literature. Further, we found that, around wounds, actin protrusion precedes wound-induced fusion. Thus, we propose that when the nuclear membrane is ruptured, the subsequent DNA damage triggers actin-dependent DNA repair. Some of the DNA repair proteins are small enough to leak into the cytosol where they carry out their actin polymerization role. The resulting actin protrusions, when combined with plasma membrane damage also inflicted by the laser wound, result in cell fusion.

B156/P1282
A novel class of therapeutic, extracellular matrix protection factor 1, has chondroprotective properties as demonstrated in a rat model of osteoarthritis
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Osteoarthritis (OA) is a common degenerative joint disease of multifactorial etiology causing pain and disability in more than 25% of the adult population. Despite affecting a vast amount of the population, there is currently no therapy available that successfully suspends or reverses the deterioration of cartilage and bone occurring in OA. Current pharmacological treatment of OA targets inflammation and pain management through non-steroidal anti-inflammatory drugs, intra-articular corticosteroids, and analgesics. Extracellular Matrix Protection Factor 1 (ECPF-1) is a novel therapeutic that inhibits specific matrix metalloproteinases (MMPs) that

are involved in degradation of the extracellular matrix (ECM) in cartilage during OA. In this study, a chemically-induced rat model of OA in the knee joint was utilized to investigate the effects of ECPF-1 at various concentrations in early-stage osteoarthritis. The progression of OA in this study was assessed using microcomputed tomography (μCT), histological analysis and gait analysis. The acute effects of OA progression were studied on a timeline of 4 and 8 weeks. The μCT data showed that at both the 4-week post-loading (4 weekly injections of ECPF-1(0.25uM – 5uM), saline control or Bone Morphogenetic Protein-7 (500 ng) control) and 8-week protection-extension phases, the ECPF-1 treated cohort demonstrated decreased ECM degradation compared to untreated controls. Improved articular cartilage histological staining of the ECPF-1 treated joints supported the μCT image data for the femur and tibia, while rat gait was improved. Trabecular thickness and number were increased following ECPF-1 treatment. These results suggest a chondroprotective property to ECPF-1 and its potential as a therapeutic that may slow the progression of OA.

B154/P1280

Alterations in articular chondrocyte extracellular matrix production in response to synovial fluid dilution, a pilot study to model exploratory arthroscopy conditions

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Exploratory knee arthroscopy is a minimally invasive procedure used for the diagnosis of knee joint pathologies. However, emerging evidence suggests that the irrigation of the joint with saline, required for visibility during this procedure, dilutes the synovial fluid and may contribute to cellular and metabolic changes consistent with early osteoarthritis pathogenesis. To address the challenge of studying cellular-level effects in vitro, our lab optimized a three-dimensional, serum-free tissue culture model for primary, human articular chondrocytes (HC-a) and measured key extracellular matrix proteins to monitor their metabolism. Optimal production of the target extracellular matrix endpoints was observed when HC-a were encapsulated in alginate beads at a density of 1.5x106 cells per 1 mL of alginate and cultured in serum-free medium for a period of 24 hours. Using this optimized culture model, we reared HC-a in varying dilutions of artificial synovial fluid (ASF), to recreate what chondrocytes experience during exploratory arthroscopy. We measured sulfated proteoglycan, type II collagen, and matrilin-3 (a potential marker of OA pathogenesis) production in cultures reared in 100% ASF, 50% saline-diluted ASF, 66% saline-diluted ASF, or saline-only conditions. HC-a increased the amount of type II collagen and matrilin-3 protein produced when cultured in several dilutions of ASF with saline and in saline-only conditions. Sulfated proteoglycan production exhibited a significant increase only when HC-a were cultured in saline alone. These data indicate that damaging cellular effects occur early when synovial fluid concentrations are altered. We are currently investigating whether prophylactic approaches can minimize these cellular effects. These data indicate a need for a more thorough investigation of the cellular alterations associated with the standard conditions for exploratory arthroscopy.

B147/P1273

Decoding the Spatial and Metabolic Control of Immune Cell Differentiation During Tissue Repair

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The spatial and metabolic cues that direct immune cell fate during tissue repair remain poorly defined. We investigated how secreted molecules, transcriptional programs, and metabolic patterns within the skin microenvironment control immune cell differentiation and function. Using single-cell secretomics and spatial multi-omics, we mapped how the local tissue environment provides metabolic cues that promote immune cell differentiation and coordinate repair. Our analyses revealed that aging disrupts these spatially organized secretomes, leading to impaired metabolic programming and defective immune cell fate transitions. To resolve the complex relationships between metabolism, spatial patterning, and cell state, we employed large language models (LLMs) trained on transcriptomic and metabolomic profiles. This approach enabled the identification of previously unrecognized spatial networks linking secreted factors, metabolic pathways, and immune cell differentiation. Restoration of secreted molecule levels and metabolic rewiring rescued immune cell differentiation and tissue function in aged animals. These findings highlight the fundamental role of spatially organized metabolism in regulating immune cell fate and tissue homeostasis and demonstrate the power of AI-guided multi-omic approaches to decode complex regenerative processes.

B150/P1276

Development of an Accelerated Glial Scar Testbed

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A continuing research issue in CNS repair is to develop strategies that overcome the pathological neuron connectivity result of glial scar formation while retaining its benefits of inflammation segregation and tissue protection. Key studies [1,2] have pursued an investigative path that correlates post-mortem animal studies with ex situ investigations designed to accurately re-create the in-situ damaged tissue and inflammatory environment. The latter is difficult due to its complexity, even at the bioreactor level. A previous investigation [3] of cerebral cortical astrocytes treated with dBcAMP [4] and cultured on planar poly-l-lysine (PLL) functionalized Aclar® produced the unexpected result that astrocytes formed chain-like structures that appeared to recapitulate key features of glial scar formation. If the glial scar mimicry response can be demonstrated to be sufficiently representative of an actual response, these results indicate that a simpler glial scar testbed can be developed. In the present work, we present investigations of the mimicry system. The timing of the ‘glial scar’ response is shown to be shorter, 24h versus 3-5 days, indicating potential for the development of an accelerated testbed. Astrocyte cell responses are characterized for differential morphometry (size, shape and alignment) at the concave ‘wound’ edge through to the opposite convex side. GFAP orientation investigated by super-resolution microscopy is also quantified as a function of differential position throughout. The mimicry system results are compared with previously reported in-situ results [2] to determine how close they are in value, as indicative of a representative situation. Cell group curvature was noted without the introduction of any external inflammatory response cells or chemistry that the ‘glial scar’ was putatively trying to segregate. Two explanations: energy minimization as a result of the top-to-bottom differential cell ‘weaving’ itself, or a response to surface characteristics of the underlying substrate, are investigated and compared. If a simple yet representative glial scar testbed can be developed, it is noted that Aclar® has desirable stability and transparency properties that could make it suitable for comparative space versus terrestrial investigations. [1] MV Sofroniew. Nature Rev. Neurosci. 16(5): 249-263 (2015). [2] IB Wanner, MA Anderson, B. Song, J Levine, A Fernandez, Z Gray-Thompson, Y Ao, MV Sofroniew. J. Neurosci. 33(31):12870-12886 (2013). [3] VM Tiryaki, VM Ayres, I Ahmed, DI Shreiber. Nanomedicine 10(4), 529-545 (2015). [4] S Fedoroff, WJ Mcauley, JD Houle, RM Devon. J. Neurosci. Res.12(1), 15-27 (1984).

B155/P1281
Effect of extracellular matrix protection factor-3, a novel inhibitor of matrix metalloprotease-12, on transforming growth factor-beta production in pulmonary fibrosis diseased fibroblasts
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Chronic obstructive pulmonary disease (COPD) is the third leading cause of death worldwide and a significant incurable global health burden. COPD refers to a group of diseases that cause blockage of airflow and breathing-related issues. Transforming growth factor-β (TGF-β) production and endogenous activation are crucial events in the progression of pulmonary fibrosis as a subset of COPD. TGF-β is considered a potent inducer of collagen production and other extracellular matrix (ECM) proteins, leading to the progression of pulmonary fibrosis. In addition to the overexpression and endogenous activation of TGF-β, matrix metalloprotease (MMP)-12 is upregulated in the pathogenesis of pulmonary fibrosis, leading to ECM dysregulation. Despite the importance of both TGF-β and MMPs in the pathogenesis of pulmonary fibrosis, little is known about the effects of inhibiting MMP-12 on endogenous TGF-β activation. Diseased Human Lung Fibroblast (DHLFB) primary cells were cultured in monolayer and were either treated with serum free media or serum free media containing 10-50 μg of a novel MMP-12 inhibitor, Extracellular Matrix Protection Factor (ECPF)-3, for 24 hours. The presence in secreted and extracellular matrix incorporated fractions of total TGF-β production and endogenous TGF-β activation were determined via enzyme-linked immunosorbent assays. DHLFB cells produced more total TGF-β in serum-free cultures than normal controls (DHLFB 109pg/culture versus NHLFB 30.93pg/culture). In addition, DHLFB cells produced a larger amount of endogenously activated TGF-β (DHLFB 18.7pg/culture versus NHLFB 3.09pg/culture), reflecting their diseased profile. Treatment of DHLFB cells with 10-50ug ECPF-3 for 24 hours in serum-free media resulted in an increase in incorporated TGF-β, both total and endogenously activated ($p<0.001$ for each) while the secreted fractions were unchanged. Our results suggest that inhibiting MMP-12 in DHLFB cells alters the activation state and storage of TGF-β. In previous studies, we have shown that ECPF-3 decreases elastin degradation and collagen production. Overall, these results indicate the potential for ECPF-3 to act as a therapeutic in slowing the progression of pulmonary fibrosis in diseased fibroblasts.

B148/P1274
In vivo imaging of muscle repair in Clytia hemisphaerica
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Muscle is a contractile tissue that undergoes constant repair as it experiences forces from movement and injury. In cnidarians, muscle tissues drive essential behaviors such as locomotion and feeding. The hydrozoan Clytia hemisphaerica medusae have two types of “muscle”: striated swimming muscle and radially oriented smooth muscle called epitheliomuscular cells. However, the cellular mechanisms underlying muscle repair in cnidarians remain poorly understood. Here, we characterize how small muscle wounds heal and how contraction influences the repair process. Using live differential interference contrast (DIC) imaging, we are establishing the in vivo dynamics of repair by examining features such as restoration of cell shape, differences between striated and epitheliomuscular cell healing, and the impact of wound orientation relative to actin filament alignment in epitheliomuscular cells. Preliminary results indicate that smooth muscle wounds close at

rates comparable to exumbrella wounds. Notably, wounds perpendicular to the radial epitheliomuscular actin fibers exhibit initial cell recoil and tissue unfurling prior to closure, whereas parallel wounds do not. During repair, actin filaments in epitheliomuscular cells become transiently disorganized but recover their orientation after healing. These results suggest a potential relationship between muscle orientation and repair mechanisms in Clytia. Future work will examine how actin orientation is reestablished.

B149/P1275
More than a stomachache: stress-primed systemic inflammation exacerbates peptic ulcers
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Psychological stress has profound effects on the body and is a risk factor for disease. However, how stress is conveyed systemically to impact specific cells, tissues and organism health is poorly understood. Our work aims to determine how acute stress impacts local cell biology to alter tissue function and regeneration, using the mouse stomach as a model. Peptic ulcer disease is a common stomach condition associated with psychological stress, affecting 4 million people annually. However, how stress affects ulcer susceptibility and pathogenesis remains controversial. To this end, we subjected mice to acute stress prior to inducing stomach ulcers. We found that stress sensitizes gastric tissue to damage, resulting in larger ulcers with more extensive mucosal damage and inflammation. Mechanistically, we found that blocking Corticotrophin-Releasing Factor (CRF) signaling partially ameliorated ulcer severity in stressed mice, but glucocorticoid manipulation did not, suggesting that peripheral CRF contributes to ulcer exacerbation under stress. Given the relationship between stress and immunity, we hypothesized there could be changes in inflammation in stressed mice. Indeed, following stress there was a proinflammatory shift in circulating cytokines, even in the absence of injury, supporting a role for innate immune cells under stress. Overall, our data suggest that stress primes systemic proinflammatory signaling that could sensitize tissues to damage via immune dysfunction. Our current experiments are aimed at further testing the mechanistic bases of these findings. This work provides important insight into how systemic conditions reprogram cell biology to alter tissue homeostasis by revealing that stress causes body-wide inflammatory changes that sensitize the stomach to injury. These findings also directly demonstrate a mechanistic link between stress and stomach ulcer pathogenesis, addressing a longstanding controversy in medical and popular discourse. Furthermore, most disease-relevant cellular changes derive from non-genetic physiological conditions, making it likely that these findings represent a conserved paradigm in other tissue types, setting the stage for future investigation of how systemic physiology regulates cell biology.

B153/P1279
Myo/Nog Cells in Pulmonary Fibrosis
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Introduction and Hypothesis: Pulmonary fibrosis is a group of lung diseases characterized by the presence of scar tissue that may arise from a variety of causes. Fibrosis impedes gas exchange by physically blocking the normally thin barrier between the alveoli and capillaries and reducing the lung's elasticity. Myofibroblasts have been implicated in the development and progression of lung fibrosis. In the eye, Myo/Nog cells are the source of myofibroblasts. Could Myo/Nog cells also be the source of myofibroblasts in the lung? Myo/Nog cells were

discovered in the early embryo by their co-expression of the skeletal muscle specific transcription factor MyoD, the bone morphogenetic protein (BMP) inhibitor Noggin, and brain-specific angiogenesis inhibitor 1 (BAI1). In the adult lens, Myo/Nog cells proliferate, migrate, and differentiate into myofibroblasts following cataract surgery. Differentiated Myo/Nog cells also are present in membranes that form on the surface of the retina in a disease called proliferative vitreoretinopathy. In both structures, their differentiation into contractile myofibroblasts produces a tractional force that impairs vision. This study aims to determine whether Myo/Nog cells are present in fibrotic lung tissue, express markers of myofibroblasts, and are phagocytic. Methods: Cadaver lung tissue samples were collected from 10 donors with and without known lung pathologies. The tissues were embedded in paraffin, sectioned, and double labeled with antibodies to BAI1 and alpha smooth muscle actin (α-SMA) or striated muscle myosin heavy chain to determine whether Myo/Nog cells had differentiated into myofibroblasts. Antibodies were visualized with species and subclass-specific secondary antibodies conjugated with rhodamine and Alexa 488. Sections were visualized with Nikon E800 epifluorescent and Evident Fluoview 400 confocal microscopes. Results: Normal lung tissue contained only few BAI1-positive (+)/Noggin+ cells that were distributed throughout the tissue. Neither α-SMA nor striated muscle myosin were detected in BAI1+ cells of the normal lung. Fibrotic lung tissue contained an abundance of BAI1+/Noggin+. In these areas, BAI1+ cells had synthesized both α-SMA and striated muscle myosin. Some Myo/Nog cells had ingested particulate matter in the diseased lung. Discussion: This preliminary study showed that Myo/Nog cells are present in the normal human lung and increase in fibrotic lung tissue. The presence of α-SMA and striated muscle myosin in Myo/Nog cells indicates that they have differentiated into myofibroblasts. Future studies will examine whether Myo/Nog cells contribute to fibrosis by depositing extracellular matrix.

B151/P1277

Polyploid cells can function as non-professional antigen presenting cells.

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My recent studies identified a novel mechanism of kidney adaptation at the cellular level: polyploidization of tubular epithelial cells (TC). Beyond well-established roles in increasing cell size/metabolic output, polyploidy in cells can also promote nonuniform genome, transcriptome, and metabolome alterations and it can be closely related to chromosomal instability. On the other hand, polyploid cells could potentially function as a buffering mechanism to prevent tumor development due to their redundant genomes. Mechanisms generating these alterations have been completely unknown in the kidney up to now. However, my studies identified YAP1 as the key regulator of TC polyploidy. Employing conditional transgenic mouse models based on the FUCCI2aR reported where kidney TC polyploidy is enhanced (SAV1 knock-out mice) or decreased (YAP1 knock-out (KO) mice), we found that SAV1 KO mice were not prone to cancer development. Accordingly, polyploid TC become senescent over time suggesting that they acquire senescence as a ploidy-limiting mechanism to prevent cancer development. Moreover, single cell RNA-seq analysis performed at different days after a direct tissue injury showed that subset of polyploid TC upregulates MHC class II, becoming semiprofessional antigen presenting cells, while diploid TC do not. This was confirmed by FACS and confocal analyses. Histocompatibility complex upregulation was linked to the presence of DNA damage, suggesting progressive accumulation of genome instability triggers MHC expression. Collectively, we demonstrated for the first time the presence of histocompatibility complex on the surface of polyploid TC. This important results indicate that MHC+ polyploid TC may act as immunomodulatory cells that can influence injury and wound healing processes.

Cell Biology Across the Tree of Life - Archae

B392/P0960
Cell membranes of the extreme: characterizing the biosynthesis and physiological roles of membrane-spanning lipids in archaea
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Archaea, the so-called “third” domain of life, are a diverse group of microorganisms that possess many unique features which distinguish them from other lifeforms on Earth. Paramount amongst these defining characteristics are the novel lipids that archaea use to construct their cell membranes. Most notably, archaea commonly fuse the canonical membrane bilayer into a monolayer of membrane-spanning lipids known as glycerol dibiphytanyl glycerol tetraethers (GDGTs) often just referred to as tetraethers. The formation and modification of these lipids is highly dependent upon radical (S)-adenosyl methionine (rSAM) enzymes. GDGTs are formed by the rSAM tetraether synthase and the subsequent cyclization of their lipid tails is performed by the B12-binding rSAM (B12-rSAM) GDGT ring synthase. We sought to identify enzymes responsible for additional GDGT modifications such as crosslinking, methylation, desaturation, and hydroxylation and to determine the physiological roles of such lipids. Utilizing three archaeal model organisms – Sulfolobus acidocaldarius, Thermococcus kodakarensis, and Methanosarcina acetivorans – we identified six enzymes responsible for these modifications, five of which are rSAM enzymes. In particular, we uncovered 1) the rSAM enzyme GMGT synthase (Gms) which covalently crosslinks GDGT tails to form glycerol monoalkyl glycerol tetraethers (GMGTs), 2) three distinct B12-rSAM GDGT methylases which introduce 1-5 additional methyl groups into the tails of specific classes of GDGT lipids (GMGTs, cyclized GDGTs, and acyclic GDGTs), 3) a B12-rSAM tetraether desaturase (Ted) which seemingly reverses previous saturation steps in the archaeal lipid biosynthesis pathway, forming 1-4 double bonds in the saturated hydrocarbon tails of GDGTs without the need for molecular oxygen in contrast to the fatty acid desaturases of bacteria and eukaryotes, and 4) a non-rSAM hydroxy-GDGT synthase (Hgs) which hydrates the double bonds introduced by Ted to form OH-GDGTs. Notably, we show that Gms proteins are widely distributed throughout archaea and unexpectedly in some bacteria, and we demonstrate that the cross-linking of GDGTs by Gms is part of a conserved heat stress response in phylogenetically diverse archaea. Further, we demonstrate that GDGT methylation participates in a novel archaeal stress response triggered by exposure to membrane-destabilizing chemical agents such as hexanoic acid and penicillin G. These results highlight the value of exploring the rSAM enzyme landscape of archaea – revealing diverse and novel rSAM enzyme activities amongst homologous proteins that invoke new paradigms such as reversibility in the archaeal lipid biosynthesis pathway.

B394/P0962
The Biochemical and Physiological Drivers of Membrane Modifications in Thermophilic Archaea
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Archaeal microbes represent a domain of life that is phylogenetically distinct from Bacteria and Eukaryotes. While archaea exhibit physiological characteristics similar to what is observed in the two other domains – for example, archaea contain single circular chromosomes like bacteria but utilize histones to package and regulate their chromosomes like eukaryotes – they also possess features that are uniquely archaeal. The most

striking biochemical distinction of the archaea is the chemical and structural composition of their lipid membranes. Archaeal membranes are composed of isoprenoid-based chains ether linked to glycerol-1-phosphate while bacteria and eukaryotes primarily have lipid bilayers composed of fatty acid chains ester linked to glycerol-3-phosphate. In addition, several archaeal species fuse their membrane bilayers to generate monolayers that can be further modified with the addition of pentacyclic rings, methylations, and cross-links across the lipid monolayer. All these membrane modifications are thought to occur as a response to harsh conditions that many archaea encounter in their native environments – high temperatures, pH fluctuations, and nutrient limitations. However, links between environmental conditions and archaeal membrane modifications have been difficult to make for a variety of reasons including the difficulty in culturing these organisms, the paucity of molecular and genetic tools to manipulate these organisms, and a lack of understanding of the biochemical and regulatory pathways involved in the synthesis and modification of archaeal membranes. Here, we identify the biosynthetic proteins necessary for the formation and methylation of cross-linked membrane spanning lipids in a variety of thermophilic archaea. Specifically, we confirm the requirement of two radical SAM proteins necessary for the synthesis and subsequent methylation of cross-linked glycerol monoalkyl glycerol tetraethers or GMGTs through heterologous expression in Thermococcus kodakarensis. Using these proteins as markers to search genomic databases, we identified a variety of archaea with the genetic potential to produce both GMGTs and methylated GMGTs. Further, we explore the taxonomic diversity of tetraether methylases and identify a variety of similar but distinct methylases in other archaea. We characterize a novel tetraether methylase from the thermoacidophile Sulfolobus acidocaldarius and explore the physiological conditions in which tetraether methylation is upregulated in this organism. Taken together, our findings demonstrate the utility of exploring archaeal membrane lipid biosynthesis to provide a connection between archaeal membrane biology and the extreme environmental conditions these organisms thrive in.

Cell Biology Across the Tree of Life - Bacteria

B397/P0965

A novel encapsulation peptide identified in Gordonia, a new addition to the synthetic biology toolkit.

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Bacterial microcompartments (BMCs) are prokaryotic organelles made of proteins, containing multiple enzymes, resulting in enhanced efficiency of the metabolic pathway. Encapsulation peptides (EP) which are about 20 amino acids at the N, or the C terminal ends of the “cargo” proteins, help in encapsulation of these enzymes. Amongst Actinobacteria, bacterial microcompartments have been reported from Rhodococcus, Mycobacterium, etc., but none from Gordonia spp. which are reported to have immense applications in bioremediation. Here, we report identification of a novel EP from G. phthalatica QH-11. Multiple sequence alignment and structural analysis of the cargo enzymes in G. phthalatica QH-11 was carried out which revealed a putative EP sequence at the C terminal of the aldehyde dehydrogenase protein. These 20-residue peptide was found to be outside the globular domain of the protein. The sequence also had comparable hydropathic index to the other reported EPs in literature. To demonstrate the functionality of the identified EP sequences, genes encoding for biodesulfurization enzymes DszA, -B, -C were fused at the C terminus with the EP tagged GFP gene. As a control, an N-terminal EP, PduD from Citrobacter freundii was cloned at the N terminus of the Dsz enzymes. Confocal microscopy of heterologous host R. erythropolis IGTS8 harboring the plasmid expressing the novel EP tagged enzymes was performed. The EP tagged enzymes were localized towards the membrane, forming a series of connected foci all throughout the periphery of the cell. The results were compared with translational fusions without EP sequences. Without EP sequences, multiple discrete foci were observed, and they did not appear aggregated. Multiple mutants of the novel EP were constructed and

assessed and the key residues responsible for driving the localization of the tagged protein were determined. The encapsulation efficiencies were also investigated for the WT versus mutant EPs. The newly identified EP is the first to be reported in any species of Gordonia genus. This novel peptide may also be used as an encapsulating tool to encase enzymes in various bacteria for advancements in the field of metabolic engineering.

B403/P1819
Biomolecular condensates as stress-responsive chemically distinct microenvironments within the bacterial cytoplasm
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Bacteria use biomolecular condensates to organize their cytoplasm, but how they shape chemical environments without membranes remains unclear. Here, we set out to determine whether ribonucleoprotein condensates (BR-bodies) in Caulobacter crescentus can generate distinct chemical niches within the bacterial cytoplasm. Using pH-sensitive reporters, fluorescence microscopy, and vitro biochemical assays, we found that BR-bodies create acidic microenvironments, with pH significantly lower than the surrounding cytoplasm despite the lack of a membrane barrier. Unexpectedly, we also observed that BR-bodies bind and buffer toxic copper from mis-metallation of associated enzyme clients, then shifting to a more solid-like state under high copper stress. These acidic conditions and metal buffering together preserve the activity of the essential enzyme PNPase when localized within BR-bodies. Our findings demonstrate that bacterial biomolecular condensates function as dynamic chemical niches within cells, adding an unrecognized layer of spatiotemporal regulation and stress protection.

B395/P0963
Characterization of IncA-mediated fusion in Chlamydia muridarum
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Chlamydia trachomatis (Ct) is an obligate intracellular bacterium that infects over 129 million people each year worldwide and is the most common sexually transmitted bacterium. When multiple bacteria infect a single cell, each organism forms its own inclusion in which it replicates. Ultimately, at 12-24 hours post-infection, the inclusions fuse into a single compartment in a process known as homotypic fusion, which requires the Ct protein IncA. Importantly, non-fusogenic clinical isolates show decreased pathology and disease severity compared to fusogenic isolates. Understanding this fusion process may reveal new therapeutic targets. Preliminary data with Ct suggests that overall fitness is decreased in non-fusogenic strains, including reduced replication, which is important for controlling acute infection in vivo. To further understand the impact of homotypic fusion on disease progression and pathogenicity, we decided to use Chlamydia muridarum (Cm) and the mouse model of infection. Cm expresses an IncA homolog, TC0396, and also undergoes homotypic fusion when cells are multiply infected. However, little is known about homotypic fusion in Cm. Thus, we obtained a TC0396 KO Cm strain, which we called CmIncAKO. We then characterized the intracellular development of CmIncAKO and its capacity to undergo fusion in vitro, compared to wild type Cm. Here we demonstrate that, similar to Ct, CmIncA is required for homotypic fusion to occur in Cm, and CmIncAKO

exhibits decreased replication. These data suggest that Cm uses machinery similar to Ct to drive homotypic fusion and that this process is important for Chlamydia development and pathogenicity.

B399/P0967
Comparative proteomic analysis of multiple Neisseria gonorrhoeae strains to identify new factors contributing to antibiotic resistance
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Neisseria gonorrhoeae is a sexually transmitted disease that infects 82 million people every year, leading to serious health problems such as infertility. Despite existing antibiotics to treat cases of N. gonorrhoeae, the pathogen is quickly developing resistance, which limits treatment options. While genomic approaches have been able to identify factors that are involved in antibiotic resistance, these methods have not been sufficient to fully understand the complex resistance mechanisms occurring in N. gonorrhoeae. Indeed, previous studies showed that transforming altered genetic regions of isolate FA6140 into related strains with lower penicillin resistance was unable to replicate the higher resistance of FA6140. Therefore, to identify the “Factor X” that is responsible for the increased antibiotic resistance, we utilized a comparative, quantitative proteomics approach. The comparative analysis was done with four isolates: FA6140, which shows a level of penicillin resistance that is conferred by the unknown “Factor X”; AZOI, an isolate that is closely related to FA6140 but exhibits even higher penicillin resistance; as well as SPE111 and SPE114, which are FA6140-related isolates that are more susceptible to penicillin. Samples were taken from these isolates at 0 h, 2 h, 4 h, and 8 h post-penicillin exposure and analyzed through label-free, quantitative proteomics. This analysis allowed for the identification and quantification of 1325 proteins, 1172 of which showed significant differential abundances in at least one condition comparison. Comparisons between different strains and time points, including hierarchical clustering and visualizations as heatmaps, allowed us to determine proteins that specifically showed significant abundance changes in the AZOI strain in response to the penicillin exposure. These proteins might correspond to “Factor X” or to pathways that are likely involved in the associated increased antibiotic resistance. These results highlight the value in utilizing proteomics alongside genomic comparisons to further understand the development of antibiotic resistance in bacteria. In addition, the identified candidate proteins could be further used as targets for the development of new therapeutic approaches.

B398/P0966
Different Protein O-glycosylation Pathway Mutants in Pseudomonas aeruginosa Exhibit Distinct Phenotypes in Motility but not Antibiotic Resistance
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Protein glycosylation is increasingly recognized as a key determinant of bacterial physiology, affecting traits such as motility and virulence. Although Pseudomonas aeruginosa is a clinically relevant, opportunistic pathogen and a member of the ESKAPE pathogens, the contributions of protein glycosylation to its cellular functions remain only partially understood. Four glycosylation pathways have been described across different P. aeruginosa strains, including fgtA-dependent and tfpW-dependent systems in PAO1 and Pa5196, respectively. While glycosylation of flagellins and pilins has been demonstrated, the phenotypic consequences

of disrupting these pathways have not been well characterized. To address this, we investigated the roles of protein glycosylation in motility and antibiotic resistance. The deletion of fgtA did not impair flagella-driven swimming but significantly reduced swarming motility, which depends on coordinated flagella and pili activity, in addition to secretion of a bio-surfactant. In Pa5196, deletion of tfpW diminished twitching motility, but had no effect on swimming or swarming motility. These distinct phenotypes of different protein glycosylation pathway mutants underscore the diverse, potentially strain-specific functions of protein glycosylation in P. aeruginosa. In contrast, neither fgtA nor tfpW mutations altered antibiotic resistance. Given the importance of different types of motility for colonization and pathogenicity, targeting glycosylation pathways may represent a novel therapeutic approach against P. aeruginosa infections.

B401/P0969
Exploring Microbial Diversity in Water and Sediment of Local Jackson, TN Ponds
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Lane College is a historically black college committed to providing research opportunities for undergraduate students. This research utilized metagenomic data generated by the Lane College Metagenomic Research Project Team in 2023 and 2025. Samples of both water and sediment were collected from four area ponds: Lane College, Fairgrounds, Campbell Street, and Muse Park. DNA was extracted from each sample and sequenced using the Nanopore Mk1C (2023) and Nanopore MinION Mk1D (2025) platforms. Analysis revealed that water and sediment samples from all ponds shared several core bacterial taxa, including Candidatus Planktophila, Streptomyces, and Bacillus. However, sediment samples consistently showed greater microbial diversity, including rare Actinobacteria such as Mycobacterium, Corynebacterium, and Nocardioides, along with spore-forming Firmicutes. Water samples were primarily populated by planktonic Actinobacteria and lactic acid bacteria, notably Lactobacillus and Leuconostoc. Within water samples, unique taxa such as Rhodoluna and Bifidobacterium were more prevalent, while sediment contained a broader range of environmental and potentially beneficial microbes. This project highlighted the importance of examining both water and sediment to fully understand pond ecosystem diversity.

B402/P0970
Exploring the effect of TcpC enzymatic activity on planaria clearance of Uropathogenic Escherichia coli
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Bacterial resistance is a threat to global health. Uropathogenic Escherichia coli (UPEC) is the leading cause of urinary tract infections, and due to antibiotic treatment, there has been a notable rise in the levels of antibiotic-resistant bacteria. Studying this bacteria’s molecular mechanisms could provide insight into how to counteract the threat of bacterial resistance. UPECs produce a protein known as TcpC. TcpC is a Toll/interkeukin-1 receptor domain-containing protein (TIR) which acts as a virulence factor by inhibiting innate immune host immune responses. To study TcpC in E. coli we used the model organism planaria. Planaria are an emerging model for host- pathogen interactions due to their efficiency at clearing bacteria. Because TcpC blocks mammalian innate immune responses, we hypothesized that TcpC would also block planarian responses, and that when fed a variant of E. coli lacking TcpC, bacterial clearance will occur faster versus than when fed E. coli with TcpC. Results in our laboratory have supported this hypothesis. We now aim to explore

aspects of the protein TcpC and effects it has on host immune response. First, we plan to test the role that TcpC enzymatic activity has on planarian immune response. In addition, we plan to study transcriptional responses of planaria to TcpC to identify specific genetic pathways that are affected by TcpC. Characterization of pathways targeted by TcpC in planarian may inform future areas for targeted therapeutics for UPECs

B400/P0968
Simplifying dynamic instability through the lens of a bacterial actin-like protein model
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Across all domains of life, cells use dynamic instability of cytoskeletal components to regulate transport and division. Dynamic instability is the process by which a filament switches from a phase of steady growth to rapid depolymerization, or catastrophic shortening. Dynamic instability was discovered over 40 years ago in microtubules, yet due to their complex nature many basic questions surrounding this process remain unanswered. To address this, we study ParM, a simpler bacterial actin-like filament that also undergoes dynamic instability. We developed a theoretical framework alongside stochastic simulations and compared this to single filament TIRF experiments to achieve a complete biophysical description of dynamic instability in ParM. We find that a simple cap model is sufficient to capture experimental results and determine biologically relevant values for model parameters. This model is the first step in understanding dynamic instability across different domains of life in bacteria, archaea, eukaryotes, and open the door to synthetically creating dynamically unstable filaments.

B396/P0964
Social avoidance of bacterial spores provides a survival benefit in the presence of other bacterial species
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Bacteria generally reside in multi-species ecosystems, however this context is often neglected in studies of cellular responses. We investigated how the decision for Bacillus subtilis spore germination is impacted by other bacterial species. Germination is commonly triggered by L-alanine and competitively inhibited by its enantiomer, D-alanine. The biological role of this enantiomer sensing remains unclear. Given that D-alanine is primarily produced by microbial organisms in contrast with the more ubiquitous L-alanine, we investigated whether enantiomer sensing plays an ecological role. We mapped the enantiomer response space for germination, which showed that spores integrate the ratio of L- to D-alanine. Next, we quantified the L- and D-alanine concentrations secreted by over 20 diverse bacterial species. Unexpectedly, alanine enantiomer ratios secreted by most species map outside the germination response range for B. subtilis spores. This result suggested that spores avoid germination when sensing another species. By forcing germination in pairwise co-cultures we uncovered that the presence of another bacterial species is often detrimental to germinating spores. Spores thus appear to exploit the L-/D-alanine ratio as a social avoidance mechanism, revealing a benefit for enantiomer sensing and the importance of amino acid handedness in ecosystems.

B390/P0958
Hierarchical Decision-making in Stentor
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Between 1902 and 1906 Herbert Jennings demonstrated that Stentor roeselii, a semi-transparent generally-sessile member of the genus, was capable of enacting different responses to the same noxious stimulus provided in consistent short bursts. Further, Jennings characterized the different response outcomes in a population of stentors and demonstrated that these organisms followed a general hierarchical decision tree; usually enacting one behavior after another in a very specific pattern. Several decades later, researchers working with S. coeruleus, a much more motile and light-sensitive species, suggested that this stentor defaulted to detachment from substrate whenever presented with the same noxious stimulant rather than exhibiting any other response before detachment, thus throwing Jennings' original work into doubt. Recent work with S. roeselii has validated Jennings' original work and brought these observations into the modern era of cell biology and behavior. We follow up on this recent re-validation of Jennings' work by asking whether other species of Stentor are also capable of demonstrating hierarchical decision patterns in response to noxious stimulants, and if so, whether these always exhibit the same general pattern or whether there is species-specific expression of these behaviors. Using a hydraulic microinjector, we introduced either of two noxious stimulants – 1um magnetic polystyrene beads, or reconstituted powdered milk – in controlled volumes and at controlled intervals of 1 minute to the frontal field of stentor cells anchored to a substrate. In this way, we analyzed the response of four stentor species – light-sensitive S. coeruleus, light-loving S. stipatus, and light-agnostic S. muelleri and S. polymorphus. Interestingly, all species, including S. coeruleus, exhibit all of the primary responses (turning, ciliary reversal, and contraction), but not all exhibit the terminal response (detachment). Furthermore, some species heavily favor certain responses over others, demonstrating that the hierarchical response may be tied to cellular, behavioral, and ecological differences amongst these species.

B393/P0961
New classes of RNA-binding proteins reveal the convergent evolution of viral satellites, the "molecular parasites"
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Satellite RNAs (satRNAs) are unique small (200-1500 nucleotides) coding or non-coding RNAs associated with various animal, plant, or fungal viruses. Despite satRNAs sharing limited to no sequence homology with their cognate helper viruses, these "molecular parasites" fully rely on helper viruses for their replication and encapsidation. Certain satRNAs enhance (e.g., human hepatitis D virus, HDV) or attenuate (e.g., cucumber mosaic virus) viral symptoms of many diseases. However, the origins and evolution of satRNAs remain a mystery to date. Trichomonas vaginalis virus (TVV) is a dsRNA virus that infects T. vaginalis, a flagellar protozoan pathogen of trichomoniasis, which is the most common non-viral sexually transmitted disease worldwide. Although TVV satRNAs (satTVV) have been identified for over 30 years, their cellular and functional properties remain largely uncharacterized. Here we report two new satTVV-encoded proteins, p7 and p14, with novel RNA- and iron-binding activities. p7 shows sequence-specific and high affinity interactions with

satTVV RNAs in the presence of Mg2+ and ATP, with binding preference to the (-) than the (+) strand (Kd=8 nM versus 73 nM). AlphaFold predicts that iron Fe3+, a virulence factor and essential mediator of T. vaginalis growth, can interact with p7 histidine/cysteine pocket (His11/15/38, Cys53). This prediction is consistent with our finding that iron (1 mM Fe2+ or Fe3+) can eliminate p7-RNA binding completely, with Fe2+ or Fe3+ binding affinity of p7 (Kd=28 microM) being >13-fold higher than with other cation ions. Conformational changes of p7 upon RNA and iron binding are also evidenced by the alteration of quantum yield and redshift of the intrinsic fluorescence of the adjacent tryptophan 42 residue. This is the first report of an iron-sensing RNA-binding protein in a virus. With heme, p7 SxIP motif is predicted to bind to conserved EB1 C-terminal helix, suggesting that p7 may also interact with T. vaginalis EB1 as microtubule plus end tracking (+TIP) protein. These data imply that the change of iron/blood concentration in the vaginal environment throughout the menstrual cycle may regulate the satTVV life cycle (translation, replication, encapsidation etc.) via p7. In contrast, p14 binds to satTVV (+) and (-) strand RNAs equally, and its binding is not affected by iron. Although p7 and p14 show no sequence homology with any known protein, p14 is predicted to form a jelly-roll motif, which is commonly found in many RNA-binding proteins (e.g. capsid or movement proteins) of plant ssRNA viruses or satRNAs. Taken together, our results not only unravel new classes of RNA-binding proteins but also give insight into the convergent evolution of viral satellites.

B387/P0955

Plasticity of invertebrate cell division in a changing ocean

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Animal reproduction requires a complex coordination of events during embryonic cell division. How will this coordination be affected in fluctuating environments, such as the ocean, where global temperatures are forecasted to increase 1-4°C across the next century? Here we use the sea stars Patiria miniata and Asterias forbesi, which reproduce by broadcast spawning eggs into the open ocean, to define the thermal resilience of early cellular events during development. With live imaging, we find that P. miniata embryos from the Pacific are acutely sensitive to temperature, as a 2°C increase from their normal temperature can cause incomplete cleavage during the first cytokinetic division. Some embryos recover in the subsequent cell cycle through a multipolar division, while those which cannot face cell death or deformities later in development. Cytokinesis failures at higher temperatures are not due to chromosome segregation errors. Instead, we find that, under heat stress, daughter nuclei progress into the next cell cycle before the cytokinetic ring fully ingresses, suggesting a loss of coordination between cell cycle progression and cytokinesis. This coordination can be rescued by cyclin A knockdown or CDK inhibition to elongate the cell cycle at elevated temperatures. We also hypothesize that altered biophysical properties of the cortex may underlie temperature sensitivity. With micropipette aspiration measurements, we find that cortical tension is reduced during failed cytokinesis at higher temperatures. To explore how cytokinesis is evolutionarily tuned to different environments, we turned to A. forbesi embryos from the warmer Atlantic. These embryos display a thermal tolerance that is 3°C warmer than the tolerance of P. miniata. Given that the eggs of A. forbesi are morphologically very similar to those of P. miniata, apart from being 40-70µm smaller, we hypothesize that this smaller egg size may contribute to higher thermal tolerance. Supporting this, we find that surgically reducing the size of P. miniata eggs to that of A. forbesi allows for successful cytokinesis at higher temperatures where full-sized embryos normally fail. Based on measured parameters for both species of sea stars, we have developed a working mathematical model which supports the idea that early cytokinetic success may be dependent on an embryo's ability to complete cytokinetic ring ingression, based on the size of the fertilized egg, within the amount of time available in the cell cycle for cytokinesis at a given temperature. These size-timing relationships may represent a conserved

principle in cell size scaling. Multipolar compensation during the second mitotic cell division may be a conserved corrective response to thermal stress.

B388/P0956
Subcellular localization and expression patterns of de novo emerged microproteins
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Recent genomic analyses have revealed thousands of previously overlooked small open-reading frames that undergo active translation, giving rise to evolutionarily novel microproteins. Despite emerging evidence that some of these microproteins form structured domains and influence cellular fitness, their subcellular localization, expression stability, and cell biology remain largely unknown. Are these novel microproteins rapidly degraded or selectively shuttled to specific cellular compartments? Do their localization patterns differ fundamentally from canonical proteins? To systematically address these questions, we engineered approximately 260 fluorescently-tagged de novo emerged microproteins for inducible expression in the model organism Saccharomyces cerevisiae. Using live-cell fluorescence microscopy, we comprehensively mapped the subcellular localization and derived protein abundance profiles of these novel microproteins. Our data reveal a remarkable diversity of localization and abundance patterns. These novel microproteins display subcellular localization distributions distinct from canonical proteins, with notable enrichment in mitochondria and ER and reduced presence in the nucleus. Moreover, we identify specific sequence features associated with distinct subcellular localizations, suggesting intrinsic cellular recognition mechanisms for these evolutionarily naive sequences. This comprehensive cellular atlas provides foundational insights into the cell biology of de novo emerged microproteins, as well as their potential impact on cellular homeostasis and evolutionary innovation.

B389/P0957
The chytrid fungus Batrachochytrium dendrobatidis differentially regulates actin networks between its animal-like and yeast-like cell types.
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Cell morphogenesis is critical for organismal development and physiology. In nearly all eukaryotes, including animal and yeast cells, shape control relies heavily on actin. While animal cells change shape with an actin cortex and protrusions, yeast remodel their walls with directed actin-mediated material delivery and endocytic actin patches. The mechanisms of cell morphogenesis have clearly diversified across animals and fungi, but how this diversification occurred has largely remained unstudied. Chytrids are deep branching fungi that have actin networks resembling those of both animals and yeast, making them great tools for studying morphogenetic diversification. Chytrids develop from motile wall-less “zoospores” into sessile, walled “sporangia” that produce the next zoospore generation. In the “frog-killing” chytrid Batrachochytrium dendrobatidis (Bd), zoospores have animal-like actin structures like a cortex and protrusions, while sporangia have more yeast-typical structures like actin patches. It is not understood if Bd actin structures are homologous to similar structures in animals and yeast, as we would need to know which actin networks incorporate animal- and yeast-like actin regulators, but when and how Bd uses any of its actin network components is unknown. Therefore, to establish when Bd expresses these components, we performed RNA sequencing throughout its lifecycle. By analyzing RNA abundance, we find that actin regulatory and endocytosis-related genes can be

divided into two groups: those highly expressed in zoospores and cellularizing stages and those highly expressed in developing sporangia. Several genes in the first category are associated with animal-typical actin structures, including the SCAR/WAVE complex and talin, actin regulators that are important for crawling motility in animal cells. Actin itself also falls into this category, suggesting that Bd may use high monomer levels in zoospores and lower levels in sporangia. In contrast, genes that are highly expressed in sporangia tend to be general actin regulators and endocytosis-associated genes. These data suggest that actin structures in Bd could be homologous to their counterparts in animals and yeast, a finding that we are confirming by determining the localization of SCAR/WAVE, talin, and select endocytosis proteins to the actin structures of interest.

B391/P0959
UT-1 Transporter Expression in the Spiny Dogfish (Squalus acanthias): UT-1 Protein Shows a Different Localization in Comparison to That of Other Sharks
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The original UT-1 transporter gene was initially identified in the spiny dogfish (Squalus acanthias), but localization of the UT-1 protein was not determined. Subsequent UT-1 expression was shown to localize to the collecting tubule (CT) of the shark nephron in other shark species, with expression in a closely related chimaera species also located additionally at a lower level in the intermediate-I segment (IS-I) of the nephron. In spiny dogfish, two UT-1 splice variants are known (UT-1 long and short), and there was also a second UT-1 gene described (here termed Brain UT). In this study, a second splice variant of the second Brain UT gene was discovered. Expression profiles (mRNA) of UT-1 long and short and Brain UT were determined in a number of spiny dogfish tissues. Quantitative PCR in kidney samples showed that the level of the short variant of UT-1 was around 100 times higher than the long variant, which was itself expressed around 10 times higher than Brain UT cDNA/mRNA (in kidney). For the long variant, there was a significantly higher level of mRNA abundance in fish acclimatized to 75% seawater. Ultimately, three UT-1 antibodies were made that could bind to both the UT-1 short and long variant proteins. The first two of these showed bands of appropriate sizes on Western blots of around 52.5 and 46 kDa. The second antibody had some additional lower molecular weight bands. The third antibody was mainly bound to the 46 kDa band with faint 52.5 kDa staining. Both the 52.5 and 46 kDa bands were absent when the antibodies were pre-blocked with the peptide antigens used to make them. Across the three antibodies, there were many similarities in localization but differences in subcellular localization. Predominantly, antibody staining was greatest in the intermediate segment 1 (IS-I) and proximal (PIb) segments of the first sinus zone loop of the nephron, with reasonably strong expression also found at the start and middle of the late distal tubule (LDT; second sinus zone loop). While some expression in the collecting tubule (CT) could not be ruled out, the level of staining seemed to be low or non-existent in convoluted bundle zone nephron segments such as the CT. Hence, this suggests that spiny dogfish have a fundamentally different mode of urea absorption in comparison to that found in other shark species, potentially focused more on the nephron sinus zone loops than the CT.

Cell Biology Across the Tree of Life - Fungi

B413/P0979
Endosomal proteins regulate light dependent reproductive development and secondary metabolite production in Aspergillus nidulans
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The motility and positioning of organelles in eukaryotic cells are primarily driven by dynein and kinesin motors that move along microtubule tracks. The proper distribution of organelles is essential for development and function across the tree of life. The filamentous fungus Aspergillus nidulans is a well-established model for studying microtubule-based transport because it relies on dynein and kinesin to position organelles and is experimentally tractable. Previously, in our genetic screen in A. nidulans, we identified a gene, PxdA, that is required for peroxisome positioning. Surprisingly, PxdA localizes to endosomes, and we discovered that peroxisomes move only when associated with a moving endosome, a phenomenon we termed “organelle hitchhiking.” Since this discovery, our goal has been to determine both the mechanism and physiological function of organelle hitchhiking. A. nidulans has a complex life cycle that includes both asexual and sexual reproduction, with the developmental decision influenced by several environmental cues, including light. Our recent work shows that in the absence of PxdA, sexual reproduction is favored even under light conditions that normally promote asexual reproduction in wild-type A. nidulans. Interestingly, deletion of HookA, an adaptor that links endosomes to microtubule motors, produces the same phenotype. These findings suggest that moving endosomes play a regulatory role in determining whether A. nidulans undergoes sexual or asexual development. In contrast, deletion of PxdB, a gene required for peroxisome hitchhiking that localizes to peroxisomes, does not alter this developmental decision. As a first step to understanding the mechanism of this developmental phenotype, we performed RNA sequencing in strains lacking pxdA, hookA, or pxdB. We observed significant changes in the expression of genes involved in development. In addition, and more surprisingly, our data revealed significant changes in the expression of seoncdary metabolite genes. Furthermore, LC-MS analysis revealed many changes in the production of secondary metabolites. For example, in strains lacking PxdA or HookA (loss of endosome motility) led to altered synthesis of Sterigmatocystin, a well-characterized carcinogenic toxin. A. nidulans produces more than 70 secondary metabolites, including antibiotics and toxins, yet the cell biology underlying their synthesis remains poorly understood. Our data implicate both endosomal proteins and endosome motility in the regulation of secondary metabolism. Current experiments are focused on understanding why motile endosomes are permissive for asexual development and how organelle trafficking and inter-organelle contacts contribute to the control of secondary metabolite biosynthesis.

B412/P0978

Living with a killer: how coevolved Saccharomyces cerevisiae become killer toxin resistant

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Some yeasts are killers. They secrete toxins that kill neighboring cells but protect themselves with an intracellular antidote. Killers have an advantage in mixed populations, although toxin production comes at a metabolic cost. Many Saccharomyces cerevisiae strains require two viral genomes to be killers. Along with sensitive cells in the environment, this is a complex genetic conflict: yeast and viral genomes alike are capable of adaptation and fitness tradeoffs abound at many levels. Our research dissects the molecular basis of adaptation in the face of these competing selection pressures to understand evolutionary outcomes. We identified beneficial mutations that arose in coevolved killer and sensitive yeast, including mutations responsible for toxin-resistance. Through bulk segregant analysis, we identified a putative gain-of-function missense mutation in the HOG osmoregulatory pathway component SSK1. Preliminary data suggest this dominant-acting polymorphism is sufficient to protect yeast from the coevolved toxin, and other killer toxins. Ongoing experiments aim to determine the mechanism by which this polymorphism creates toxin-resistance

and any host tradeoffs associated with the coevolved allele. Preliminary data suggest this mutation may affect protein-protein interactions, leading to crosstalk with the Cell Wall Integrity (CWI) pathway. We are pursuing the transcriptional and signaling impacts of the resistant allele. SSK1 is required for multiple drug resistance in Candida auris, and our ongoing work will address how this mutant allele might impact phenotypes of fungi associated with human pathogenicity and drug resistance. In future, we aim to understand how killer yeasts counter-adapt in the face of resistant competitors, and how viral genomes evolve as competing host population composition changes. Understanding how fungi adapt, defend themselves, and kill one another has important implications for health during climate change, as fungal pathogens emerge and drug resistance spreads.

B411/P0977
Organelle inheritance and scaling in a multi-budding yeast
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Cells typically divide in half producing two daughters, but there are diverse examples of more elaborate division strategies that give rise to multiple daughters. These include multiple fission of some green algae and bacteria, cellularization during embryogenesis of plants and insects, and similar strategies in Ichthyosporeans, Chytrids, and Apicomplexans. In some multinucleate yeast species, large mother cells can produce multiple buds (daughters) in a single cell cycle. Here we investigate whether mother cells confer an equal inheritance to each bud or whether sibling buds receive unequal contents during multibudding yeast proliferation. Using live cell probes, we imaged the inheritance of cytoplasm, nuclei, mitochondria, endoplasmic reticulum (ER), vacuoles, and peroxisomes in multibudded Aureobasidium pullulans. Mother cells grew sibling buds at similar rates, and delivered organelles to each bud at stereotyped times. On rare occasions when buds grew at different rates, organelle inheritance generally correlated with growth rate. While cortical ER and mitochondria were evenly partitioned across daughters, nuclei, vacuoles, and peroxisomes could vary >2-fold across sibling buds. Across a population of yeast cells, cell volume varied up to ~100-fold, and we found that ER and mitochondria content was directly proportional to volume, while vacuole and peroxisome content was more weakly correlated with volume. Nevertheless, daughter cells inheriting unusually high or low numbers of vacuoles or peroxisomes appeared to adjust organelle content during subsequent growth. Our findings suggest that this unconventional fungus has developed accurate mechanisms to evenly partition cortical ER and mitochondria among sibling buds, and compensatory pathways to adjust the content of other organelles so as to maintain an optimal content across an impressive range of cell sizes.

B414/P0980
The Role of Potassium in Initiating Pressure Gradients of Filamentous Fungi
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Filamentous fungi move cytoplasm and organelles through their hyphae by establishing pressure gradients that lead to bulk flow. While the properties of this flow have been described, the mechanisms by which the fungus initiates a gradient are unknown. One hypothesis is that maximal ion uptake and osmolyte production occurs at a point behind the leading edge of the colony and the osmotic potential drives water intake that pushes cytoplasm towards the hyphal tips. Using the model fungus Neurospora crassa we aim to elucidate the role of ion distribution in initiating the pressure gradient required for mass flow. We chose to initially focus on

potassium (K+), as N. crassa maintains a 4-5-fold higher intracellular concentration of K+ than a minimal media substrate. Using the extracellular indicator dye Ion Potassium Green 1- TMA+, we mapped changes in extracellular K+ distribution with a biomolecular imager during the growth of N. crassa mycelia on agarose plates of defined Vogel's N media. Another set of colonies were imaged with 3,3'-diethyloxacarbocyanine iodide (DiOC2(3)) membrane potential indicator. Fluorescent imaging revealed that 24 hours post-inoculation, an extracellular K+ gradient had been established with a peak near the basal region of the colony. 48 hours after inoculation the extracellular K+ gradient was maintained but the peak shifted 10mm towards the growing edge. K+ signal was not maintained on the basal side of the peak. DiOC2(3) imaging revealed that dye consistently accumulated within the first 5 mm of the hyphal tips, with little dye accumulation occurring basally in the colony, indicating a steeper membrane potential at the tips than elsewhere in the hyphae. These findings suggest that K+ influx occurs within the first 5 mm of the tips, resulting in the linear decrease seen in extracellular K+ from the edge of the plate to the hyphal tips. Coupled with the apparent efflux of K+ at the base of the colony, the results suggest a potential mechanism for pressure gradient initiation counter to current thinking. We propose that K+ efflux basal to the growing tips increases hydrostatic pressure in the external environment, which overcomes the osmotic potential at that point and drives water into the cell, increasing pressure inside the cell and driving the mass flow of cytoplasm. To further test this mechanism, future work will include hourly time-series measurements and the mapping of intracellular K+ gradients using membrane-permeable IPG1-AM dye, as well as mapping of ions, such as sodium and chloride, and of osmolytes. Additionally, we will endogenously tag the known N. crassa K+ transporter genes, trk-1 and hak-1, with fluorescent proteins and utilize fluorescent microscopy to visualize and quantify plasma membrane localization along the length of the hyphae.

Cell Biology Across the Tree of Life - Protists

B407/P0973
Bioinformatic, cell localization and phylogenetic analyses indicate a novel family of lipases in Tetrahymena thermophila
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Lipid droplets are important and dynamic organelles in all cells. Work in our lab has shown that the freshwater ciliate Tetrahymena thermophila, like other organisms, increases lipid droplet formation during nutrient challenges such as starvation. Proteomic studies have shown that lipid droplets are decorated with up to hundreds of different proteins that modulate their formation, function, and interaction with other organelles. Results of a T. thermophila proteomic screen identified lipid droplet-associated proteins, one of which (TTHERM_00784270, or 4270), is a hypothetical protein of no known function. A protein-protein BLAST of 4270 identified 15 other T. thermophila proteins, nine of which are identified as alpha/beta hydrolases, and six hypothetical proteins in other Tetrahymena species (three in T. malaccensis and three in T. utriculariae). All of the genes are sequentially arranged along macronuclear chromosome 55 and share several conserved amino acid sequences, including a pentapeptide GXSXG motif characteristic of lipases. Phylogenetic analysis of the protein sequences reveals subgroups that share chromosomal proximity, suggesting a history of gene duplication. Fluorescent tagging revealed that four members of this family colocalize with populations of lipid droplets. Molecular modeling corroborates the bioinformatic evidence that we have identified a novel family of lipases in Tetrahymena.

B405/P0971

Cellular Transformers: Surface Glycans Mediate Robust Assembly of Silica Cages in Loricate Choanoflagellates

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Controlled, bottom-up assembly of micron-scale 3D structures remains a fundamental challenge in engineering and physics. Loricate choanoflagellates provide a striking counterpoint: within 10 minutes, a single cell constructs an intricate extracellular basket (loricae) from hundreds of biomineralized silica strips through a process that has to balance opposing requirements of free movement and strong adhesion. The building blocks (curved, rod-shaped strips 2–3 µm long and ~100 nm wide) must first remain relatively non-adhesive to move into position yet later adhere strongly to form stable structures. How does a single cell modulate adhesion and microscale manipulation to build robust extracellular architectures in a fluctuating marine environment? Using the loricate D. grandis as a model, we generated high-resolution 3D datasets of intact loricae and quantified key geometric features, confirming the fidelity of assembly. We found that fully assembled loricae are highly resilient to mechanical stress, withstanding significant compressive strain without detectable damage, consistent with strong adhesion between constituent strips. Inspired by other microbial adhesive structures, we hypothesized that glycans (cell-secreted polysaccharides) mediate this adhesion. Using fluorescent lectin labeling and super-resolution microscopy, we identified distinct spatial patterns: O-glycans showed uniform coverage, while N-glycans were enriched at strip junctions, suggesting adhesive roles in guiding and anchoring strips, respectively. In vitro experiments on isolated strips revealed that controlled proteinase treatment sequentially “unmasks” adhesive N-glycan sites at distinct locations, indicating a layered architecture. These surface glycans also impart key biophysical properties, including strong negative surface charges that may enable mobility of the strips during assembly. Interestingly, we found that perturbing strip surface properties during assembly—by altering protein or glycan composition (e.g., charge or sialylation)—produced striking assembly failures, underscoring the importance of surface biophysical properties in lorica assembly. Together, these findings reveal how a single cell encodes information for reliably building complex extracellular 3D structures through glycan-mediated, spatiotemporal control of nanoscale adhesion.

B408/P0974

Characterization of an Unknown Tir Gene in Dictyostelium discoideum, through CRISPR-Cas9 Technology

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Mammalian macrophage cells contain Toll-like Receptors (TLRs) that have cytoplasmic Toll/Interleukin-1 receptor (TIR) domains aiding immune cascades and pathogen recognition. Though full TLRs are absent in the model system Dictyostelium discoideum, its genome encodes for TIR domain-containing proteins. TirA in D. discoideum has been characterized and shown to play a role in immune related phagocytic signaling. Here we aim to characterize the function of an under-studied D. discoideum Tir domain-containing protein (DDB_G0283429), which shows sequence similarity to bacterial Tirs. Though this Tir’s functions are widely unknown, our results with a recombinantly expressed protein suggest that this Tir may exhibit enzymatic activity. We successfully created two Tir knockout cell lines in D. discoideum using the CRISPR-Cas9 gene editing methodology to observe gene function. Disruption of the gene sequence was captured at AA62 and AA73 in each respective cell line causing a frameshift mutation and the incorporation of a premature stop codon. To begin basic functional characterization of the Tir mutant, we are performing cell growth and developmental assays. Our preliminary results suggest that there may be alterations in multicellular

development in our Tir knockout lines, and we are exploring if the enzymatic activity of this Tir may contribute to its function. Further characterization will be required to confirm the role of this Tir in D. discoideum function and to assess TIR conservation in mammalian systems.

B409/P0975
Identification of TTHERM_00497200 as a putative seipin in Tetrahymena thermophila
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Lipid droplets are essential organelles responsible for fat storage and metabolism. Seipin, a conserved transmembrane protein localized in the ER is critical to lipid droplet formation. It is thought that all eukaryotic cells have seipin; however, the gene for seipin has not been identified in Tetrahymena thermophila, a single-celled freshwater ciliate. Using DELTA-BLAST, we identified a seipin domain in the protein TTHERM_00497200. Molecular modeling indicates structural similarity between TTHERM_00497200 protein and human BSCL2/seipin and Saccharomyces cerevisiae SEI1/seipin. Two lines of evidence suggest that TTHERM_00497200 may be a Tetrahymena seipin. First, we expressed a fluorescently tagged TTHERM_00497200 in Tetrahymena, and its localization is consistent with the ER. In addition, overexpression of TTHERM_00497200 in Tetrahymena results in increased triacylglycerol accumulation, as it does in other organisms. To determine if TTHERM_00497200 functions as seipin, we asked whether the TTHERM_00497200 protein could rescue the phenotype of S. cerevisiae sei1Δ mutants, which exhibit fewer, larger lipid droplets and altered lipid accumulation. We constructed yeast expression plasmids with codon-optimized TTHERM_00497200, and transformed them into sei1Δ yeast. Fluorescence microscopy of lipid droplets with BODIPY 493/503 did not rescue the sei1Δ yeast lipid droplet phenotype. Our current experiments are aimed at determining if TTHERM_00497200 rescues the altered lipid accumulation in yeast and further characterizing its function in Tetrahymena.

B410/P0976
Modulating morphogenesis in a single cell: localized RNA encoding transcription factors in Stentor coeruleus
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The giant ciliate Stentor coeruleus is a classical model system used to study morphogenesis and regeneration at the single-cell level. Prior work revealed that approximately 6300 RNAs are localized to the anterior or posterior, many of which encode Myb DNA-binding proteins. In this study, we investigated the role of transcription factors that are regionalized on the anterior-posterior axis with RNAi. We performed RNAi knockdowns via microinjection to determine whether candidates are required for proper cell structure or regeneration in Stentor coeruleus. This approach opens up a new window into understanding the relationship between patterned transcription factors and complex morphological structures in cells.

B406/P0972
Signaling pathways governing the trigger of regeneration in Stentor coeruleus
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A key question in the biology of regeneration is what triggers the process? While multicellular organisms often rely on reactivation of developmental gene networks, it remains unclear whether single cells employ similar strategies. Regeneration in the giant ciliate Stentor coeruleus, not only involves the reassembly of complex structures such as the oral apparatus but also recapitulates many of the morphogenetic events typically associated with cell division. Previously, it has been suggested that Stentor cells are either in a state of activation or inhibition depending on its stage of development, which may correlate to cell cycle stages. Transcriptional profiling has identified an E2F homolog (SteCoe_12750) that is upregulated early in regeneration, even without new protein synthesis, indicating it is a primary response gene in the regenerative signaling cascade. Pharmacological inhibition of CDK4 with Palbociclib impairs regeneration and downregulates expression of E2F pathway components, including Rb, cyclins, CDKs, and downstream targets. Phosphoproteomic analyses further implicate MAPK signaling in even earlier signaling regeneration events, where MAPK inhibition during regeneration produces abnormal morphologies and ectopic structures, suggesting that the MAPK signaling may be part of the cell's inhibitory state. These results indicate that canonical cell cycle regulators function as molecular switches linking external injury cues to transcriptional programs that control regeneration. Thus, Stentor demonstrates how cell cycle machinery can be repurposed for non-canonical roles, highlighting that regeneration depends on the strategic redeployment of developmental pathways in a spatiotemporally regulated manner.

Cell Biology Across the Tree of Life - Viruses

B417/P0983
Membrane and Protein-Bound Organelles Compartmentalise Genomes During Phage Infection
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Bacteriophages of the Chimalliviridae family assemble a proteinaceous phage nucleus that sequesters the viral genome from host defenses. The phage nucleus also functions similarly to a eukaryotic nucleus, importing proteins for phage DNA replication and separating transcription from translation and metabolic processes. Until recently, it was unclear how phage DNA is protected from host defense systems before phage nucleus formation. Our recent work using CRISPRi-ART, fluorescence microscopy, and cryo-electron tomography revealed that preventing phage nucleus assembly arrests Goslar infections at an early stage in which the incoming phage genome resides within a lipid membrane–bound vesicle, termed the early phage infection (EPI) vesicle. Transcription of early phage genes by the virion-packaged RNA polymerase occurs inside the vesicle, establishing the first known example of a lipid-bound organelle in prokaryotes that separates transcription from translation. Following nuclear shell assembly from early phage proteins, viral DNA is transferred into the phage nucleus, where genome replication and late transcription proceed. Thus, nucleus-forming phages require two distinct compartments of different composition and function to complete their life cycle. Building on these results, we are characterizing the role of essential phage proteins of unknown function during early stages of infection.

B416/P0982
Spatially resolved thermal profiling uncovers HSV-1-driven regulation of proteostasis via nuclear sequestration of translation machinery
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The cellular response to viral infections involves cell-wide remodeling of the interactions and localizations of host proteins. Herpes simplex virus type-1 (HSV-1) initiates extensive competition for essential resources and cellular factors, remodeling the nucleus by requisitioning histones and RNA polymerases, reprogramming splicing complexes, and dissolving the nucleolus, a site responsible for the bulk of ribosome biogenesis. Considering this multi-dimensionality, we sought to globally measure infection-driven remodeling of protein-protein interactions (PPIs) with spatial resolution by integrating nuclear/cytoplasmic fractionation with thermal proximity coaggregation analysis (N/C-TPCA). In this way, we monitored the compartment-specific assembly status of PPIs in a global manner throughout infection with an immunostimulatory strain of HSV-1. This highlighted the diverse ways by which HSV-1 infection modifies the shuttling and assembly status of protein complexes involved in DNA repair, replication, chromosome maintenance, nuclear actin networks, and innate immune signaling to suit virus growth. Furthermore, we discovered that host chaperones are broadly recruited to the nucleus following infection. This finding to correlated with the appearance of virus-induced chaperone-enriched (VICE) domains, a unique feature of HSV-1 infection whose composition and functional significance are incompletely understood. We leveraged our subcellular PPI analysis to investigate VICE domain composition, finding that proteins exhibiting infection-dependent interactions with chaperone proteins in the nucleus were enriched in ribosome subunits and biogenesis factors. Follow-up experiments involving live-cell imaging, polysome profiling, and RNA FISH led us to establish that VICE domains sequester ribosome biogenesis factors from ribosomal RNA, accompanying a cell-wide defect in ribosome supply. Furthermore, using pulse-chase imaging to follow ribosomal proteins of varying molecular ages, found that VICE domains recruit not only nascent ribosomal proteins, but even ribosomal factors of mature, cytoplasmic origin. These findings highlight VICE domains as nodes of translational remodeling in the competition between virus and host for essential resources during infection and moreover demonstrate the utility of N/C-TPCA to study dynamic biological contexts.

Cell Death - Apoptosis

B253/P1377
A gated hydrophobic funnel within BAX binds bioactive lipids to potentiate pro-apoptotic function.
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Mitochondria maintain a distinct biochemical environment that cooperates with pro-apoptotic BAX and BH3-only proteins (e.g., BIM) to promote mitochondrial outer membrane permeabilization (MOMP), the key event to initiate physiological and pharmacological forms of apoptosis. The sphingosine-1-phosphate metabolite 2-trans-hexadecenal (2t-hexadecenal) is a bioactive lipid described to support BAX-dependent MOMP, but structural and molecular mechanisms remain largely unknown. Using integrated structural and computational approaches, we determine that 2t-hexadecenal binds within a previously undefined dynamic region – a hydrophobic cavity formed by core-facing residues of α5, α6, and gated by α8 – we now term the "BAX actuating funnel" (BAF). Complementary biochemical and biophysical techniques reveal that 2t-hexadecenal non-covalently interacts with the BAF, and cooperates with BIM to stimulate intramolecular activation of monomeric BAX prior to membrane association. BAX α8 mobility and proline 168-mediated allostery are critical determinants for 2t-hexadecenal synergy with BAX and BIM, as is alkenal length to stimulate BAF function. Collectively, this work imparts detailed molecular insights into how pro-apoptotic BCL-

2 proteins and bioactive lipids cooperate to initiate the mitochondrial pathway of apoptosis with implications for biological and therapeutic regulation*. *Revision submitted to Nature Communications on August 27, 2025.

B255/P1379
Anti-apoptotic protein Bcl-xL directly binds to mitochondrial ATP synthase and modulates its activity
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Bcl-xL is an anti-apoptotic protein of the BCL-2 family that is abundantly expressed in cardiomyocytes. Bcl-xL localizes to the outer mitochondrial membrane and interacts with pro-apoptotic protein Bax to inhibit apoptotic cell death. Reduced Bcl-xL expression, in contrast, correlates with increased cardiomyocyte cell death. Bcl-xL has also been shown to localize in the mitochondrial inner membrane and regulate the metabolic function of the cell through its interaction with mitochondrial ATP synthase. This study investigated the molecular nature of Bcl-xL interaction with ATP synthase. We have optimized the large-scale purification of fully assembled and catalytically active ATP synthase from porcine heart mitochondria. The porcine Bcl-xL was overexpressed and purified from E.coli. Here, we found a direct and high-affinity interaction between purified Bcl-xL and ATP synthase (KD=65 nM) using the microscale thermophoresis. The assay was also performed in the presence of a small molecule ABT-737, a BH3-mimetic compound, which binds to the p2 and p4 hydrophobic pockets of BCL-xL within its hydrophobic groove. The binding affinity of BCL-xL to ATP synthase was reduced in a dose-dependent manner in the presence of ABT-737, which further validates direct binding and formation of the Bcl-xL/ATP synthase complex, and suggests the involvement of p2 and p4 hydrophobic pockets in this interaction. Furthermore, we show that Bcl-xL significantly enhances the catalytic activity of ATP synthase. Lastly, we used single-particle cryo-EM to obtain the molecular structure of the Bcl-xL/ATP synthase complex. This study will facilitate the structure-based drug design of therapeutic compounds mimicking Bcl-xL function to regulate catalytic and leak channel activities of ATP synthase.

B251/P1375
Elucidating Molecular Mechanisms Governing Reversal of Apoptosis
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Apoptosis has long been assumed to be an intrinsically irreversible programmed cell death process ending in demise, because it causes rapid and massive cellular demolition. Challenging this general dogma, recent studies demonstrated that apoptotic dying cells can recover from the brink of death, even after the generally accepted “point of no return”, such as mitochondrial outer membrane permeabilization (MOMP) and caspase-3 activation, and their subsequent cellular damages, including cell shrinkage and formation of apoptotic bodies, as demonstrated by live-cell imaging in mammalian primary cells and cancer cell lines. This cell recovery mechanism is called “anastasis”, which means “rising to life” in Greek. Promoting anastasis to rescue terminally differentiated cells and tissues that are difficult to replace such as neurons in brain may be beneficial for treating brain injury. Conversely, suppressing anastasis could arrest the recovery of apoptotic brain cancer cells after cell death-inducing anti-cancer therapy, and therefore inhibits brain cancer recurrence. To harness the therapeutic potential of anastasis, we applied time-lapse microarray and RNA-sequencing to elucidate the molecular signatures and identify the druggable targets of anastasis in vitro. Our study reveals

upregulation of regulators that arrest apoptotic cell death machineries in anastatic cells, including XIAP to inhibit caspase-3 mediated cell execution, MDM2 to suppress p53-mediated apoptotic cell death signaling, PARP to repair DNA damage, and ATG12 to remove the caspase-cleaved and damaged cellular components by autophagy. Importantly, interfering the function of these regulators directly or indirectly with pharmacological approaches suppresses anastasis, indicating that they are the druggable targets of anastasis. This work lays a strong foundation for studying the consequences and mechanisms of anastasis, a critical step towards controlling anastasis to develop new therapeutic interventions by mediating reversibility of apoptosis.

B256/P1380

Impact of Fas ligand application and caspase inhibition on Rankl expression in IDG- SW3 cells

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Kratochvilova A 1 , Kostolanska K 1,2 , Sedlackova B 1,2 , Kytkova L 1 , Doubek J 2 , Matalova E 1,2 1 Laboratory of Odontogenesis and Osteogenesis, Institute of Animal Physiology and Genetics, CAS, v.v.i., Brno, Czech Republic 2 Department of Physiology, University of Veterinary Sciences, Brno, Czech Republic Bone consist of 3 key types of cells: matrix producing osteoblasts which can differentiate into osteocytes, the prevalent cell population in the adult bones, and osteoclasts displaying osteolytic activity during life-long bone remodelling. Fas ligand (FasL) belongs to cytokines associated with this process since FasL positive osteoblasts can induce apoptosis of osteoclasts. The number of osteoclasts is regulated also via the cytokine Rankl, which impacts differentiation of their precursors. Notably, FasL can work in both, apoptotic and non- apoptotic manner, the latter cases have been reported recently. The apoptotic effect engages caspase activation, the mechanisms of non-apoptotic pathway are not yet known. The aim of this investigation was to analyze the impact of FasL on Rankl expression in osteocytic cell line, IDG-SW3. The design consisted of 3 parallel groups including stimulation of cells by FasL, stimulation by FasL plus inhibition of caspases and caspase inhibition only. The IDG-SW3 cells, the common in vitro model of osteoblasto/cytogenesis, were differentiated for 28 days based on the common protocol. Additionally, expression of FasL and Rankl was followed using flow cytometry. In this pilot experiment, expression analysis (RNA level) revealed significant increase of Rankl after FasL application (1.5 x), significant increase (9.2 x) after FasL and simultaneous caspase inhibition but decrease (2.2 x) after caspase inhibition only. The findings indicate different effects of FasL in presence/absence of caspases and provide new evidence to FasL- Rankl cytokine networks involved in osteogenesis. Supported by the Internal Agency of the University of Veterinary Sciences Brno (ITA VETUNI), project 2022ITA13.

B252/P1376

Investigating candidates of neuronal death signaling in the larval nervous system of the tunicate Ciona robusta

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Programmed cell death (PCD) is a biological mechanism that helps maintain healthy cell populations and proper development; however, dysfunction of PCD can lead to diseases such as cancer or neurodegenerative diseases. Understanding the mechanisms for PCD in the context of neurodegeneration helps us understand the circumstances for when PCD mechanisms do not work properly in human health. As marine invertebrates,

tunicates are the closest living relatives to us vertebrates. The tunicate Ciona robusta metamorphoses from a swimming larva to a sedentary adult. After metamorphosis, larval cell types and structures die to make way for adult tissues, including the adult nervous system. This makes them great organisms to study the mechanisms of PCD in neurons, which invariantly happens during these drastic body plan changes. We are interested in exploring the various death signaling pathways involved in signaling neuronal death in the larval Ciona nervous system. Using tissue-specific CRISPR/Cas9 mutagenesis to eliminate the larval sensory neurons that trigger metamorphosis, we found that PCD in the larval nervous system is independent of an internal timing mechanism, as larval neurons did not undergo PCD when metamorphosis was blocked. We also found that blocking vesicular trafficking in neurons by expressing a dominant negative Sar1 similarly disrupted typical larval neuronal PCD, further suggesting that neuronal cell death depends on extrinsic signals. Promising candidate effectors of PCD downstream of this yet unidentified extrinsic death signal are the caspases, which are proteases that are well known for their role in apoptosis. When we knocked out Caspase8/10 via tissue-specific CRISPR/Cas9, we observed disruption of normal metamorphic processes of tail retraction. We are currently using a candidate-gene approach to identify other key players within this extrinsic pathway for PCD in the Ciona nervous system.

B254/P1378

Mitochondrial Resilience Mechanisms: Structure-Function Studies of ATP Synthase Leak Channel in Different Species

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Mitochondria are complex organelles that regulate not only cellular metabolism but also cell death. Mitochondrial calcium overload leads to the opening of the mitochondrial permeability transition pore (mPTP), a non-selective 1.5 kDa channel that forms within the otherwise impermeable inner mitochondrial membrane. Under prolonged opening of the mPTP, mitochondria undergo an osmotic imbalance, leading to mitochondrial swelling and subsequent rupture, followed by the release of apoptotic factors and cell death. Although mPTP is crucial in regulating cell death, its identity, structure, and gating mechanism are poorly understood. Mitochondrial ATP synthase has been recently discovered to possess a leak channel within its c-ring with similar biophysical characteristics of the mPTP, such as conductance, probability of channel opening, and sensitivity to calcium. Different species of the animal kingdom have evolved to possess diverse properties of mitochondrial permeability transition. Species such as the brine shrimp, Artemia franciscana, and the fruit fly, Drosophila melanogaster, lack the calcium-regulated mPTP. Here, we used comparative structural and functional analysis of ATP synthase across species to understand the gating mechanism of the ATP synthase c-subunit leak channel (ACLC) and its role in mPTP. Synthetic peptides resembling unique structural features of A. franciscana and D. melanogaster ATP synthase were developed to target the ACLC in mammals and assess their inhibitory properties. The therapeutic potential of these peptides to alter ATP synthase and ACLC activity are being assessed using structural and functional approaches.

B257/P1381

Novel SINE inhibitor induces apoptosis in Jurkat leukemia cells

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Selective inhibitors of nuclear export (SINE) drugs are a class of cancer drug that inhibits the action of nuclear export protein XPO1, thus blocking the export of nuclear proteins and RNAs. In 2020, the FDA approved the first SINE inhibitor drug, Selinexor (also known as KPT-330), to treat multiple myeloma and B cell lymphoma. Selinexor is associated with substantial toxicities and side effects emphasizing the need for continued exploration and development of novel SINE drugs. We examined the cancer cell cytotoxicity of a newly developed SINE drug, SINE-2. SINE-2 was prepared via a biomimetic Michael addition in which silver carbonate acts as a Lewis acid to form the key alkene in the desired Z configuration. Metabolic activity, trypan blue exclusion and Annexin V binding were used to investigate human Jurkat leukemia cell susceptibility to SINE-2 mediated cell death. Jurkat cell viability decreased in a dose-dependent manner after 24 hours of SINE-2 treatment. The cell death observed was regulated and not necrotic in nature. Our data show that SINE-2 induces regulated cell death in human Jurkat leukemia cells. Current studies include investigating SINE-2-mediated oxidative stress in treated Jurkat cells and normal non-malignant cell SINE-2 susceptibility.

B258/P1382
Phosphorylation of JNK by Cypress Essential Oil in both Fibroblasts and FIbrosarcoma Cells
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Cypress oil is an essential oil derived from evergreen coniferous trees native to Southern Europe and Western Asia. The components of cypress essential oil include a total of 20 constituents which represent 98.1% of the oil. These include: α-pinene (48.6%), δ-3-carene (22.1%), limonene (4.6%) and α-terpinolene (4.5%) which are the main components comprising 79.8% of the oil. Some of these components have demonstrated anti-cancer properties, but little is known about their effects in fibroblasts. Previous work in our laboratory demonstrated that both HT-1080 fibrosarcoma cells and CUA-4 normal human fibroblasts displayed reduced proliferation and viability after treatment with cypress essential oil. Apoptosis was provoked, as shown by PARP cleavage, in the two cell types. Our focus for this project was to test the involvement of the stress signaling protein JNK in the trigger of apoptosis. Both the fibrosarcoma cells and fibroblast were treated with cypress oil. Proteins were then extracted and used for western blot analysis to determine whether JNK was activated. The JNK proteins remained constant after treatment in normal fibroblasts while the level of JNK decreased after fibrosarcoma cells were treated. Activation of JNK in both cell types was still able to be identified although the level of total JNK was low in the fibrosarcoma cells. For further research, it may be possible to impede the activity of JNK with the help of chemical inhibitors or overexpression of phosphatases in order to test whether apoptosis could be reduced.

Cell Death - Emerging mechanisms of cell death

B267/P1391
C. elegans Myelin Regulatory Factor 1 primes a migrating cell for non-apoptotic cell death via the polyglutamine gene pqn-41
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Migration and cell death are important for normal development and are both deregulated in cancer, especially in metastatic cells, which contribute to 90% of cancer deaths. How cell migration and death are intertwined in development and in disease is not well understood. To address this question, we study the C. elegans male linker cell: a migrating leader cell that undergoes Linker Cell-type Death (LCD), a novel non-apoptotic morphologically conserved cell death program, upon completion of migration. Morphological evidence hints at similarities between the ultrastructures of cells undergoing LCD and metastatic cancer cells. We hypothesize that migrating metastatic cancer cells may actively suppress LCD, allowing them to evade death. To identify new migration and LCD regulators, we performed 10x single-cell RNA sequencing on FACS-purified migrating and dying linker cells. We conducted a candidate linker cell-specific RNAi screen on 257 genes upregulated in the dying cell and scored animals for abnormal linker cell survival. We identified several novel classes of LCD regulators: chromatin factors, ESCRT III components, and a highly conserved gene, myrf-1, encoding a membrane-anchored transcription factor implicated in many human developmental disorders. We show that the cleaved N-terminus of MYRF-1 translocates to the nucleus during early migration. Surprisingly, linker cell-specific auxin-inducible degradation of MYRF-1 at these early times, but not at the transition to death, blocks LCD, suggesting that MYRF-1 primes the linker cell for death. 67% of the genes upregulated in dying linker cells harbor MYRF-1 binding motifs, including pqn-41, encoding a glutamine rich aggregating protein required for LCD. ChIP-seq experiments reveal that MYRF-1 physically binds several sequences within a large pqn-41 intron, and CRISPR/Cas9-mediated deletion of one of these is sufficient to promote inappropriate linker cell survival. We propose that MYRF-1 binding to pqn-41 is an important component of LCD priming. By understanding how the migrating linker cell initiates its programmed death pathway, we hope to determine a mechanism to activate death in migrating metastatic cancer cells.

B270/P1394

Controlling NETosis through nuclear envelope regulation attenuated UVB-Induced Inflammation in the Skin and Distant Kidneys in Lupus-Prone Mice

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Background/Purpose: Ultraviolet B (UVB) overexposure is a trigger for lupus, provoking skin and systemic inflammation in organs like the kidneys through poorly understood mechanisms. Recent studies have implicated neutrophils as mediators connecting skin inflammation to kidney injury in context like skin thermal burns or UVB irradiation, the specific mechanisms by which UVB exposure triggers this pathogenic cascade remain not fully understood. Here, we sought to explore if neutrophil extracellular traps (NETs) are involved in this pathological processes in lupus prone mice as we have reported that UVB exposure induces skin inflammation with NET formation in wildtype mice. Methods: Exposure of asymptomatic young female lupus-prone mice to UVB, to study the concurrent skin and kidney inflammation with proteinuria, as well as the involvement of neutrophils and NETosis in these processes. Results: To study this, we exposed asymptomatic young female lupus-prone mice to UVB, inducing concurrent skin and kidney inflammation with proteinuria, characterized by neutrophil infiltration, NETosis, and elevated pro-inflammatory cytokines and complement C3. Controlling NETosis through nuclear envelope regulation via PKCα deletion ameliorated skin and kidney inflammation and proteinuria by reducing NET-associated cytokines/C3. To explore the mechanism mediating skin and kidney inflammation, we found that UVB irradiation of neutrophils from lupus-prone mice increased proinflammatory cytokines and the transmigration receptor CXCR4, suggesting a pathway for communication between the skin and kidneys. Using a photoconvertion tracking approach, we found that genetic deficiency of CXCR4 reduced neutrophil transmigration from the skin to the kidneys and decreased proteinuria in UVB-irradiated kikGR;CXCR4-/+ mice, indicating a role for CXCR4 in skin-kidney crosstalk. Furthermore,

pharmacological inhibition of CXCR4 attenuated acute kidney inflammation and proteinuria, confirming its role in ameliorating the renal accumulation of neutrophils, NETs, and NET-associated cytokines/C3 in UVB-irradiated lupus-prone mice. Conclusion: This study reveals that neutrophil transmigration and NETosis drive UVB-triggered skin and kidney inflammation, highlighting them as potential therapeutic targets. Remarkably, nuclear envelope regulation represents as promising strategy for controlling NETosis in lupus.

B269/P1393

Reperfusion Time Mediates the Crosstalk Between Apoptosis, Necroptosis, Ferroptosis, Autophagy, and Pyroptosis in Ischemia-Reperfusion Injury in Rat Hearts

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Reperfusion injury is characterized by mitochondrial dysfunction and the activation of multiple cell death pathways, following blood flow restoration after myocardial infarction. In this study, we assessed apoptosis, necroptosis, ferroptosis, autophagy, and pyroptosis during ischemia and at various time points during reperfusion to evaluate the role of reperfusion time in the activation and interaction between these mechanisms. Langendorff-mode retrograde perfusion using normal Krebs-Henseleit solution was performed using isolated hearts from adult male Sprague Dawley rats (200-250 g). Hearts were subjected to 20 min equilibration, and 25 min of global ischemia followed by short (5 min), moderate (30 min) or long (60 min) reperfusion. At the end of each experiment, mitochondria were isolated and used to analyze Ca2+-induced swelling and ROS production. Protein levels of death markers for apoptosis, necroptosis, ferroptosis, autophagy, and pyroptosis were measured using SDS-PAGE and western blotting. Our results showed a 77% ($p<0.001$) decrease in cardiac function, measured by LVDP during reperfusion compared to pre-ischemic values. Mitochondrial Ca2+-induced swelling and ROS production showed a significant time-dependent increase ($p<0.01$, and $p<0.005$ respectively) during reperfusion. Western blot analysis demonstrated a significant increase in necroptosis, indicated by elevated MLKL phosphorylation during ischemia ($p<0.01$), which inhibited extrinsic apoptosis, as reflected by reduced Caspase-8 cleavage ($p<0.05$). Caspase-3 cleavage, an apoptosis marker, was increased during ischemia and all reperfusion time points ($p<0.005$), while the pyroptosis marker GSDMD showed reduced cleavage ($p<0.05$) during early reperfusion. Caspase-3 can act as a molecular switch between apoptosis and pyroptosis, and it may inhibit pyroptosis during very early reperfusion. Glutathione peroxidase 4, an endogenous ferroptosis inhibitor, showed decreased expression from ischemia ($p<0.05$) to moderate reperfusion ($p<0.05$) compared to control. Autophagy marker LC3B, showed a significant time-dependent accumulation ($p<0.005$) compared to the control group. Autophagy inhibition at later reperfusion might reduce ferroptosis potentiation due to a reduction in iron availability and GPX4 degradation. Our data demonstrates an association between cardiac and mitochondrial dysfunction, and the distinct activation of five cell death pathways depending on reperfusion time. Additionally, our results point to a potential time-sensitive interaction between these pathways that could influence future therapeutic strategies for the treatment of myocardial infarction patients.

B268/P1392

Understanding the Role of the Retromer Complex in Apoptotic Cell Clearance

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Approximately one million cells undergo apoptosis every second in adult humans, requiring professional and non-professional phagocyte-mediated clearance of apoptotic cells (ACs). Efficient clearance of ACs relies on the conserved process of efferocytosis to avoid unnecessary immune activation and maintain tissue homeostasis. During efferocytosis, AC-containing endosomes acquire the autophagic protein LC3 on their membranes—a process termed “LC3-associated phagocytosis” (LAP) to promote their fusion with lysosomes for cargo degradation. The retromer protein complex, composed of Vps26, Vps29, and Vps35, is crucial for endosomal protein sorting and recycling; it interacts with key autophagy components such as ATG5 and influences lysosomal function. Despite its established role in endosomal trafficking, the involvement of the retromer in LAP remains poorly understood. To examine the role of the retromer complex in LAP, we performed siRNA-mediated knockdown of the retromer components in primary mouse bone marrow–derived macrophages (BMDMs). Retromer loss led to an accumulation of undigested apoptotic corpses but did not substantially impair the recognition or uptake of ACs. Retromer deficiency markedly impaired LC3 recruitment to phagosomes; si-control macrophages showed strong LC3 rings around AC-containing phagosomes, while knockdown reduced LC3 localization, highlighting retromer's essential role. Mechanistically, no direct VPS35–LC3 interactions were detected; however, the GTPase-activating protein TBC1d5 co-immunoprecipitated with VPS35 and colocalized on phagosomes, suggesting it bridges retromer to LAP. Retromer knockdown significantly decreased membrane-bound NOX-2 protein levels without affecting mRNA levels, indicating enhanced protein degradation. These findings were further confirmed by cycloheximide chase and CAG-RFP-EGFP-LC3 reporter assays, which demonstrated reduced membrane LC3 and NOX-2 following retromer loss. Functionally, retromer deficiency impaired phagolysosomal maturation, increased dead cell accumulation, and elevated proinflammatory cytokines. Treatment with the retromer-stabilizing chaperone TPT-260 restored apoptotic cell clearance in vivo. Collectively, these results identify the retromer complex as a key regulator of LC3 lipidation, phagosomal maturation, and immune homeostasis during LAP, acting through TBC1d5 and modulating NOX2 stability. This study advances our understanding of how retromer integrates membrane trafficking with noncanonical autophagy pathways to preserve tissue homeostasis. Future investigations will assess the role of retromer-mediated cargo degradation in disease models.

Cell Death - Ferroptosis

B265/P1389
Comprehensive Analysis of Iron Dynamics with Fluorescence Probes
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Iron is essential for intracellular metabolism and redox reactions, and excessive iron accumulation impairs cellular function, leading to a type of cell death known as ferroptosis, which is caused by lipid peroxidation. Ferroptosis has been identified as a cell death pathway distinct from apoptosis, which is well known as programmed cell death. Furthermore, ferroptosis has attracted attention as a potential target for novel cancer therapies. Additionally, the association between ferroptosis and various diseases, including neurodegenerative disorders, stroke, and metabolic dysfunction-associated steatohepatitis (MASH), has been reported. The dynamics of iron, which induces ferroptosis, are of particular interest in cancer and neurodegenerative disease research, highlighting the need for methods to monitor iron dynamics in living cells. We detected labile ferrous iron in living cells subjected to drug stimulation using the fluorescent probe, FerroOrange, and a novel fluorescent prove for labile ferrous iron in lysosome, which can be applied to living cells without the need for

genetic modification. As a result, we successfully detected changes in iron dynamics in cells treated with the xCT inhibitor, erastin and doxorubicin, which induces cellular senescence. Furthermore, by combining FerroOrange with various fluorescent probes, we simultaneously detected oxidative stress and lipid peroxidation associated with changes in iron dynamics in living cells. In addition, we report findings on changes in mitochondrial membrane potential and nutrient uptake influenced by the amount of intracellular free iron. We expect this analytical method will contribute to the future development of iron research.

B266/P1390

Ferroptosis induces heterogeneous death profiles that are controlled by lysosome rupture

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Ferroptosis, a well-studied lipid peroxide–dependent form of regulated cell death, occurs in degenerative diseases and cancer, however its mechanisms of execution at single-cell resolution remain poorly understood. Here, we show that the induction of ferroptosis through any of numerous mechanisms leads to heterogeneous death profiles, where individual cells die through different modes of execution, from necrotic to apoptotic forms of death. We find that starvation for amino acids and depletion of the anti-oxidant glutathione sensitizes to homogeneous necrotic execution that further exhibits propagation, a unique non-cell-autonomous feature that leads to death spreading in spatiotemporal waves across cell populations. We further identify that the rupture of lysosomes is as a key intracellular feature of propagative death that releases lysosomal cathepsin enzymes that regulate necrotic death execution. Collectively, our findings reveal two parallel but mechanistically distinct routes of ferroptotic death—non-propagative, heterogeneous death induced by GPX4 inhibition, versus homogeneous, lysosome-dependent propagative ferroptosis triggered by glutathione depletion—and establish lysosomes as central regulators of propagative ferroptosis. These insights advance our understanding of ferroptosis and provide a framework for dissecting its complex cellular dynamics.

B264/P1388

Forging Diabetes: The Critical Role of Mitochondrial Iron-Glutathione Homeostasis in Beta Cell Development and Function

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Background: Pancreatic beta cells are essential for insulin production and maintaining glucose homeostasis, but they are highly susceptible to oxidative stress, which leads to dysfunction and loss central to the development of both type 1 and type 2 diabetes. The delicate balance between glutathione (GSH) and iron (Fe) homeostasis is critical for beta cell survival and function. Reactive oxygen species (ROS), generated by labile iron overload, can damage these cells, while GSH acts as a crucial buffer against this oxidative stress. Iron is also a key component of iron-sulfur cluster (Fe-S) proteins, which are vital for the electron transport chain in mitochondria. Therefore, maintaining the Fe-GSH balance is crucial for beta cell health. Aim: Our study aimed to understand the role of Iron-GSH homeostasis in pancreatic beta cell development and how its dysregulation leads to beta cell dysfunction and diabetes. While BOLA3, a mitochondrial protein, is a known component of the iron-sulfur (Fe-S) cluster biogenesis machinery, its specific role in beta cell development and function had

not been elucidated. Methods and Results: We identified BOLA3 as a critical regulator of cellular iron homeostasis and oxidative stress. Disruptions in iron metabolism and mitochondrial Fe-S cluster biogenesis impair beta cells' ability to produce and secrete insulin in response to elevated blood glucose. Using CRISPR-Cas9 to knock out BOLA3 in both murine and human stem cell-derived beta cells during development resulted in cell death. In mature beta cells, BOLA3 loss led to progressive glucose intolerance and functional decline. Single-cell RNA sequencing and proteomic analysis of BOLA3 knockout islets revealed transcriptomic changes linked to decreased GSH levels, increased iron accumulation, oxidative stress, and lipid peroxidation, ultimately triggering ferroptosis. This effect could be rescued by supplementing with GSH and small molecule inhibitors for the ferroptosis pathway. Additionally, we explored how BOLA3 protects cells by maintaining mitochondrial function, regulating iron metabolism, and modulating key ferroptosis regulators. Understanding the effect of BOLA3 in maintaining Iron-GSH homeostasis in beta cells opens new avenues for therapeutic interventions to preserve beta cell mass and improve glycemic control in diabetes. Conclusion: In conclusion, our work provides compelling evidence that BOLA3 is a central regulator of iron-GSH homeostasis, protecting pancreatic beta cells from the damaging effects of labile iron and subsequent ferroptosis. By elucidating the intricate molecular mechanisms linking BOLA3 to beta cell dysfunction, we have identified a potential therapeutic target for preserving beta cell mass and improving glycemic control in diabetes.

B263/P1387
Iron-ROS axis as a molecular bridge between ferroptosis and inflammasome activation induced by particulate matter
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Particulate matter (PM) is a global environmental hazard that affects human health through free radical production, cell death induction, and immune responses. PM activates inflammasomes, leading to excessive inflammatory responses, and also induces ferroptosis, an iron- and lipid peroxidation–dependent form of cell death. Despite ongoing research on the correlation among PM-induced ferroptosis, immune response, and inflammasomes, the underlying mechanism of this relationship has not been elucidated. In this study, we demonstrated the levels of PM-induced cell death and immune responses in murine macrophages, J774A.1 and RAW264.7, depending on the size and composition of particulate matter. PM2.5, containing extraction ions, induced significant levels of cell death and immune responses; it induces lipid peroxidation, iron accumulation, and reactive oxygen species (ROS) production, which characterize ferroptosis. Additionally, inflammasome-mediated cell death occurred as a result of excessive activation of inflammatory responses. PM-induced iron accumulation activates ferroptosis and inflammasomes formation through ROS production; similar results were observed in vivo. These results suggest that the link between ferroptosis and inflammasome formation induced by PM, especially PM2.5 with extraction ions, is mediated through the iron-ROS axis. Moreover, this study can effectively facilitate the development of a new therapeutic strategies for PM-induced immune and respiratory diseases.

Cell Death - Necroptosis

B259/P1383
An anti-inflammatory mechanism regulating the crosstalk between TNF-induced apoptotic and necroptotic pathways and its role in inflammatory bowel disease

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Necroptosis is a form of regulated cell death characterized by membrane disruption mediated by the necrosome complex, which comprises RIPK1, RIPK3, and MLKL. It is triggered when the extrinsic apoptotic pathway is inhibited by pathogenic infections or genetic defects. This pathway leads to the release of DAMPs, thereby provoking robust inflammation and contributing to the pathogenesis of various inflammatory diseases. Caspase-8 (CASP8), a key initiator of extrinsic apoptosis, serves as a checkpoint molecule that inhibits necroptosis by cleaving and inactivating RIPK1 and RIPK3. While numerous regulators of apoptosis and necroptosis have been identified, molecules that control the crosstalk between these pathways remain poorly understood. Here, we identify LAP1 (LUBAC-associated protein 1), a novel LUBAC-binding ubiquitin ligase, as an anti-inflammatory regulator that promotes apoptosis and inhibits necroptosis downstream of TNF signaling. Mechanistically, LAP1 facilitates CASP8 activation through RIPK3 and cooperates with LUBAC to suppress necrosome formation via heterotypic ubiquitin chain assembly on RIPK1. Lap1-deficient mice exhibited exacerbated pathology in the DSS-induced colitis model, which was alleviated in Lap1/Ripk3 double-deficient mice, indicating a necroptosis-dependent pathogenesis. Moreover, LAP1 expression was significantly reduced in intestinal tissues from patients with inflammatory bowel disease (IBD), suggesting that LAP1 loss may represent a potential risk factor for IBD.

Cell Death - Pyroptosis

B261/P1385

Alix-Mediated Internalization of Gasdermin-D Membrane Pores Governs Membrane Repair in Pyroptosis

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Background Pyroptosis, an inflammatory-regulated cell death, is triggered by damage and pathogen-associated molecular patterns (DAMPs and PAMPs). These molecular patterns activate inflammasomes, leading to the activation of inflammatory caspases, which cleave gasdermin-D (GSDMD), generating an N-terminal GSDMD (N-GSDMD) fragment that oligomerizes and forms membrane pores. However, N-GSDMD pore formation does not always result in cell death. While sustained N-GSDMD pore activity causes lytic cell death, signaling mechanisms determining cell survival versus death remain unclear. Results Using pyroptotic stimuli and time-course membrane integrity assays, we demonstrate that transient N-GSDMD membrane pore formation (≤2 hours) is compatible with cell survival. Removal of the stimulus within 1-hour triggers rapid membrane pore clearance and restoration of membrane integrity. In contrast, prolonged membrane pore activity (>2 hours) causes irreparable damage and death. Mechanistically, we identify dynamin-dependent endocytosis as the primary pathway for N-GSDMD removal. Confocal microscopy (N-GSDMD/EEA1 co-labeling) revealed pore internalization via early endosomes. Crucially, ALIX, an ESCRT-associated protein, orchestrates this repair process. Thus, ALIX knockdown exacerbates N-GSDMD pore persistence and cell death, whereas ALIX overexpression promotes N-GSDMD pore removal and enhances survival. Implications Our work establishes N-GSDMD membrane pore duration in the membrane as the critical switch between cell recovery and pyroptotic death and identifies ALIX-driven endosomal clearance as a repair mechanism for gasdermin pores. This reveals an unexpected self-healing process in pyroptotic cells. Targeting ALIX or membrane pore dynamics could shift outcomes in chronic inflammation, opening new therapeutic avenues to promote cellular resilience

B260/P1384

Caspase-2 plays a critical role in impairing membrane repair in pyroptotic cell death.

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Caspases, a family of cysteine proteases, are key mediators of programmed cell death, with distinct subsets governing apoptotic (e.g., caspase-3, -8, -9) and inflammatory (e.g., caspase-1) pathways. Caspase-2, though structurally an initiator caspase (such as caspase-9), displays substrate-cleaving properties, as effector caspases (such as caspase-3), and remains one of the least understood members of this family. Here, we uncover a novel role of caspase-2 in regulating pyroptosis, a lytic and highly inflammatory form of cell death triggered by gasdermin-D (GSDMD) membrane pore formation. Pyroptosis is initiated when caspase-1 cleaves GSDMD, releasing its N-terminal fragment (N-GSDMD), which oligomerizes to form membrane pores. While GSDMD pores are central to pyroptosis, the molecular determinants that dictate whether pore formation leads to cell death or survival remain poorly studied. We demonstrate that caspase-2 is activated by potassium efflux induced by pyroptotic stimuli, including Staphylococcus aureus α-toxin and nigericin, and that it disrupts a critical membrane repair pathway. Short-lived N-GSDMD pores (<1 h) allow cells to restore membrane integrity upon stimulus withdrawal, whereas prolonged pore presence leads to irreversible damage and cell death. Using dynamin inhibition and confocal microscopy co-labeling N-GSDMD with the early endosomal markers, we show that N-GSDMD is internalized via an endocytic mechanism. Moreover, we identify ALG-2–interacting protein X (ALIX), an accessory protein of the Endosomal Sorting Complex Required for Transport machinery, as a key regulator that promotes N-GSDMD clearance and facilitates membrane repair. Stable ALIX knockdown enhanced pyroptosis, while ALIX overexpression suppressed pore persistence and improved cell survival. Strikingly, caspase-2 leads to ALIX cleavage, inactivating its repair function and tipping the balance toward pyroptotic death. Our findings reveal that the duration of N-GSDMD pore residence is a decisive factor in cell fate, governed by a caspase-2–ALIX axis that determines whether cells undergo membrane repair or pyroptosis. This work establishes caspase-2 as a novel modulator of inflammatory cell death and highlights a therapeutic avenue for chronic inflammatory diseases and infections by enhancing the cells' inherent membrane repair potential.

B262/P1386

DPP9-mediated restraint of CARD8 C-terminal fragment controls inflammasome assembly

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CARD8 detects intracellular danger signals and forms a caspase-1 activating inflammasome. Like the related inflammasome sensor NLRP1, CARD8 autoprocesses into noncovalently associated N-terminal (NT) and C-terminal (CT) fragments and binds the cellular dipeptidyl peptidases DPP8 and 9 (DPP8/9). Certain danger-associated signals, including the DPP8/9 inhibitor Val-boroPro (VbP) and HIV protease, induce proteasome-mediated NT degradation and thereby liberate the inflammasome-forming CT. We report cryoelectron microscopy (cryo-EM) structures of CARD8 bound to DPP9, revealing a repressive ternary complex consisting of DPP9, full-length CARD8, and CARD8-CT. Unlike NLRP1-CT, CARD8-CT does not interact with the DPP8/9 active site and is not directly displaced by VbP. However, larger DPP8/9 active-site probes can directly weaken this complex in vitro, and VbP itself nevertheless appears to disrupt this complex, perhaps indirectly, in cells. Thus, DPP8/9 inhibitors can activate the CARD8 inflammasome by promoting CARD8 NT degradation and by

weakening ternary complex stability Our findings provide a structural framework for understanding CARD8 inflammasome regulation and suggest that its activation can be modulated by DPP8/9 inhibitors through multiple mechanisms. It remains unclear whether CARD8 selectively drives pro-inflammatory cytokine release in specific immune cell subsets or if its activation intersects with other inflammasome pathways, such as NLRP3. Additionally, exploring small molecules that fine-tune CARD8 activity could offer novel therapeutic strategies for modulating excessive inflammation in autoimmune conditions. Structural insights into CARD8 interactions with other immune regulators may further refine our understanding of its function in health and disease. We plan to capture additional CARD8 conformational states that will be critical in elucidating its precise role in immune homeostasis and autoimmunity.

Cell Division and Cell Cycle Control - Cell cycle enzymology and checkpoints

B474/P0456
AlphaFold 2 screen reveals novel G1 cyclin docking modalities
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The cell cycle is a series of tightly regulated events that drive cellular growth, DNA replication, and division. Regulation of this cycle is tightly controlled by cyclins and CDKs, which function together to phosphorylate a specific set of substrates. Understanding these interactions involved in cell cycle transitions is crucial for discovering the molecular mechanisms of cell cycle regulation. However, identifying specific docking interfaces has been challenging due to the dynamic nature of these interactions. Traditional methods, such as alanine scanning, are time-consuming and rely on low-throughput, one-by-one validation approaches, making it difficult to systematically identify these interactions. We present a computational pipeline leveraging AlphaFold2 to predict docking interfaces between yeast cyclin, Cln3, and the full nuclear proteome of Saccharomyces cerevisiae. This allows for identification of candidate docking interactions at a proteome-wide scale. Predicted AlphaFold2 complexes were analyzed based on spatial and confidence features to define potential docking regions. To validate, we generated yeast strains expressing mutant versions of Cln3 with targeted disruptions in the predicted interfaces. Functional impact was assessed using a cell size assay as it's a proxy for Cln3-activity during G1 phase progression. Mutations in select docking regions resulted in altered cell size distributions, supporting their role in mediating Cln3's function in driving cell cycle progression. Our results provide proof of principle for using AlphaFold2 to identify novel docking interfaces between cyclins and their target proteins across model systems. This allows for the development of targeted therapeutics that can disrupt specific docking interactions to halt cell division.

B485/P0467
Cause for speckle-ation: Excess cell size re-organizes the genome to drive alternative splicing programs that enforce senescence
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Cellular senescence refers to a permanent state of cell cycle exit and plays a key role in blocking the proliferation of damaged or otherwise compromised cells. Despite diverse drivers for senescence, senescent cells often share common features, one of which is their increased size. Though previously viewed as a passive

outcome of cellular growth in the absence of division, recent work from our group and others suggests that excess cell size can actively promote senescence via the p53-p21 pathway, meaning that excess cell size is both a consequence and a cause of cell cycle arrest. Still, how cells sense their own size and communicate this information to the cell cycle control apparatus remains unresolved. Our group has recently shown that excess cell size perturbs nuclear speckle-genome interactions. Nuclear speckles are dynamic hubs for mRNA splicing, and the proximity between genomic coding loci and nuclear speckles directly impacts the efficiency of mRNA splicing. Based on this, we examined how excess cell size impacts normal splicing patterns. We found that cellular enlargement profoundly impacts splice isoform usage, indicating that altered nuclear speckle function may drive alternative splicing programs when cells become too large. Notably, we found that MDM4—a critical negative regulator of p53 signaling— is differentially spliced to yield a non-functional variant when cells become enlarged. Re-introducing correctly-spliced MDM4 transcripts restores cell cycle progression, thereby reverting senescent phenotypes in enlarged cells. Together, these data indicate that perturbations in mRNA splicing patterns resulting from a reorganized genome may link cell size to cell cycle exit.

B484/P0466

CDC7 and APC/CCdh1 Gate Distinct Routes to Initiate DNA Replication

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Precise temporal control of DNA replication initiation is essential for faithful cell division, yet there are conflicting prevailing models for how cells trigger origin firing. Here, we demonstrate that human origins are not fired at fixed thresholds of cell cycle regulators E2F, APC/CCdh1, CDK2/1, and CDC7. Instead, origin firing is triggered at a tunable CDK2/1 threshold that is gated by either CDC7 or APC/CCdh1 activity. CDC7 phosphorylates MCM helicase, enabling Cyclin E-CDK2 to trigger origin firing at low CDK2/1 activity, independent of APC/CCdh1 inactivation. In contrast, APC/CCdh1 inactivation enables Cyclin A-CDK1 to phosphorylate MCM at distinct CDK2/1-specific sites and trigger origin firing, independent of CDC7 activity. Strikingly, this requires much higher CDK2/1 activity, which CDK2 cannot normally reach alone. Thus, having two distinct routes necessitates blocking both routes, through inhibition of CDC7 or CDK2 together with reinforcing APC/CCdh1 activity or inhibiting CDK1, to prevent S phase.

B482/P0464

Cell cycle dependent localization of the DNA replication protein ORC1 during multiple fission in Chlamydomonas

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Chlamydomonas reinhardtii is a unicellular green alga that divides by multiple fission, wherein DNA replication and cell division are repeated without intervening growth. To uncover how DNA replication is controlled during these rapid S/M cycles, we visualized components of the pre-replicative complex in live cells. While CDC6, Cdt1, and MCM4 remained nuclear in G1 and S, ORC1 had dynamic localization: nucleolar when it accumulates in late G1, cytoplasmic in S phase, and chromosome-bound during mitosis before re-export to the cytoplasm for the subsequent S phase. ORC1 remained cytoplasmic when mitotic entry was blocked in cdkb and cycb temperature-sensitive mutants, and was stably localized to chromosomes when cells were arrested at metaphase in cdc27 and cdc20 mutants. We also observed partial nuclear pore complex disassembly during

mitosis, similar to some fungal and protist species. Inner ring but not outer ring nucleoporins were dispersed from the nuclear envelope coincident with de-localization of nuclear proteins. Localization of ORC1 to the nucleus always co-occurred with both partial nuclear pore complex disassembly and nuclear protein de-localization. Our data suggest a model in which transient loss of the nuclear permeability barrier during mitosis allows cytoplasmic ORC access to chromosomes to initiate DNA replication for the subsequent S phase.

B481/P0463
Coiled-coil interactions drive ectopic condensation of overexpressed MAD1 to promote mitotic slippage in cancer cells
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The mitotic checkpoint protein MAD1 is overexpressed in cancers, weakening the checkpoint and promoting mitotic slippage. Overexpressed MAD1 forms ectopic foci in mitotic cells, but the biophysical nature of these foci and their roles in mitotic slippage remain unclear. Here, we report that MAD1 ectopic foci are phase-separated condensates that shorten mitosis by sequestering checkpoint proteins. We show that MAD1 ectopic foci display dynamic condensate behaviors, and Mad1 phase separation is driven by interactions between its coiled-coil and disordered domains at the N-terminus. By inducing MAD1 ectopic condensates in mitotic cells with low MAD1 levels, we decouple the effects of condensation from concentration and demonstrate the causal role of condensation in mitotic slippage. Mechanistically, MAD1 ectopic condensates trap the diffusive pool of MAD2, weakening the MAD2 conversion cycle needed for a robust checkpoint. Our work highlights a loss of function caused by ectopic condensates in cancer cells.

B479/P0461
Inhibitor-2 Coordinates Handoff of Protein Phosphatase 1 to Adapters with Inhibition Release
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The catalytic subunit of Protein phosphatase 1 (PP1c) mediates a major fraction of Ser/Thr dephosphorylation events in cells. To achieve its many functions, PP1c binds a diverse collection of adapters, forming active holoenzymes that function at specific cellular sites in a spatiotemporally controlled manner. PP1c activity is controlled by three conserved accessory proteins that promote holoenzyme formation—Sds22, Inhibitor-2, and Inhibitor-3. Here, we employ the early C. elegans embryo to investigate Inhibitor-2 (referred to as Inh2; C. elegans gene name SZY-2). Inh2 interacts with PP1c through two docking motifs–SILK and RVxF–similar to those present in many PP1c adapters, as well as an active-site binding inhibitory motif containing the conserved HYNE sequence. In contrast to the severe defects resulting from depletion of the PP1 catalytic subunits, Sds22, or Inhibitor-3, depletion of Inh2 resulted in modest defects in PP1c functions in meiosis and mitosis. Specifically, Inh2 depletion led to delays in chromosome alignment and anaphase onset, similar to those caused by disruption of the transient PP1c docking onto kinetochores during mitosis mediated by the adapter KNL-1. These results suggested that Inh2 might function as a reservoir that holds PP1c in an inhibited state that is available for controlled handoff to mitotic adapters like KNL-1. Unexpectedly, mutation of the RVxF docking motif of Inh2 resulted in meiotic and mitotic phenotypes that were more severe than Inh2 depletion and instead resembled the broad loss of PP1c function. The severe effect of the Inh2 RVxF mutant was

suppressed by an additional linked mutation in the active site-binding inhibitory HYNE motif. Neither wild-type nor RVxF-mutant Inh2 localized to meiotic or mitotic chromosomes where PP1c interacts with its adapters MEL-28 and KNL-1. Instead, the presence of the Inh2 RVxF mutant prevented PP1c from binding to adapters at these sites. Yeast 2-hybrid analysis showed the single RVxF and HYNE mutants retained the ability to interact with PP1c while the double mutant abolished binding. These results suggest that the RVxF mutant of Inh2 traps PP1c in a HYNE motif-bound inactive state that can no longer be handed off to adapters to execute its functions. Overall, these data indicate that Inh2 functions as a dynamic reservoir for holding mature PP1c and facilitating its handoff to adapters in a tightly controlled manner. In addition, the RVxF motif of Inh2 appears to be critical for releasing the linked HYNE motif from the active site, suggesting an intramolecular coupling between handoff to adapters and relief of activity inhibition.

B476/P0458

Neural stem cells exhibit permissive checkpoints during brain development

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DNA damage checkpoints and the DNA damage response (DDR) ensure the timely detection and repair of DNA lesions, impeding the proliferation of cells with an abnormal genome and thus allowing for the maintenance of genetic stability. During development, checkpoints and the DDR are essential to ensure homeostasis and organism viability, yet the specific responses and actors at play are far from being understood. We have addressed this question in neural stem cells (NSCs) in the developing brain, as germline mutations in the DDR machinery have been linked to human developmental disorders such as Seckel Syndrome, Fanconi Anemia, Bloom Syndrome and Werner Syndrome. These disorders predominantly present neurodevelopmental phenotypes such a microcephaly, suggesting that the developing brain is particularly vulnerable to DNA damage. From a cell-cycle point of view, the brain is assembled during a short developmental window, requiring NSCs to undergo fast cell cycles to allow for the generation and differentiation of multiple cell types. Taken together, NSC vulnerability to DNA damage and fast cell cycles pose the question of DNA damage checkpoint strength and function throughout brain development. To tackle this question, we have utilized Drosophila and mouse developing brain tissue and a combination of gene expression analysis, functional studies and fixed and live imaging. We have found that in the developing brain, only high levels of DNA damage are sufficient to partially inhibit cell cycle progression, as some NSCs can still progress into mitosis despite exhibiting abnormal chromosome morphology and DNA damage. Even if key checkpoint regulators such at Chk1 and Chk2 are expressed at the mRNA level, these proteins were not detected in NSCs. In agreement, the knockdown of Chk1 and Chk2 did not reveal any implication in checkpoint response. However, their overexpression specifically in NSCs results in cell cycle arrest, suggesting that strong DNA damage checkpoints are incompatible with the fast cell cycles typical of NSCs. To identify actors of the partial DNA damage checkpoint response observed in response to high levels of DNA damage in NSCs, we performed a candidate screen. We identified 9-1-1 checkpoint complex protein Rad9 as a key actor of the checkpoint response, as its depletion lifts the partial cell cycle arrest observed under conditions of high DNA damage. Altogether, our work shows that NSCs have permissive checkpoints that rely on alternative checkpoint pathways, to ensure the fast cell cycles required for brain development.

B483/P0465

Persistent DNA breaks attenuate global transcription for mitotic re-entry via the Kinase Module of the

Mediator of Transcription
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The double strand breaks (DSBs) are the most deleterious type of DNA lesions which threaten genomic integrity. To protect genomic integrity in the face of DSBs, eukaryotic cells activate the DNA damage response (DDR), an elaborate and evolutionarily conserved signaling pathway. We find that an essential component of DDR upon the induction of DSBs is the global attenuation of transcription. Supporting this, global transcription upregulation interferes with proper DDR and inhibits mitotic re-entry. Through a genetic interaction screen, we identified the Cdk8 kinase module (CKM) as the modulator of this global attenuation. CKM is a non-obligate and dissociable subcomplex of Mediator of transcription, a key regulator of RNA polymerase II (RNAPII). Upon DDR activation, CKM breaks apart the RNAPII complex, thereby reducing the transcriptionally-engaged RNAPII pool. In the absence of CKM or its kinase activity, transcription upon DNA damage is increased, which leads to the spreading of damage-specific histone mark g-H2AX into gene bodies. In addition to its global role we show that CKM localizes to DNA breaks to prevent aberrant RNAPII recruitment to break sites, potentially reducing mutagenic end-joining repair events. Importantly, we demonstrate that the role of CKM on DDR and transcriptional attenuation is conserved from yeast to mammals. CKM inhibition in human cell lines leads to aberrant DDR, elevated RNAPII chromatin retention, increased g-H2AX foci and heightened sensitivity to DNA damaging reagents. Together, these findings establish CKM as a conserved and essential regulator of the DDR.

B480/P0462
Perturbation of replication timing control by early activation of late replication origins increases transcription-replication conflicts in human cells.
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The faithful and complete duplication of the genome during the DNA replication is critical to maintaining genome integrity. The regulatory mechanisms controlling the process of DNA replication determine when each part of the genome is replicated during the S-phase, and perturbation of this replication timing control program appears to increase genome instability. However, how alteration in replication timing control induces genome instability is still elusive. In this study, we induced early activation of late replication origins by tethering RECQL4 protein on the pre-replicative complex in U2OS cells, and investigated the replication stress induced in these cells. Immunostaining of U2OS cells expressing RECQL4-ORC4 proteins showed significant increase in FANCD2 foci and S9.6 foci, suggesting increase in replication fork stalling and R-loops, respectively. Increase in FANCD2 foci in these cells were almost completely reversed by expression of RNaseH1, which resolves R-loop. Furthermore, Proximity ligation assay (PLA) using anti-PCNA and anti-Pol II CTD pS5 antibodies showed increase in PLA foci in RECQL4-ORC4 expressing cells. Collectively, these results suggested that early activation of late replication origins increases transcription-replication conflicts in human cells. Analyses using an ATR inhibitor and a FANCD2K561R ubiquitination mutant showed that ATR activation and FANCD2 monoubiquitination in the Fanconi anemia pathway are required for the proper cellular response to TRCs which are induced by perturbation of replication timing control. Furthermore, depletion of Rif1, a well-established replication timing control factor, also led to increase in TRCs. Therefore, precise control of the timing of late-replication origin firing is critical for preventing TRCs, and TRCs appear to be responsible for the genome instability induced by perturbation of replication timing control in human cells.

B477/P0459

Role of growth rate on the accumulation mitotic cyclin Cdc13

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Eukaryotic cells control their size by coordinating growth and division. In the fission yeast S. pombe, cells enter mitosis and divide at a reproducible size due to regulated activation of the cyclin-dependent kinase Cdk1 in complex with its cyclin subunit Cdc13. Recently, we showed that the nuclear concentration of Cdc13/cyclin increases in a time-dependent manner to promote Cdk1 activity as cells grow. Here, we used nutrient limitation to uncouple the Cdc13/cyclin timer from cell growth rate. Nutrient limitation is known to reduce cell growth rate and overall cell size. We used static cell imaging and microfluidics-based live cell imaging to measure Cdc13/cyclin accumulation in limited nutrient conditions. We found that low glucose availability reduced cell growth rate and cell size at division but did not affect time-dependent accumulation of Cdc13/cyclin. This uncoupling of cell growth rate and cyclin accumulation means that cells grown in low glucose divide with significantly higher concentrations of nuclear Cdc13/cyclin despite their reduced size. This coincides with a decrease in the activating phosphorylation T167 on Cdk1. To determine if these findings apply broadly to limited nutrient conditions, we also tested Cdc13/cyclin accumulation in poor nitrogen media, which similarly reduces cell growth rate and overall cell size. In contrast to our findings in low glucose, Cdc13/cyclin accumulated at a reduced time-dependent rate in poor nitrogen. Together, our results show that Cdc13/cyclin accumulates in a time-dependent manner that is independent of cell growth rate but can be altered by nutrient-specific mechanisms. By combining these experimental results with mathematical modeling, we propose a model for a nutrient-modulated Cdk1 activity threshold that controls entry into mitosis.

B475/P0457

Structural insights into AMBRA1-mediated degradation of D-type cyclins in cell cycle control

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AMBRA1 is a tumor suppressor protein that functions as a substrate receptor in the ubiquitin conjugation system, with critical roles in autophagy and cell cycle regulation. This study aimed to elucidate the structural mechanism by which AMBRA1 regulates the stability of D-type cyclins and controls cell cycle progression. Despite its intrinsic disorder, AMBRA1 was shown to stabilize upon interaction with DDB1, the adaptor of the Cullin4A/B E3 ligase. These findings suggested that AMBRA1 can function as a dynamic scaffold for substrate recognition and ubiquitination. Using cryo-electron microscopy, we determined the structure of the AMBRA1-DDB1 complex at 3.08 Å resolution, revealing that AMBRA1 N- and C-terminal regions fold into a functional WD40 domain. This domain interfaces with the double-propeller fold of DDB1, creating a stable platform for substrate recruitment. To further explore substrate recognition, we solved the structure of the AMBRA1-DDB1 complex bound to cyclin D1 at 3.55 Å resolution. The interaction between AMBRA1 and cyclin D1 is mediated by the phosphorylation of the Thr286 residue in the phosphodegron motif of cyclin D1. This motif folds into a turn-like conformation followed by a 310 helix, enabling high-affinity binding to AMBRA1 WD40 domain. Functionally, AMBRA1 mutants that disrupt binding to either DDB1 or cyclin D1 impair cyclin D1 ubiquitination, leading to its accumulation, enhanced cell cycle progression, and increased DNA damage. These mechanistic

insights suggest that AMBRA1 plays a critical role in regulating regulating cell cycle progression by promoting the timely degradation of D-type cyclins. In conclusion, this study provides a detailed molecular framework for understanding AMBRA1-mediated regulation of stability of D-type cyclins, uncovering its tumor-suppressive functions and highlighting its potential as a therapeutic target. Dysregulation of cyclin D activity is a hallmark of many cancers, and targeting the AMBRA1 pathway could offer new strategies to enhance the degradation of cyclin D and overcome resistance to CDK4/6 inhibitors. These findings advance our understanding of the molecular interactions that govern cell cycle regulation and their implications for cancer therapy.

B478/P0460
Wee1 opposes APC/CCdh1 activity to promote S-phase entry
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Cyclin-dependent kinases (Cdks) drive the eukaryotic cell cycle, ensuring division and genomic stability. Cdk4/6, Cdk2, and Cdk1 act sequentially from quiescence exit to mitosis. Cdks require timely activation through Cyclin binding and phosphorylation. Wee1 kinase phosphorylates tyrosine-15 (Tyr15) on Cdk1 and Cdk2, inhibiting Cdk activity, a process reversed by Cdc25 phosphatases. In cancer therapy, Wee1 inhibitors (e.g., Adavosertib) combined with DNA-damaging agents exploit the G2 checkpoint in TP53-deficient cells, to promote mitotic catastrophe and apoptosis. Despite conservation of Tyr15 in Cdk2, how Wee1 regulates Cdk2 and its role in early cell-cycle events, such as G1 progression and S-phase entry, remain unclear. This is critical, as Wee1 inhibitor trials face suspension due to uncharacterized toxicities, underscoring the need to understand Wee1 across the cycle. We investigated the role of Wee1 during S-phase entry as cells re-enter cycle from quiescence. Contrary to our predictions that Wee1 inhibition in G1 would accelerate S-phase entry via premature Cdk2 activation, it instead delayed S-phase entry, despite premature Cdk2 activation. We hypothesized this arose from APC/CCdh1 hyperactivation upon Wee1 inhibition. Using single-cell timelapse imaging, modelling, and chemical and genetic perturbation, we show that Wee1 promotes S-phase entry by opposing APC/CCdh1 and directly phosphorylating Cdh1. Consistently, levels of G1 APC/CCdh1 substrates, Cdc25A and Cdc6, are reduced upon Wee1 inhibition, and Cdh1 depletion in Wee1 inhibitor-treated cells restores a normal G1 length. These observations represent a new mechanism that enables Wee1 to coordinate the G1-S transition. Moreover, Wee1 inhibition shortens the replication licensing window, impairing DNA replication. Consistent with these observations, we find aberrant accumulation of p21 during S-phase in Wee1-inhibited cells, attributable to increased DNA damage and premature termination of p21 degradation due to increased Cdk1 activity. Finally, cells with reduced p21 or Cdh1 expression are more sensitive to Wee1 inhibitors, suggesting biomarkers for patient selection. Our work reveals a previously unappreciated role for Wee1 in G1-S control, highlighting the importance of understanding the full complement of Wee1 cell-cycle functions to safely and effectively exploit its therapeutic potential.

Cell Division and Cell Cycle Control - Cell division, cytokinesis and abscission

B576/P0557
A Novel Fluorescent Timer, ELDR-Glo, for Tracking Cell Cycle Progression and Cell Age.
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Determining the precise age of individual cells is essential for understanding cell cycle dynamics and quiescence, as well as the regulatory processes that govern cell fate. However, current approaches rely on labor-intensive live-cell imaging and manual tracking. To address these limitations, we developed a fluorescence timer (FT)-based reporter that provides a time-efficient alternative, while also offering new insight into how cellular age influences key regulatory events and correlates with processes throughout the cell cycle. We engineered two FT variants derived from a slow-folding mCherry that accumulate in G0/G1 and are degraded in S phase, enabling readout of cell age, defined as hours since mitotic exit. We stably expressed these reporters in retinal pigment epithelial cells co-expressing a PCNA marker of cell cycle progression. Using live-cell imaging, we observed a strong correlation between FT normalized intensity and cell age for up to 30 hours post-birth ($R^2 = 0.9799$, n = 180), demonstrating that fluorescence intensity reliably predicts cell age in untracked populations. We next examined FT behavior in cells arrested under distinct conditions for several days. While absolute intensity varied depending on the arrest signal, correlations among cells within the same condition remained robust. When combined with quiescence depth assays, FT intensity further distinguished shallow from deep quiescence: dim cells re-entered the cell cycle more rapidly than bright cells. These findings establish FT-based reporters as powerful, time-efficient tools for quantifying cell age and defining quiescence dynamics when live-cell imaging is impractical. More importantly, ELDR-GLO enables direct assessment of quiescence depth without forcing cells back into the cell cycle, providing a more accurate measure of quiescence in its native state. This capability opens the door to more comprehensive downstream assays, such as RNA-seq and proteomics, to investigate how quiescence contributes to cell regulation. Overall, this approach offers broad utility for studying cell cycle regulation and holds significant implications for developmental biology and regenerative medicine.

B577/P0558

Acetylation cycle disruptions result in chromosomal instability and cell division defects

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Lysine deacetylases (KDACs or HDACs) reverse lysine acetylation, which occurs on thousands of human proteins, although the biological relevance of this cycle is not well characterized. To probe the functional consequences of acetylation/deacetylation, we created HT1080 derived-cell lines in which the genomic locus of a single KDAC, either KDAC6 or KDAC8, has been mutated to inactivate the enzyme, as well as knockout cell lines. Gene expression analysis revealed that inactivating either KDAC8 or KDAC6 resulted in downregulation of genes involved in several cell cycle related processes. Inactivation of either enzyme, but not the corresponding knockout, resulted in slower doubling times than wild-type HT1080 cells. DAPI imaging in fixed cells, live cell imaging, and flow cytometry revealed increased chromosomal instability (CIN) and apparent defects of mitosis in the mutant cell lines, while knockout lines were little changed from wild-type. Specifically, KDAC8 inactivation caused genome duplication events, multinucleate cells, and proliferation of micronuclei. This effect appears to be mediated through increased SMC3 acetylation, a known substrate of KDAC8 that is required for proper chromosome segregation. Inactive KDAC8 acted in a dominant-negative fashion, apparently leading to less functional SMC3 associating with DNA, and ultimately causing chromosomal instability after several generations. In contrast, KDAC8 knockout cells were able to compensate for the disruption of SMC3 deacetylation through altered expression of cohesin genes. KDAC6 inactivation similarly resulted in greater frequency of multinucleate cells and micronuclei, but not genome duplication events. These cells often took much longer to complete cytokinesis than other cell lines, and displayed mitotic defects such as failed abscission and multipolar cell division, which could account for the slower doubling time and observed CIN in

this line. These defects cannot be explained by increased acetylation of tubulin, the best characterized KDAC6 substrate, because the KDAC6 knockout also caused enhanced tubulin acetylation. Future work will focus on identifying relevant KDAC6 targets and understanding the mechanism by which KDAC6-dependent deacetylation regulates cell division.

B583/P0564

Adhesion-Cortical Flow Coupling Drives Spinning Cytokinesis to Establish Embryonic Chirality

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Chirality is one of the fundamental properties of living systems, wherein the external and internal body structures are asymmetric and cannot be superimposed on their mirror images. In animals, tissue- and organismal-scale chirality emerge during morphogenesis, termed chiral morphogenesis. In frogs, snails, and Caenorhabditis elegans, previous studies have proposed that asymmetric movement of the cell cortex during early embryonic divisions, termed chiral cortical flow, is regulating chiral morphogenesis. However, the link between chiral cortical flow and chiral morphogenesis has remained correlational. Here, we found that adhesion-cortical flow coupling at the cell-cell interface drives cellular rotation in early C. elegans embryos. Embryonic chirality in C. elegans is specified during cytokinesis of the ABa and ABp cells at the 4-cell stage. The division axes of these cells are orthogonal to the anterior-posterior axis until early anaphase, then undergo a clockwise tilt during cytokinesis. By isolating the individual ABa/p cell, we found that both chiral cortical flow and division axis tilt require neighboring cells. When cultured on artificial adhesive substrates, both phenomena were induced, indicating a crucial role of adhesion during chiral morphogenesis. Consistently, disruption of major cell adhesion proteins cadherin and L1CAM led to defects in embryonic chirality in vivo. Together, we show that adhesion-cortical flow coupling is central to establish embryonic chirality. Interestingly, embryonic chirality is also characterized by the chiral arrangement among the daughter cells of ABa and ABp after cytokinesis, where ABar is in contact with ABpl while ABal is separated from ABpr. However, theoretically, such chiral arrangement cannot be achieved by simple rotation alone. We found that extracellular matrix, such as the embryo's eggshell, is essential for chiral morphogenesis. Indeed, mathematical simulation confirms that steric hinderance imposed by the eggshell, together with cellular rotation and adhesion, can recapitulate the chiral cellular arrangement observed in vivo. By combining biomimetic model, in vitro assays, and mathematical simulations, we demonstrate that cellular rotation and deformation as the primary and secondary mechanisms leading to embryonic chirality. Our study establishes the interplay between cortical flow, adhesion, and extracellular confinement as the key mechanism in regulating chiral morphogenesis, which is pivotal in shaping the embryonic body plan.

B565/P0546

An inhibitory role of Rho-associated protein kinase in cortical excitability

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Cytokinesis is accomplished by the activation of the small GTPase Rho at the cell equator, which drives the assembly of a contractile ring by linking spindle microtubules to actin and myosin-2. However, during this process, active Rho does not follow a conventional linear signaling pattern, but instead appears as "waves" of activity. This dynamic behavior results from rapid positive feedback by the Rho guanine nucleotide exchange factor (GEF) Ect2 facilitating propagation of active Rho, as well as delayed negative feedback by the Rho GTPase

activating protein (GAP) RGA-3/4 suppressing active Rho. The combination and precise timing of these feedback loops makes Rho activity appear wave-like. This dynamic behavior, along with other dynamic behaviors of the cortex, is referred to as cortical excitability. While evidence supporting this feedback model has grown in recent years, the specific mechanisms remain unclear. Here, we propose that Rho-associated protein kinase (ROCK) also plays a role in these dynamics. ROCK is present in the cleavage furrow during cytokinesis, and multiple phosphorylation consensus sites are located within the polybasic cluster (PBC) of Ect2. We hypothesize that ROCK phosphorylation of Ect2 at these consensus sites alters the positively charged nature of the PBC and therefore the ability of Ect2 to interact with the membrane. Furthermore, this affects Ect2's ability to recruit and activate Rho, essentially functioning as an additional form of negative feedback. To test this, we first characterized the patterning of ROCK in relation to active Rho and Ect2 during induced cortical excitability in immature Xenopus laevis (frog) oocytes. We found that fluorescently tagged ROCK trails waves of active Rho, supporting our hypothesized model where active Rho recruits ROCK. Additionally, we found that fluorescently tagged ROCK trails waves of fluorescently tagged Ect2, supporting our model that increased amounts of ROCK leads to less membrane-bound Ect2. We also investigated the role of a single consensus site (Ser839) for ROCK phosphorylation within the PBC of Ect2. We found that the S839A Ect2 lead to waves with longer periods and widths. These characteristics suggest that Ect2 remains active on the membrane longer, consistent with our hypothesis that ROCK normally disrupts Ect2's ability to associate with the membrane. Collectively, these results provide evidence that ROCK is part of a previously uncharacterized negative feedback mechanism that directly inhibits Ect2, and therefore Rho activation, during cytokinesis.

B586/P0567

Biological interactions of KLHDC5 with various proteins in yeast and mammalian cells

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In an effort to learn more about the biological functions of KLHDC5, we performed a yeast two-hybrid screen. KLHDC5 was used as bait to identify interacting proteins. The screen revealed several interacting partners for KLHDC5 in yeast. We selected four proteins for further study including succinate dehydrogenase complex iron sulfur subunit B (SDHB), plexin C1 (PLXNC1), centrosomal protein 57 (CEP57) and an unknown gene (Bac Clone from Chromosome #12). We subcloned the gene for each KLHDC5-interacting protein from the pGADT7 yeast plasmid into the pCMV-HA vector, which is compatible with expression in mammalian cells. We next transfected HeLa cells with each gene of interest along with Myc-tagged KLHDC5. After transfection, the cells were harvested to make lysate samples for protein analysis by SDS-Page followed by Western blotting. Membranes were probed with HA and Myc antibodies to confirm expression of each protein. Immunoprecipitation followed by Western blotting revealed interactions of KLHDC5 with some of the proteins of interest in mammalian cells.

B570/P0551

CAMSAP2 regulates the minus end depolymerization activity of Kif2a in human cells.

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Following chromosome segregation in anaphase, microtubules in the spindle midzone shorten, forming the midbody. This transition is a critical process during cell division that requires precise regulation of microtubule dynamics and organization. To understand the mechanism of this transition we examined the localization and

activity of the microtubule depolymerase Kif2a and minus-end binding protein CAMSAP2 in HeLa cells. Immunofluorescence staining demonstrates that Kif2a localizes to both microtubule plus and minus ends, while CAMSAP2 localizes specifically to minus ends. Following RNAi-mediated depletion of Kif2a, midzone microtubules were bent, buckled and elongated and cells frequently had enlarged asters, consistent with prior work. Because CAMSAPs promote minus-end microtubule stability, we next performed RNAi of CAMSAP2, the major CAMSAP in HeLa cells. Unexpectedly loss of CAMSAP2 showed similar phenotypes as cells depleted of Kif2a, including bent and elongated midzone microtubules. Live-cell imaging of mCherry-tubulin expressing cells further showed that both Kif2a and CAMSAP2 contribute to proper midzone shortening in HeLa cells. Although CAMSAPs have been shown to mediate minus-end growth in vitro, our data suggest that at the midzone, CAMSAP2 may function to localize Kif2a for minus-end shortening. To examine this possibility, we performed localization studies in depleted cells and found an asymmetric interdependence between these proteins. CAMSAP2 remained present and extended along elongated microtubules in Kif2a depleted cells, however, Kif2a localization specifically at microtubule minus ends was completely lost in CAMSAP2 depleted cells. Together, our results support the hypothesis that CAMSAP2 regulates the minus end depolymerization activity of Kif2a in human cells.

B588/P0569

Coarse-grained simulations of cytokinetic ring anchoring protein Cdc15 phase separation

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The assembly of Cdc15 into cytokinetic nodes plays a significant role in the organization and anchoring of the cytokinetic actomyosin ring to the plasma membrane. The intrinsically disordered region (IDR) of Cdc15 is regulated by multiple cell polarity kinases and dephosphorylation has been shown to induce liquid-liquid phase separation [1]. A computational model of single Cdc15 dimer suggested how IDR phosphorylation may inactivate Cdc15 by blocking its membrane binding and oligomerization via its F-BAR domain [1], but did not explore the phase separation of many proteins. Here we investigate the phase separation of multiple IDRs with coarse-grained molecular dynamics simulations. We use the hydrophobicity scale (HPS) model, in which aminoacids are represented by single beads and their interactions quantified with a hydrophobicity factor. To confirm the different phase separation properties of phosphorylated and dephosphorylated Cdc15, we simulated condensation of the IDRs in a slab geometry (elongated rectangular box with periodic boundary conditions) and quantified the separation of proteins into condensed and dilute phases. The results show that phosphorylation decreases the phase separation critical temperature T_c value, indicating that it inhibits phase separation. The HPS coarse-grained model is thus in agreement with the experimentally observed difference between the phase separation of dephosphorylated Cdc15 and its highly phosphorylated state. [1] Bhattacharjee, R., Hall, A.R., Mangione, M.C., Igarashi, M.G., Roberts-Galbraith, R.H., Chen, J.S., Vavylonis, D. and Gould, K.L., 2023. Multiple polarity kinases inhibit phase separation of F-BAR protein Cdc15 and antagonize cytokinetic ring assembly in fission yeast. Elife, 12, p.e83062.

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Coarse-Grained Simulations of Myosin II Coiled-Coil Tail Interactions

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Non-muscle myosin II, essential for cytokinesis, cell migration, and cytoskeletal organization, forms dimers with coiled-coil tails. Human non-muscle myosin II leads to robust bipolar filaments mediated through periodic charge distribution patterns. However, current evidence suggests that fission yeast Myo2 assembles into the cytokinetic nodes for the contractile ring in a bouquet arrangement with weak or no filament bundle formation. To better understand the mechanisms underlying non-muscle myosin II organization, we used coarse-grained simulations that account for electrostatic interactions at a resolution of one bead per aminoacid. We constructed straight coiled coils by concatenating short tail segments of Alphafold3 predictions that otherwise predicts self-folding of whole tails. We use an elastic network model to allow for tail flexibility. OpenMM and the OpenABC framework was used to investigate the interaction between myosin II dimers aligned in parallel and antiparallel orientations and varying degree of overlap. To sample the binding landscape, we implemented adaptive external pulling sliding forces, dynamically scaled based on center-of-mass separation velocities. This allows overcoming barriers while maintaining quasi-equilibrium sampling during simulated tail sliding. Our results confirm stable overlapping lengths reported in prior human non-muscle myosin II studies, with electrostatic forces playing a pivotal role in stabilizing tail interactions. Ongoing work investigates Myo2 tails.

B581/P0562

Cytokinesis failure can be predicted before mitosis in mammary epithelial cells

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The stability of the cell division cycle is essential for maintaining genomic integrity. Perturbations in the mechanisms that regulate the cell cycle can result in abnormal cellular phenotypes, such as multinucleated cells, which cause genomic instability, cancer progression, and metastasis. We hypothesized that cytokinesis failure and multinucleation are preceded by measurable changes in cellular morphology and behavior. To test this hypothesis, we took advantage of MCF10A human mammary epithelial cells, which undergo epithelial–mesenchymal transition (EMT) and cytokinesis failure when treated with transforming growth factor-beta (TGFβ). We performed long-term time-lapse imaging with fluorescent reporters to track mitochondrial activity, cell-cycle progression, nuclear morphology, and motility at single-cell resolution. This analysis revealed distinct phenotypic alterations in mother cells that undergo cytokinesis failure and produce multinucleated progeny as compared to those that divided normally and produce mononucleated daughters. While mitochondrial membrane potential remained preserved, mothers of multinucleated cells displayed irregular cell-cycle durations, namely prolonged G2 and M phases, alongside nuclear pleomorphism and aberrant size regulation. Motility analysis revealed a higher frequency of persistent migration and altered polarization among mothers of multinucleated progeny. Our findings reveal that multinucleation is preceded by disruptions in cell motility, nuclear morphology, and cell-cycle duration, providing a functional signature that predicts cytokinesis failure. These insights suggest that the observed variations could serve as predictive markers for aneuploidy, offering insights into the cellular instabilities that initiate cancer progression.

B568/P0549

Decoding the Functions of Rab5 Isoforms in Cytokinesis

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Cytokinesis is the final step of cell division wherein the daughter cells split their cytoplasmic volumes following chromosome segregation. We had reported that Rab5, a master regulator of mammalian endocytosis, interacts and colocalizes with the conserved exocyst complex at the cytokinetic midbody in the cytokinetic intercellular bridge and is required for abscission. The three mammalian Rab5 isoforms - Rab5A, B and C - share over 85% sequence identity; however, their potential division of labour during cytokinesis is not understood. Using stable human cell lines expressing each of the three Rab5 isoforms individually, we performed functional complementation experiments by depleting all three endogenous isoforms, enabling investigation into the function of the individual isoforms. Our results surprisingly reveal that two of the isoforms promote cytokinetic progression, while the third impedes cytokinesis. Using the well documented QL and SN mutants of all three isoforms in similar complementation experiments, we discovered that the GTPase activity of all three isoforms is required to either promote or impede cytokinesis, respectively. These observations reveal an unexpected tug of war between the three Rab5 isoforms in maintaining optimal rates of cytokinetic progression. Our investigations into the molecular mechanism(s) underlying the functions of the three isoforms revealed an exclusive interaction of the cytokinesis-promoting isoforms with a conserved microtubule-binding protein, the depletion of which also impeded cytokinesis. Functional analysis uncovered further complexity, whereby only one of the two cytokinesis promoting isoforms collaborates with this protein, while the other isoform independently promotes cytokinesis. Our current investigations are focused on uncovering the details of the molecular mechanisms that differentially govern the cytokinetic roles of the Rab5 isoforms.

B589/P0570

F-actin as a positive regulator of cell and chloroplast division in Chlamydomonas green algae

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During cell division, organelles are accurately distributed to daughter cells; failure to properly position and segregate the organelles often results in reduced fitness of the daughter cells. In photosynthetic eukaryotes, chloroplast is segregated during cytokinesis but the mechanism is largely unknown. The unicellular green alga Chlamydomonas reinhartdii contains a single, large chloroplast that lies along the cell division plane and divides once every cell cycle during cytokinesis. The precise mechanism involved in the tight coordination between cell and chloroplast division remains unclear. To explore this phenomenon further, we generated genetic mutants for the core components of the FtsZ ring, a constricting structure located in the stroma that drives chloroplast division. Knockout mutants of FtsZ1 (FtsZ ring subunit) and ARC6 (transmembrane tethering protein) resulted in the absence of FtsZ ring during chloroplast division. Surprisingly, disruption of the FtsZ ring not only delayed chloroplast division but also caused the division plane of the organelle and the rest of the cell to become asymmetrical. Closer examination of these mutants revealed that the cleavage furrow cleaves through the undivided chloroplast in ftsz1Δ, often in an asymmetric fashion. When cleavage-furrow ingression was blocked by microtubule perturbation, the chloroplast in ftsz1Δ can no longer divide, indicating that the FtsZ ring is essential for autonomous chloroplast division and its coordination with furrowing. Recent work from our laboratory implicates filamentous actin (F-actin) in Chlamydomonas chloroplast division, as the process is delayed in cells lacking F-actin. Therefore, we investigated the relationship between F-actin and the FtsZ ring, and found F-actin loss diminished the proper localization of FtsZ1/2 and ARC6 in the chloroplast. Preliminary data implicated F-actin to be involved in the efficient transport or translocation of the chloroplast division proteins from cytosol into the stroma. Furthermore, to identify additional components involved in chloroplast division that may be dependent on F-actin, we performed a forward genetic screen for mutants hypersensitive to the loss of F-actin. From a collection of 33,000 UV-mediated mutants, we isolated a collection of mutants that are blocked in chloroplast division and cytokinesis under mild disruption of F-actin polymerization. Pinpointing the driver mutations in the isolated mutants should further elucidate F-actin's role

during chloroplast division and illuminate how photosynthetic eukaryotes coordinate cell division with that of the essential organelle, the chloroplast.

B578/P0559
Fission yeast cells use distinct cell size control mechanisms for size adaptation to osmotic, oxidative, or low glucose conditions
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Cells maintain their size to function properly within their ecological and organismal contexts. While environmental factors are known to influence cell size across both unicellular and multicellular organisms, the molecular mechanisms that allow cells to adapt their size under changing conditions remain poorly understood. In fission yeast, cells divide at a threshold size due to size-dependent activation of cyclin-dependent kinase (Cdk1). Recent work suggests that this control is achieved through scaling of mitotic activators with distinct aspects of cell size or growth: Cdr2, a Wee1 inhibitory kinase, accumulates with surface area; Cdc25, a mitotic phosphatase, accumulates with cell volume; and Cdc13 (mitotic Cyclin) scales with cell cycle time. Previous studies have primarily focused on these pathways under nutrient-rich conditions. Here, we investigate how these cell size control pathways contribute to size adaptation under environmental stress. Using high-throughput image analysis, we quantified the geometry of dividing cells grown under osmotic stress, oxidative stress, and low-glucose conditions. To test the contributions of surface area, volume, and time-sensing pathways, we modulated the protein levels of Cdr2, Cdc25, and Cdc13, respectively. Our data show that wild-type cells increase their surface area-to-volume (SA:Vol) ratio under low glucose conditions but decrease it under oxidative or osmotic stress. We find that the volume-sensing pathway (Cdc25) is required for proper size adaptation under oxidative and osmotic stress, while the time-sensing pathway (Cdc13) is necessary for size adaptation in osmotic stress. In contrast, the surface area-sensing pathway (Cdr2) is necessary for an increase in SA:Vol ratio under low glucose. These findings reveal how distinct cell size control pathways contribute to environmental adaptation and highlight the modularity of cell size control systems.

B063/P0645
Hot tags in acid: A thermostable rhodamine-binding system for real-time protein dynamics in thermoacidophilic archaea
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Studying protein dynamics in extremophiles like Sulfolobusacidocaldarius, which thrive at 75 °C and pH 2 requires molecular tools that can withstand extreme conditions However, traditional protein tags including fluorescent proteins (e.g., GFP) and self-labeling tags (e.g., HaloTag, SNAP-tag) fail to function reliably at these high temperatures due to denaturation or loss of fluorescence. This lack of suitable tools has left a major gap in live-cell imaging and the study of thermophile physiology. To address this, we used Rhobin9, a de-novo designed rhodamine-binding tag with nanomolar affinity, high photostability, and exceptional thermostability. When paired with JF dyes, Rhobin9 enables specific and high-contrast labeling of proteins in live S. acidocaldarius cells. Rhobin9 enables real-time imaging of protein dynamics under extreme conditions, opening new opportunities for cell biology research in high-temperature organisms.

B567/P0548
How the Rho GAP RGA-3/4 contributes to the negative feedback of cortical excitability
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Cortical excitability – the ability of cell cortex to generate propagating waves of actin assembly and Rho GTPase activity – has been implicated in many essential biological processes such as cell division, cell locomotion, and morphogenesis. During cytokinesis in amphibian and echinoderm embryos, propagating waves of Rho activation and inactivation, and complementary waves of F-actin assembly and disassembly are amplified and focused at the equatorial cortex by the mitotic spindle. Such GTPase waves are proposed to result from fast positive feedback based on Rho and the Rho GEF, Ect2, and delayed negative feedback based on F-actin and the Rho GAP, RGA-3/4. Currently, this model is tenuous, in part because the relationship between F-actin and RGA-3/4 is not well understood. To further our knowledge about the negative feedback of cortical excitability, I performed structure and function analysis of RGA-3/4 in Xenopus oocytes. I found that RGA-3/4, when expressed alone in Xenopus oocytes, localizes to and reorganizes the cortical F-actin into large cables. F-actin reorganization is independent of its GAP activity but nonetheless requires interaction with Rho. Additionally, the first 41 amino acids as well as IG232,233 residues are necessary for F-actin reorganization. When co-expressed with Ect2 in Xenopus oocytes, RGA-3/4 induces cortical excitability. In contrast to F-actin reorganization, cortical excitability requires GAP activity as well as the portions of the protein needed for F-actin reorganization. However, an RGA-3/4 mutant comprising the GAP domain and regions required for F-actin reorganization fails to support cortical excitability. Complete induction of cortical excitability requires a minimum length of ~120 residues of the intrinsically disordered carboxyl terminal region of RGA-3/4. Since intrinsically disordered regions (IDRs) can self-associate and promote biomolecular condensate formation, it could be that RGA-3/4 needs to self-associate in order to fully support cortical excitability. Indeed, an RGA-3/4 chimera with its carboxyl terminus replaced with heterologous IDRs was able to promote cortical excitability. To better understand the innate properties of RGA-3/4, I also performed in vitro studies. Purified RGA-3/4-GFP protein binds directly to and bundles F-actin in a manner that was correlated with self-association. In fact, RGA-3/4-GFP with shortened IDRs displays a reduced ability to bind and bundle F-actin. In conclusion, the results indicate that the negative feedback of cortical excitability depends on RGA-3/4 binding to and bundling F-actin, inactivating Rho, and self-associating, and may require highly coordinated and dynamic tripartite interactions among Rho, RGA-3/4 and F-actin.

B590/P0571
Investigating the Role of Importin α Palmitoylation in Cytokinesis
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Cytokinesis is the final sequence of mitosis in which cells are cleaved into two daughter cells. This process is tightly regulated to ensure appropriate separation of chromosomes; failures or disruption of cytokinesis can result in aneuploid cells that can drive tumorigenesis. Regulators of this process include the protein anillin which binds to contractile ring proteins and drives their constriction, and ECT2, a RhoGEF which transduces signals to regulate actin polymerization at the cytokinetic furrow. Importin β, a nuclear transport protein, has previously been found to regulate anillin furrow localization. Importin β has a well-characterized role in mediating nuclear transport by binding to importin α and RanGTP, where importin α acts as an adapter to bind to nuclear localization signal (NLS)-containing protein cargo and RanGTP binding to importin β causes the

release of cargo. Recent work has shown that importin α can be lipid-modified with palmitate, which allows for association with the plasma membrane. The role of palmitoylated importin α in anillin and ECT2 transport was observed in immunostained cells treated with palmitoylation-disrupting drugs. By altering the palmitoylation pathway, changes in the localization of anillin and ECT2 were observed during anaphase. These findings suggest an novel role for palmitoylated importin α as a mediator of cytokinesis.

B571/P0552

Kinesin-5 Contributes to Cleavage Furrow Ingression and Delivers Transmembrane Protein PKHD1 to Further Determine the Post-Division Cell Polarity

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This study aims to examine the non-spindle role of kinesin-5 in membrane trafficking and extracellular matrix (ECM) deposition during cytokinesis and the establishment of post-division polarity. Kinesin-5 motors are well characterized for their role in bipolar spindle formation. In addition, kinesin-5 can be found at the division site in post-anaphase cells of various organisms, including human Eg5 in the abscission body, and Arabidopsis KRP125c and Phycomitrella kinesin 5-c in phragmoplasts. The green alga Chlamydomonas reinhardtii is phylogenetically related to plants and divides like animals by cleavage furrow ingression. Unlike animals, Chlamydomonas cytokinesis is temporarily separated from mitosis, allowing observation of any kinesins in the furrow region without interference from the spindle microtubules. We found that KIF11, the sole kinesin-5 in Chlamydomonas, localizes to the furrow. A kif11 temperature-sensitive mutant showed pleiotropic defects in mitosis and furrowing. Chlamydomonas furrowing is not dependent on F-actin. However, we found that combining the loss of KIF11 and F-actin blocked furrow formation. Thus, KIF11 may play complementary roles with F-actin in cytokinesis, and the cell can tolerate a loss of either pathway but not both. A co-immunoprecipitation mass-spectrometry experiment using KIF11 identified proteins that are transmembrane or secreted, suggesting a role for KIF11 in membrane trafficking and deposition of ECM. One of the identified proteins, Polycystic and Hepatic Disease 1 (PKHD1)/fibrocystin, is a 500-kD single-pass transmembrane glycoprotein. In humans, mutations in PKHD1 cause polycystic kidney disease through an unknown molecular mechanism. We found that Chlamydomonas PKHD1 co-localized with KIF11 specifically in the furrow. The PKHD1 furrow localization was lost in the kif11-TS mutant, suggesting that KIF11 is required for delivering PKHD1 to the furrow. After completion of cytokinesis, PKHD1 remained polarized during the multiple fission cycles. Loss of PKHD1 altered cell polarity after the first cytokinesis and disrupted the orientation of subsequent divisions. In mammals, PKHD1 is essential for the proper development of renal tubules and establishment of planar cell polarity during kidney development; however, its involvement in cytokinesis has not been tested. Through Immunofluorescence of HsPKHD1 in the human embryonic kidney (HEK293) and mouse inner medullary collecting duct (mIMCD-3) cells, we showed that HsPKHD1 also localizes to the furrow during cytokinesis and is retained in the midbody during abscission. The evolutionarily conserved role of PKHD1 in determining the post-division cell polarity may be granted by its furrow targeting during cytokinesis in both green algae and humans.

B584/P0565

Mechanism of Aurora B-mediated relief of centralspindlin inhibition during cytokinesis

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The chromosomal passenger complex (CPC), whose active subunit is the kinase Aurora B, localizes to the central spindle after anaphase onset and is essential for cytokinesis. The current view on CPC action in cytokinesis is that it relieves inhibition of centralspindlin, a complex of kinesin-6 and CYK-4, by 14-3-3 family proteins. Centralspindlin activates the RhoGEF ECT2, which generates active RhoA to drive contractile ring assembly and furrow ingression. Consistent with this model, inhibition of either the CPC or centralspindlin in C. elegans embryos produces nearly identical cytokinesis defects, and our data suggests the CPC is required for centralspindlin recruitment to the equatorial cortex. Prior work in C. elegans embryos suggested that the 14-3-3 protein PAR-5 binds kinesin-6 on phosphorylated Ser682 to inhibit centralspindlin function, and that the CPC phosphorylates an adjacent residue, Ser680, to counter this inhibition by displacing PAR-5. To test this idea, we used a validated kinesin-6 replacement system to examine the effect of mutating Ser680 to a non-phosphorylatable alanine. Contrary to the prediction of persistent centralspindlin inhibition, mutation of Ser680 to alanine had no impact on cytokinesis. Previous literature on other 14-3-3 interactions and AlphaFold modeling instead suggest that phosphorylation of Ser680 might collaborate with Ser682 phosphorylation to strengthen rather than inhibit 14-3-3/PAR-5 binding. This model predicts that mutating both Ser680 and Ser682 to alanine would prevent PAR-5 inhibition of centralspindlin, potentially bypassing the CPC requirement. Supporting this, double mutation of Ser680 and Ser682 strongly rescued furrow ingression in Aurora B–depleted embryos, while single mutations gave partial rescue. These results suggest that phosphorylation of kinesin-6 Ser680 and Ser682, by an as-yet-unknown kinase(s), promotes PAR-5 binding and centralspindlin inhibition. Mutation of these two sites to alanine prevents PAR-5 binding and largely bypasses the need for Aurora B for furrow ingression. We therefore propose that the CPC promotes cytokinesis not by phosphorylating kinesin-6 on Ser680 but by phosphorylating PAR-5 itself to disable its inhibitory function. PAR-5 contains two candidate Aurora B sites whose phosphorylation is predicted to prevent dimerization and the ability to engage client proteins like centralspindlin. We are currently building tools to test whether phospho-null or phosphomimetic mutations in these putative sites cause PAR-5 inhibition of centralspindlin to be constitutive or absent. This work clarifies the mechanism by which Aurora B/CPC promote cytokinesis and suggests that further understanding of PAR-5/14-3-3 proteins in cell division may be of significant interest.

B582/P0563

Phosphoproteomics identifies a new role for Cdr2 kinase in cell division

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Signaling pathways coordinate the events of cell division in time and space. Proteins that act in multiple steps of cell division act as master regulators of this process. In the fission yeast Schizosaccharomyces pombe, the protein kinase Cdr2 is required for timely entry into mitosis and proper formation of the division septum. Cdr2 regulates mitotic entry through Wee1, but its targets in other processes including septation are unknown. We performed a large-scale phosphoproteomic screen to identify new targets of Cdr2 signaling. From multiple candidates, we focused on Cnt6 as a potential substrate of Cdr2. Cnt6 belongs to the centaurin family of ADP-ribosylation factor GTPase-activating proteins (Arf-GAP). Cnt6 phosphorylation was reduced in cdr2Δ but increased by Cdr2 overexpression. Mutation of phosphorylation sites identified in our phosphoproteomic screens prevented this regulation. cnt6Δ mutants exhibited septation defects similar to cdr2Δ, suggesting that Cdr2-Cnt6 signaling regulates septation. By live-cell microscopy, we found that Cnt6 localized to the cell periphery and the spindle pole body (SPB). During mitosis, Cnt6 localized asymmetrically to old SPB but not the

new SPB. This asymmetry is similar to components of the Septation Initiation Network (SIN), a signaling pathway that regulates the timing and position of cytokinesis. Consistent with this possibility, a series of genetic and live-cell imaging experiments indicated that Cnt6 functions as a negative regulator of SIN signaling. Our data identify Cnt6 as a novel target of Cdr2 signaling with functions in the SIN pathway and septation. These findings suggest that Cdr2 acts as a master regulator of cell division through distinct targets for mitotic entry and septation.

B573/P0554

Rho GTPase regulation in neighboring cells during vertebrate epithelial cytokinesis

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Cytokinesis poses a challenge to the integrity of epithelial tissues: as the diving cell and its neighbors undergo significant shape change, they must maintain their cell-cell junctions and preserve the tissue's barrier function. Using the epithelium of Xenopus laevis embryos as an intact vertebrate epithelial tissue model, we demonstrated that during cytokinesis, neighboring cells respond mechanosensitively to furrow ingression by generating their own RhoA-mediated actomyosin arrays adjacent to the cleavage furrow, which plays an important role in the determining speed and success of cytokinesis. Furthermore, mechanically uncoupling the dividing cell from its neighbors disrupts cell packing following cytokinesis and leads to increased barrier leaks during cytokinesis. Here, we test the hypothesis that decreasing actomyosin contractility in neighboring cells will cause cytokinesis defects. Xenopus embryos were mosaically microinjected with dominant negative RhoA. At late blastula stage, we imaged cytokinesis events where one or both neighbor cells express dominant negative RhoA, whereas the dividing cell does not. We find a higher incidence of "asymmetric" cytokinesis events in dividing cells with one dominant negative RhoA neighbor and one control neighbor. Additionally, we observed changes in cell packing post-cytokinesis and increased incidence of early cytokinesis failures. These data provide another piece of evidence supporting the idea that a mechanosensitive response in neighboring cells during cytokinesis is important for maintaining tissue homeostasis. In ongoing work, we are identifying upstream regulators of RhoA activation in neighboring cells Preliminary data suggest that accumulation of GEF-H1 precedes RhoA accumulation in neighboring cells during cytokinesis. Together, this research will further shed light on the mechanisms of epithelial cytokinesis as a fundamentally multicellular process.

B569/P0550

Rho, Rac, and Cdc42 GTPases regulate mesoscale cytokinetic F-actin oscillations in an artificial cell cortex

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The small GTPase Rho promotes F-actin polymerization during cell division, building an equatorial contractile array that constricts inwards to create two daughter cells. In Xenopus laevis embryos, active Rho and F-actin are dynamically patterned in an oscillatory manner at the division plane and throughout the cell cortex. This patterning is known as cortical excitability, and is driven by a molecular circuit comprised of fast positive feedback by the Rho activator Ect2 and delayed negative feedback by F-actin recruitment of a Rho inhibitor RGA3/4. While the exact role of cortical oscillations in cytokinetic signaling is not known, cortical oscillations within the division plane have unique dynamics relative to the polar regions, suggesting that cortical oscillations support cytokinetic signaling and the assembly of cytokinetic machinery. Moreover, the molecular mechanisms that regulate the spatiotemporal dynamics of cortical oscillations during cytokinesis are not

understood. There is some evidence that division plane specific F-actin polymerization is governed by spatial segregation of Rho-family GTPases, with active Rho enrichment at the division plane excluding active Cdc42 and active Rac. However, whether Cdc42 and Rac regulate Rho activity or F-actin assembly in the cortical excitability circuit has not been established. Here we used an in vitro "artificial cortex" system made of X. laevis extract and supported lipid bilayers to investigate the role of Cdc42 and Rac in cortical oscillations. We find that active Cdc42 and active Rac display robust oscillatory behavior in the artificial cortex and precede F-actin oscillations in a manner similar to active Rho. This suggests that all three Rho-family GTPases regulate cortical F-actin oscillations and are inhibited by F-actin polymerization. We found that these GTPase dynamics are interdependent, as inhibition of Rho or F-actin alters Cdc42 and Rac oscillatory dynamics. We considered spatial cues that organize the division plane in vivo and therefore investigated how microtubule plus-end overlaps affect cortical oscillations. We find that active Rho and F-actin dynamics are altered at sites of microtubule plus-end overlap, suggesting that microtubule-dependent signaling regulates dynamics of oscillations at the division plane. These findings indicate that multiple pathways, including additional Rho-family GTPases and microtubule plus end overlaps, regulate the dynamics of cortical oscillations in dividing cells. Our ongoing studies are aimed at characterizing how Cdc42 and Rac oscillations are organized in relation to microtubule plus-end overlaps, as well as manipulating upstream regulators of the Rho-family GTPases to disentangle additional mechanisms of feedback between the GTPases.

B572/P0553

RNA splicing is required for timely completion of abscission and is modulated by the abscission checkpoint

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The last stage of cell division is regulated by the abscission checkpoint, which halts essential steps in membrane remodeling at the midbody that must occur to execute abscission. Abscission checkpoint activity is maintained under various conditions, including heightened intercellular tension between dividing daughter cells, replication stress earlier in the cell cycle, chromatin bridges through the abscission site, and nuclear pore defects. Our previous studies indicated Abscission Checkpoint Bodies (ACBs) arise in the cytoplasm during cytokinesis under conditions that lead to sustained abscission checkpoint activity. ACBs harbor a unique coalescence of membrane remodeling factors, checkpoint signaling proteins, and, surprisingly, the RNA splicing factors SRRM1, SRRM2, and SON. Here, we investigate the role of RNA splicing in abscission progression and regulation. Upon mitotic exit, RNA spliceosome catalytic activity recovers to interphase levels before cells undergo abscission, and efficient abscission was found to rely on RNA splicing. We went on to identify unique patterns in RNA splicing at this time in cell division and alterations that occur when the abscission checkpoint is active. These results reveal a previously unrecognized level of transcriptome regulation during cytokinesis and its modulation by abscission checkpoint activity. This new insight adds to a growing appreciation of an intimate connection between RNA and midbody form and function.

B566/P0547

Role of pHi in Regulating TPX2 Abundance During Cytokinesis

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Intracellular pH is shown to be a critical factor in regulating normal cell behaviors, including cell division. Previous studies have established that pHi oscillates during cell division, with marked increases in pHi in mid-S phase and G2 while decreased pHi marks the G1/S transition and mitosis. Our prior work showed that lowering pHi shortens G1, whereas raising pHi elongates it. However, the functional role for decreased pHi in mitosis and cytokinesis has not been explored. Here we show that raising pHi in normal breast epithelial cells (MCF10A) increases cytokinesis defects, mediated by pH-dependent stabilization of Targeting protein for Xklp2 (TPX2), an effector of branching microtubule nucleation in the mitotic spindle. In normal dividing cells, TPX2 is rapidly degraded during late anaphase by anaphase-promoting complex (APC), enabling microtubule depolymerization in the midbody for cytokinesis. However, the mechanism of temporal TPX2 degradation is poorly understood. Using confocal microscopy approaches, we quantified TPX2 abundance in control MCF10A cells and found that TPX2 abundance increases up until metaphase and then rapidly decreases after anaphase. We hypothesized that the observed low pHi during cytokinesis promotes TPX2 degradation and is necessary for successful cytokinesis. We showed that raising pHi in MCF10A cells stabilized TPX2 abundance during telophase and increased cytokinesis defects. Furthermore, TPX2 depletion rescues successful cytokinesis under high pHi conditions, suggesting TPX2 is the critical regulator of pHi-dependent cytokinesis. We have also initiated investigations into the pH-sensitive molecular mechanism of TPX2 stabilization. We used a computational approach to identify six residues within the TPX2 spindle assembly and microtubule-binding domains that could function as pH-sensitive nodes. Future work will further elucidate the molecular mechanisms of TPX2 as a pH-dependent regulatory factor for normal mitotic cells and leverage those functions to gain insight into diseases with dysregulated pHi, including cancer where pHi is constitutively increased.

B587/P0568

Septins and cytokinesis in a polymorphic fungus

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During cytokinesis of animals and fungi, a contractile actomyosin ring (CAR) assembles at target locations and constricts to drive cell separation. In many animal cells, the position of the CAR is determined by the mitotic spindle, so that the cleavage plane is perpendicular to the mitotic axis to ensure accurate partitioning of chromosomes. Similarly, CAR assembly in the fission yeast, Schizosaccharomyces pombe, is determined by the position of the mitotic nucleus at the cell middle. However, in budding yeasts including Saccharomyces cerevisiae, the location of CAR assembly at the mother-bud neck is specified well before mitosis, and the mitotic spindle is oriented so as to segregate chromosomes perpendicular to the predetermined cleavage plane. The mother-bud neck is marked by a cortical septin cytoskeleton that recruits CAR components to the neck. Septins also mark the site of septation in hyphal fungi, although septin roles in cytokinesis can vary between species. In the polymorphic fungus, Aureobasidium pullulans, septins assemble at mother-bud necks but their role in cytokinesis is unknown. This fungus undergoes multi-budding growth in its yeast form, but also forms hyphae and large bulbous cells whose mechanisms of cytokinesis are uncharacterized. We found that CARs are assembled and constrict to drive cytokinesis at both narrow bud necks (~1 µm diameter) and at much larger septa (~4-15 µm diameter). Septins were recruited to both structures but with different kinetics. Surprisingly, cells lacking individual septins still formed CARs at most bud necks, although CAR assembly and cytokinesis stochastically failed at a subset of necks. Analysis of septin localization in cells lacking other septins suggested that septins act primarily as canonical octamers for most of their activities, but that smaller complexes with partial function can form in the absence of one septin. Construction of cells lacking multiple septins suggested that although septins contribute to CAR assembly, they are not required, indicating that there is a mysterious alternative mechanism to assemble CARs at mother-bud necks. Together these findings

indicate the roles of septins in CAR assembly are organism specific, and that budded cells with very similar geometries differ in their mechanisms of CAR placement.

B579/P0560

Spatial and Temporal Regulation of Cortical Excitability via Optogenetic in Xenopus Oocytes

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The cell cortex, defined by the plasma membrane and a thin layer of cytoskeletal structures underneath, responds to diverse external and internal stimuli via cortical excitability – a behavior that generates propagating waves of actin assembly and Rho GTPase activity at the cell cortex. Cortical excitability has been implicated in various morphogenesis such as cell locomotion and cell division. In frog and starfish embryos during cytokinesis, waves of Rho activation and inactivation, and complementary waves of F-actin assembly and disassembly are amplified and focused at the equatorial cortex. These waves are thought to arise from rapid positive feedback loops between Rho and Rho GEF Ect2, coupled with delayed negative feedback mediated by F-actin and Rho GAP RGA-3/4. To further study cortical excitability, we are using Xenopus oocytes. Xenopus oocytes are arrested in meiotic interphase and are not naturally excitable. However, a recent finding revealed that co-expression of Ect2 and RGA-3/4 can induce cortical excitability, resulting in Rho and F-actin waves that propagate across the entire cortex over a prolonged period of time. Using this Xenopus oocyte system, I employed optogenetics to precisely control the spatial and temporal induction of cortical excitability. SsrA is a light control binding site for its partner protein, SspB. We fused SsrA with CAAX, a plasma membrane binding domain, and SspB with Ect2 or RGA-3/4 to enable photoactivation-dependent recruitment of the proteins to the cortex. This optogenetic system successfully induced propagating Rho waves within a defined region of interest (ROI) at the cortex only during photoactivation. Interestingly, the directions of Rho waves seem to be more uniform as we reduce the size of ROI. In conclusion, these findings demonstrate that cortical excitability can be spatially and temporally controlled using the optogenetic system.

B564/P0545

The centriculum, a membrane reticulum adjacent to the C. elegans centrosome, acts as a microtubule filter

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During mitosis of most animal cells, centrosomes serve as the primary microtubule-organizing centers. Each centrosome contains a pair of centrioles surrounded by pericentriolar material (PCM). Although generally considered membraneless, a dense endoplasmic reticulum (ER)-derived membrane surrounding the centrosome has been observed in embryonic cells of C. elegans, Drosophila, sea urchin and the fish Medaka, as well as in some mammalian culture cells. Using conventional and volume electron microscopy, we previously investigated the structure and function of this centrosome-proximal membrane in the C. elegans early embryo. We found that this membrane forms a dense reticular network, now called the centriculum. We further demonstrated that the centriculum influences centrosome structure and its microtubule-nucleating capacity. Here, we investigated the role of the centriculum in microtubule organization. Previouly published electron tomograms of the C. elegans 1-cell embryo revealed the presence of many short peri-centrosomal microtubules. Reconstruction of the centriculum in these datasets showed that a fraction of these

microtubules collided with the centriculum, suggesting that the centriculum may function as a microtubule filter, limiting the number of microtubules that can extend beyond it. We hypothesized that if the centriculum resists microtubule elongation, microtubule collisions might exert force on the centriculum, thereby influencing centriculum size. Indeed, increasing microtubule stability or number increased centriculum size. Interestingly, decreasing microtubule number led to a smaller centriculum and smaller PCM, without reducing the amount of the PCM protein SPD-5, suggesting that the PCM is compresable. Moreover, that while the centriculum is normally adjacent to the PCM, restricting PCM expansion without affecting microtubule nucleation did not change centriculum size, suggesting that centriculum size is affected by microtubules rather than the PCM. We also show that during metaphase, the centriculum is more permissive to microtubules extending toward the chromosomes than toward the cortex, consistent with a higher density of spindle microtubules compared to astral microtubules. We therefore propose that the centriculum acts as a microtubule filter, affecting microtubule distribution, abundance, and orientation.

B592/P0573

The myosin II clusters that template the cytokinetic contractile ring in an early embryo mediate actin filament assembly and alignment

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We have investigated the architecture and dynamics of the cytokinetic contractile ring (CR) in early sea urchin embryos using light and electron microscopy. To date our studies suggest that the CR initiates as an equatorial stripe of clusters containing myosin II, actin, septin and anillin, which then congress over time into patches and ultimately a linearized and contractile array which mediates cleavage furrow formation. A fundamental assumption of this CR structural progression is that actin filaments assemble from the myosin II clusters and that they undergo a fission yeast-like "search, capture, and pull mechanism" - a process we have modeled using computer simulations. However, despite our detailed characterization of the localization of other CR-proteins, consistent and clear imaging of the actin filament organization has remained elusive. In the present study, we applied a modified fixation method in localizing actin filaments in the CRs present in cortices isolated from dividing embryos. Confocal imaging clearly indicated that the band of myosin II clusters that template the CR assembled arrays of actin filaments and that over time these filaments interconnected the clusters into a network which then transformed into a dense and linearized organization. In experiments using the myosin II contraction inhibitor para-aminoblebbistatin (PAB) we showed that the aligned actin filaments in the late-stage CR of control embryos were replaced by misaligned and disorganized actin filaments. We hypothesize that the presence of pre-polymerized actin filaments associated with the myosin II clusters in PAB-treated embryos explain how polynuclear PAB treated embryos can undergo rapid cytokinesis following drug photoinactivation. Finally, we demonstrated that PAB treatment can dissociate alpha-actinin from contractile actomyosin structures such as the CR and stress fibers. Taken together these results highlight the evolutionary connections between the CR mechanisms driving fission yeast and animal cell cytokinesis. They also indicate that myosin II contraction is necessary for the proper alignment of CR actin filaments and the associated binding of alpha-actinin, but not required for the assembly of actin from the myosin II clusters.

B575/P0556

The roles of positive feedback and autoinhibition during Ect2-mediated cortical excitability

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Cortical excitability – the ability of the cell cortex to support propagating waves of protein concentration or activity – plays a key role in processes such as cell migration, cell repair, development, and cell division. During cytokinesis, propagating waves of Rho GTPase activity and F-actin assembly/disassembly are concentrated in the equatorial cortex where they direct the formation and constriction of the contractile ring. Rho wave propagation is proposed to result from an excitable circuit that contains interdependent positive and negative feedback loops. Positive feedback is thought to depend on the Rho Guanine nucleotide exchange factor (GEF) Ect2, while negative feedback depends on the Rho GTPase activating protein (GAP) RGA-3/4. Here, we examine how Ect2-mediated positive feedback and autoinhibition shape Rho activity dynamics during cortical excitability. We first compared the phasing of Ect2 and Rho-GTP in cortical excitability. Fluorescently tagged Ect2 consistently precedes Rho-GTP activity by ~7 seconds, consistent with a role in positive feedback. We next focused on a proposed Rho-GTP–PH domain binding site in Ect2, which has been proposed to be the primary component of a direct positive feedback loop. Disruption of this interaction abolished the ability of Ect2 to initiate wave propagation. We further verified this result with an optogenetic approach. Wild-type Ect2 tail (which contains the PH domain and polybasic cluster) recruited to sites of optogenetically activated Rho, whereas an Ect2 tail mutant that lacks Rho-GTP binding failed to localize. Together, these findings support that direct positive feedback through Rho-GTP binding to the Ect2 PH domain is required for cortical excitability. We next investigated Ect2 autoinhibition and its contribution to cortical excitability. Removal of PH domain-mediated autoinhibition modestly increased Rho wave amplitude, temporal width, and period, without preventing Rho wave initiation. Removal of BRCT domain-mediated autoinhibition produced a similar but stronger effect, suggesting that BRCT domains provide the dominant form of autoinhibition. Combining both mutations further increased these effects beyond removal of BRCT-autoinhibition alone. Consistent with these results, tagged Ect2 PH- and BRCT-autoinhibition mutants were present at the membrane for longer and more so overlapped with Rho-GTP than wild-type Ect2. These results suggest that both PH- and BRCT-mediated autoinhibition regulate Ect2 activity, with BRCT-autoinhibition serving as the primary form. Together, these findings support a model in which Ect2 integrates direct positive feedback through Rho-GTP binding with autoinhibition by its PH and BRCT domains to regulate cortical excitability.

B585/P0566

TPGS1 Regulates Central Spindle Microtubule Glutamylation and Remodeling During Telophase and Abscission

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Microtubules are a key cytoskeletal component affecting many different cell functions. During cell division, microtubules make up the mitotic spindle to facilitate chromosome separation. After completion of anaphase, the central spindle population is remodeled and compacted within the intercellular bridge (ICB), arranged antiparallel at the ICB center where the midbody forms. These microtubules serving as scaffolds that aid in the trafficking of abscission machinery and must later be severed for the final abscission event to occur near the midbody. Tubulin post-translational modifications controlled by the tubulin tyrosine ligase-like (TTLL) family of enzymes confer properties that affect microtubule dynamics and associated protein interactions. The majority of the TTLL family are glutamylases, proteins that add glutamate groups onto the c-terminal tail of tubulin. Polyglutamylation has been linked to the activity of microtubule severing enzymes like spastin and katanin in vitro, but the cellular context for this connection has remained poorly understood. We examined microtubule glutamylation during mitosis with a focus on telophase and the central spindle microtubule population. We found that glutamylase TTLL1-EGFP is targeted to the midbody in late telophase, and that its trafficking associated complex member TPGS1 is required for its localization there. We also found that TPGS1-EYFP

localizes predominantly closer to the ICB minus ends in telophase but appears in the main ICB and midbody in later stages. Knockout of TPGS1 resulted in delayed in cell division and complications in both microtubule minus end remodeling (termed “fan-out”) and spastin severing for abscission. These phenotypes were accompanied by a significant reduction of glutamylation in the ICB, showing a link between glutamylation and microtubule organization in telophase and abscission.

B591/P0572

Understanding cortical activity with synthetic biology

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Dynamic changes in the cell cortex, the plasma membrane and underlying cytoskeleton, are the driving factors for many morphologic cell processes. During cytokinesis in amphibian and echinoderm embryos, these cell shape changes are driven by cortical excitability – propagating waves of Rho activation and inactivation and F-actin assembly and disassembly at the equatorial cortex. These waves are maintained by the fast positive feedback between Rho and the Rho GEF Ect2, and the delayed negative feedback based on F-actin and the Rho GAP RGA-3/4. In Xenopus Laevis, cortical excitability is regulated in cell cycle-dependent manner in early stages embryos. Immature Xenopus oocytes, which are arrested in meiotic interphase, do not naturally support cortical excitability, since the key cytokinetic regulators are not expressed at this stage. Recent findings have revealed that cortical excitability can be induced in Xenopus oocytes when Ect2 and RGA-3/4 are co-expressed. We hypothesized that there are components within the positive and negative feedback necessary for cortical excitability. GEF activity and direct association between active Rho and Rho GEF are likely necessary for positive feedback. The negative feedback may require GAP activity and direct interaction between F-actin and Rho GAP. To test this hypothesis, we built synthetic Rho GEF using GEF domain of LARG fused to active Rho binding domain of Rhotekin (rGBD). We also built synthetic Rho GAP using GAP domain of p190RhoGAP fused to different F-actin binding proteins or domains (i.e. fimbrin, cofilin, and tropomyosin). We co-expressed these synthetic constructs in Xenopus oocytes and found that the synthetic Rho GEF and GAP could indeed induce Rho waves at the cell cortex. Interestingly, there were significant phenotypic differences between the natural Rho waves (those induced by Ect2 and RGA-3/4) and synthetic Rho waves (those induced by synthetic Rho GEF and GAP). The waves produced in the natural system are relatively small and uniform, while the waves produced in the synthetic system are large, irregular, and often form a compact “Rho spot”, which is not observed in the natural system. These results indicate that we have correctly identified necessary components of cortical excitability but are still missing elements that produce the natural phenotype of Rho waves. To better understand the synthetic regulation of Rho waves, we plan to look at the expression of the synthetic Rho GEF and GAP at the protein level with the overall goal of creating a synthetic system that closely mimics natural cortical excitability. In conclusion, we have correctly identified key components to positive and negative feedback of cortical excitability using synthetic system, providing more insight into cell division as a whole.

B574/P0555

Using Fluorescence Correlation Spectroscopy to measure changes in chromatin occupancy of Chk1 in Drosophila development

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Chk1 is a vital component of cell cycle control through the DNA damage response (DDR) pathway across metazoans. Though Chk1 interacts with downstream regulators in the nucleoplasm, several upstream components in the DDR bind chromatin prior to activating Chk1. Studies in mammalian systems have shown that a fraction of chromatin-bound Chk1 is important for transcriptional control of cell-cycle related genes including Cyclin B1 and CDK1. Moreover, Chk1 dissociates from chromatin upon activation of the DDR. Despite strong conservation between organisms, Chk1's association with chromatin has not yet been documented in Drosophila. Chk1 plays a vital role in the Drosophila embryo, Prior to gastrulation, Drosophila have remarkably rapid cell cycles that lack growth phases and many of the canonical cell cycle checkpoints. Maternally supplied Chk1 (grapes in flies) is required to slow the cell cycle during the Mid-Blastula Transition (MBT) and Zygotic Genome Activation (ZGA), which occur concurrently with gastrulation. Maternal Chk1 mutants have shortened S-phases and experience mitotic catastrophe in the final rapid cell cycle prior to the MBT. The importance of Chk1 in regulating the MBT provides an excellent opportunity to investigate the presence and contribution of chromatin-bound Chk1 in normal embryonic development. Fluorescence Correlation Spectroscopy (FCS) allows the robust and reproducible separation of chromatin-bound and "free" Chk1 in the nucleoplasm of living embryos. FCS is a microscopy technique that uses fluctuations in fluorescence in a small detection volume to calculate the diffusion rate, concentration, and relative abundance of different fluorescent species. Preliminary results using FCS have revealed that nearly half of Chk1 within the early embryonic nuclei diffuses at a rate consistent with chromatin-bound dynamics, but that this fraction is reduced as the embryo reaches the MBT. This movement of Chk1 off chromatin may represent the activation of Chk1 by ATR (Mei-41 in flies) which in turn allows correct timing of cell cycle slowing at the MBT. Future work will describe the change in Chk1 diffusion patterns across developmental time and explore the role of upstream DDR components in freeing Chk1 from chromatin. This work will enhance our understanding of Chk1 activation through changes in Chk1 concentrations and upstream DDR elements prior to Chk1 activation.

Cell Division and Cell Cycle Control - Centromeres and Kinetochores

B517/P0500
Availability of highly conserved architectural proteins explains the rapid evolution of centromeric DNA satellites
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Long-read sequencing has captured the extraordinarily rapid evolution of highly repetitive centromeric DNA satellites. The next challenge lies in explaining the origins of this rapid evolution. Divergent satellite arrays are thought to emerge from short-repeat libraries through stochastic expansions, followed by the accumulation of beneficial mutations in satellite-associated proteins to maintain centromere function. Here, we provide experimental support for the selective expansion of centromeric satellites, dependent on the availability of highly conserved architectural proteins that support faithful chromosome segregation. We find that mouse "major" satellite arrays can withstand spindle forces in female meiosis because they are recognized and packaged by two DNA shape-recognizing proteins, HMGA1 and HMGA2 (high mobility group AT-hook 1 and 2). Strikingly, although the amino acid sequence of HMGA2 is conserved, its amount is higher in oocytes from Mus musculus, where major satellite has expanded, than in Mus spretus, where major satellite abundance is low. By hybridizing musculus and spretus, we introduced expanded major satellite arrays into the HMGA2-low background. We demonstrate that HMGA2 no longer supports major satellite packaging in this hybrid, consistent with observed major satellite packaging defects. We propose that major satellite expansion in musculus was dependent on the availability of both HMGA1 and HMGA2, which are required to package it to

maintain faithful chromosomal segregation. Our work provides a mechanistic framework to explain the rapid evolution of centromeric satellites across taxa.

B510/P0493
CENcyclopedia: dynamic landscape of kinetochore architecture throughout the cell cycle
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The kinetochore is a large, multiprotein complex assembled on centromeric chromatin that plays an essential role in ensuring accurate chromosome segregation. Functioning as a dynamic signaling and mechanical interface, the kinetochore mediates microtubule attachment, monitors attachment fidelity, and transmits forces required for chromosome movement. Comprised of over 100 distinct proteins, present in multiple copies, the kinetochore undergoes tightly regulated compositional changes throughout the cell cycle to fulfill stage-specific functions. Despite its importance, the mechanisms governing these dynamic changes remain incompletely understood. Previous studies have primarily focused on a limited subset of kinetochore components or their turnover during mitosis, leaving the broader temporal landscape of kinetochore assembly and disassembly unresolved. Here, we employ advanced quantitative immunofluorescence microscopy to systematically characterize the dynamic behavior of 37 kinetochore proteins and associated kinases across the entire cell cycle, including the relatively understudied G1, S, and G2 phases. Our comprehensive analysis reveals distinct temporal patterns of protein recruitment, retention, and dissociation, providing an integrated view of kinetochore architecture and its regulation. These findings significantly enhance our understanding of the molecular mechanisms that coordinate kinetochore assembly with cell cycle progression and ensure faithful chromosome segregation.

B516/P0499
Chromosomal core domain proteins, BAF and emerin, are required for centromere assembly
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Many proteins that are key constituents of the nuclear lamina assemble at the “core” domains of chromosomes during nuclear envelope (NE) reformation and mitotic exit. However, the identity and function of the chromosomal core domains remain ill-defined. Here, we show that a distinct section of the core domain overlaps with the centromeres/kinetochores of chromosomes during mitotic telophase. The core domain can thus be demarcated into an outer or kinetochore-proximal core on one side of the segregated chromosome mass that is close to the mitotic spindle and the inner or kinetochore-distal core on the opposite side, close to the central spindle. Based on this observation, we tested whether centromere and core assembly are inter-connected. We find that centromere assembly is markedly perturbed after the inhibition of the core-associated proteins, BAF, emerin and lamin A/C. We also find that these core proteins exhibit multiple biochemical interactions with centromeric components. Consistent with this, normal mitotic progression and chromosome segregation was severely impeded after the inhibition of the core-associated proteins. Intriguingly, the inhibition of centromere function also interfered with the assembly of BAF and emerin at the core domain, suggesting a mutual dependence of the core-associated proteins and centromeres for their assembly at the core domains. As a result, the localization of many NE components to the reassembling nuclear lamina were altered suggesting that centromere assembly and function is indeed linked to NE reformation. Finally, we find

that the localization of key proteins involved in the centromeric loading of CENP-A, including the Mis18 complex and HJURP, were markedly affected in BAF- or emerin-inhibited cells. Our findings suggest that the assembly of the core domain proximal to the centromeres serve a key function in loading new copies of centromeric proteins during or immediately after mitotic exit.

B513/P0496

Epigenetic centromere identity and nucleosome phasing is maintained through DNA replication, independent of CENP-B

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Centromeres are indispensable guardians of genome integrity as their function is essential in preventing aneuploidy, which is implicated in inherited syndromes, infertility, and cancer. CENP-A is the histone H3 variant that serves as the heritable epigenetic determinant of human centromeres. How such an essential sequence-independent epigenetic mark is maintained precisely over innumerable generations remains unclear. CENP-B, the only sequence-dependent human centromeric protein, binds a conserved 17-base-pair motif termed the CENP-B box, and has been claimed to serve a role in the phasing (positioning) of CENP-A nucleosomes. Despite being sequence dependent and its potential role in phasing CENP-A nucleosomes, CENP-B is not essential for mitosis as CENP-B null mice are viable. Here we aim to determine the role of CENP-B in CENP-A phasing and maintenance across the cell cycle. We also aim to determine if CENP-B knockout induces aneuploidy in vivo. We used CRISPR-Cas9 genome editing to knockout CENP-B in HeLa cells. Knockout of CENP-B resulted in reduced total CENP-A binding at centromeres and significantly increased doubling time. CENP-A ChIP-sequencing in cells enriched in G1 and G2 showed that CENP-A is precisely positioned between CENP-B boxes and is precisely maintained at these positions before and after DNA replication and independent of CENP-B binding. Thus, retention and phasing of CENP-A are maintained during DNA replication, independent of CENP-B. Finally, single-cell whole genome-sequencing of blood, testis and uterus cells from CENP-B null mice revealed a significant increase in DNA breaks and aneuploidy in uterus cells. Our results are the first in vivo evidence for a role of CENP-B in maintaining genome integrity. Our results may also explain the small uterus and infertility previously observed in CENP-B null females.

B512/P0495

Hybrid female sterility due to cohesin protection errors in oocytes

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Hybrid incompatibility can lead to lethality and sterility of F1 hybrids, contributing to speciation. The cell biological basis underlying hybrid incompatibility remains largely unknown, especially in mammals. Using cell biological approaches, we found that female hybrids between Mus musculus domesticus and Mus spicilegus mice are sterile due to the failure of homologous chromosome separation in oocyte meiosis I, producing aneuploid eggs. This non-separation (nondisjunction) phenotype was driven by the mis-localization of the cohesin protector, SGO2, along the chromosome arms instead of its typical centromeric enrichment, resulting

in cohesin over-protection. The upstream kinase, BUB1, showed a significantly higher activity in hybrid oocytes, explaining SGO2 mis-targeting along the chromosome arms. We further determined that the increased BUB1 activity was due to a synergistic effect of BUB1 increasing its expression level and localizing to the chromosome arms. Ectopic localization on the chromosome arms was not restricted to BUB1: all the tested kinetochore proteins were spreading to the arms, including the core centromere protein, CENP-A. Therefore, we propose that this hybrid incompatibility in cohesin protection stems from centromere mis-regulations. In contrast, BUB1 showed normal activity in mitosis, consistent with viable hybrid mice. Defective cohesion in oocytes is a leading cause of reduced fertility. Our work provides evidence that a major cause of human infertility may play a positive role in promoting mammalian speciation.

B521/P0504

Intrinsic Centromere-Driven "Spring"' Mechanism Cooperates with Microtubule Forces to Ensure Chromosome Segregation

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Faithful chromosome segregation during cell division relies on the precise interaction between sister chromatids and spindle microtubules. Although the roles of kinetochores and microtubules in mediating chromosome congression and anaphase separation have been extensively characterized, the adaptive capacity of centromeres in response to kinetochore-microtubule dynamics remains an open question. Here, through live-cell imaging with high spatiotemporal resolution, we report the discovery of "Spring"—a dynamic, intrinsic movement between sister centromeres that occurs independently of microtubule-generated forces. This phenomenon is conserved in diverse mammalian cell types and may be driven by inner centromere stress. We demonstrate that Spring works synergistically with microtubule-mediated tension to promote efficient chromosome alignment and accurate segregation. Our findings unveil a previously unrecognized mechanism by which centromeres autonomously regulate their architecture, thereby offering a new perspective on the maintenance of genomic integrity.

B520/P0503

One-step epigenetic centromere formation with a 2-Mb human artificial chromosome

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Human artificial chromosomes (HACs) are inherited through cell divisions alongside natural chromosomes, serving as tools for interrogating chromosomal elements and as vectors for large genetic cargoes. Despite recent progress, large (i.e., multiple Mb) HACs have not been reported. Further, HAC formation via epigenetic seeding of centromeric chromatin currently requires prior engineering of recipient human cells, hampering their potential deployment in many useful cell types. We report advances that address these limitations. First, we designed, built, and delivered to human cells a 2-Mb HAC-precursor YAC, which is ~3 times larger than the prior generation. Second, we developed a robust epigenetic centromere seeding approach that bypasses genetic engineering of target cells to form HACs that can be readily detected several weeks after YAC delivery. Beyond advancing HAC technologies, our findings shed light on rapid centromere acquisition through “protein-only” seeding of nucleosomes harboring the histone H3-variant CENP-A.

B511/P0494
Regulation of kinetochore assembly via the centromeric histone chaperone protein Scm3 in budding yeast
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A specialized centromeric nucleosome is required for the assembly of the kinetochore, a megadalton protein complex that is responsible for equal partitioning of chromosomes during cell division. The centromeric nucleosome forms after the deposition of the centromeric histone H3 variant to the centromeric DNA. In budding yeast, the centromeric histone variant, Cse4 is deposited by a conserved and essential histone chaperone protein, Scm3. The Ndc10 protein recruits Scm3 to the centromeric DNA to form the specialized nucleosome and thus initiate kinetochore assembly. However, it is unclear how Scm3's localization to Ndc10 is regulated to ensure accurate nucleosome assembly. We found that there are two Aurora B phosphorylation sites in the N-terminal region of Scm3 that binds to Ndc10. We therefore hypothesize that phosphorylation of Scm3 by Aurora B regulates the interaction between Scm3 and Ndc10 affecting Cse4 deposition to the centromeric DNA. To test this idea, we generated phosphomimetic and phosphodeficient mutants of Scm3 and analyzed Cse4 recruitment to centromeres using a single molecule de novo kinetochore assembly assay. We found a significant reduction of Cse4 co-localization to the centromeres in the phosphomimetic mutant in comparison to the phosphodeficient mutant, suggesting that Aurora B-mediated phosphorylation disrupts the Scm3 interaction with Ndc10. We are currently performing in vitro binding assays to examine the interaction between Ndc10 and the recombinant Scm3 mutants. We are also further characterizing these mutants through phenotypic assays in vivo to determine whether the localization of Scm3 to the centromere is phosphoregulated. Together these experiments will elucidate whether a phosphorylation event controls the recruitment of Scm3 and effects overall deposition of Cse4 leading to a regulation of nucleosome assembly.

B509/P0492
Satellite DNA shapes dictate pericentromere packaging in female meiosis
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The abundance and sequence of satellite DNA at and around centromeres is evolving rapidly despite the highly conserved and essential process through which the centromere directs chromosome inheritance. The impact of such rapid evolution is unclear. Here we find that sequence-dependent DNA shape dictates packaging of pericentromeric satellites in female meiosis through a conserved DNA-shape-recognizing chromatin architectural protein, high mobility group AT-hook 1 (HMGA1). Pericentromeric heterochromatin in two closely related mouse species, M. musculus and M. spretus, forms on divergent satellites that differ by both density of narrow DNA minor grooves and HMGA1 recruitment. HMGA1 binds preferentially to M. musculus satellites, and depletion in M. musculus oocytes causes massive stretching of pericentromeric satellites, disruption of kinetochore organization and delays in bipolar spindle assembly. In M. musculus × spretus hybrid oocytes, HMGA1 depletion disproportionately impairs M. musculus pericentromeres and microtubule attachment to their kinetochores. Thus, DNA shape affects both pericentromere packaging and the segregation machinery. We propose that rapid evolution of centromere and pericentromere DNA does not disrupt these essential processes when the satellites adopt DNA shapes recognized by conserved architectural proteins (such as

HMGA1). By packaging these satellites, architectural proteins become part of the centromeric and pericentromeric chromatin, suggesting an evolutionary strategy that lowers the cost of megabase-scale satellite expansion.

B514/P0497

Submicroscopic mapping of Plk1 in the presence and absence of CENP-U

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Proper kinetochore formation and microtubule attachment are essential for accurate chromosome segregation, a process tightly regulated by Polo like kinase 1 (Plk1). While Plk1 is known to stabilize kinetochore microtubule interactions, the full set of its molecular interactors and their contributions to kinetochore assembly remains unclear. The objective of this study is to identify Plk1 proximal proteins that contribute to kinetochore assembly and function. Plk1 has its role in mitosis through phosphorylation of multiple substrates, yet the specific mechanisms linking these phosphorylation events to kinetochore organization are not fully understood. we also investigate how loss of the inner kinetochore protein CENP-U affects the Plk1 interactome and affects kinetochore integrity. To address these questions, we employed TurboID-based proximity labeling, in which a biotin ligase (TurboID) fused to Plk1, Bub1, Kif2C and CENP-U, covalently label neighboring proteins in the presence of biotin. Biotinylated proteins were enriched using streptavidin pulldown and validated by Western blotting. Confocal microscopy further confirmed the proximally labeled proteins near the protein of interest in relation to the kinetochore. Additionally, we have developed CENP-U CRISPR-mediated knockouts. Preliminary data revealed robust labeling of proteins in the Plk1 microenvironment, including Bub1, Kif2C, and CENP-U within the kinetochore. Furthermore, Mass spec experiments with the biotinylated proteins, achieved by the pulldown assay with the streptavidin beads will generate a high-resolution map of the Plk1 interactome under both wildtype and CENP-U knockout conditions. In conclusion, TurboID-based proximity labeling enables systematic identification of previously uncharacterized Plk1-associated proteins and reveals new insights into the molecular mechanisms governing kinetochore assembly and faithful chromosome segregation. These findings have important implications for understanding mitotic fidelity and developing strategies to target chromosome segregation errors in cancer.

B518/P0501

Template matching-guided approaches to characterize chromatin clearings and the inner kinetochore of the human centromere

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Chromosome segregation is an essential biological process that ensures the faithful transfer of genetic material from parents to offspring. This process is directed by the centromere, a chromosomal locus defined by the presence of CENP-A nucleosomes, a histone H3 variant. The centromere recruits the constitutive centromere-associated network (CCAN) and, in mitosis, outer kinetochore proteins connect the chromosome to the spindle microtubules, enabling chromosome motion and segregation. Recently, we utilized cryogenic electron tomography combined with correlative light and electron microscopy to resolve the architecture of the human centromere on purified mitotic chromosomes (Kixmoeller et al., 2025, Cell). These analyses revealed that the

centromere is characterized by regions of markedly reduced nucleosome density relative to the surrounding chromatin, which we refer to as chromatin clearings. Moreover, the clearings harbor 20–25 nm nucleosome-associated complexes, which likely correspond to the CCAN subunits. Using emerging analytical tools, we have now enhanced both the accuracy and systematic rigor of our analysis by employing GAPSTOP, a GPU-accelerated framework for template matching. This image analysis tool detects structures by comparing experimental data to a reference pattern, enabling unbiased particle mapping on a much larger scale than is possible manually. We initially applied this framework to a subset of our data to evaluate its feasibility. We found that this approach is well-suited for analyzing the centromere, allowing us to build detailed maps of nucleosome localization within the tomogram. These maps allow us to estimate the density of nucleosomes throughout the imaged chromosomal region and within the centromere clearings, revealing boundaries and geometric parameters. In the future, we plan to expand this approach to aid in automated particle picking for structural determination (e.g. subtomogram averaging for determining the structure of the CCAN in the context of the mitotic chromosome). By applying this generalizable framework of automated, large-scale particle detection with molecular precision, we aim to generate precise spatial maps and systematically resolve the molecular organization of the human centromere.

B519/P0502

Tension-dependent regulation of centromere function by flanking DNA and chromatin remodelers

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Precise regulation of centromeric tension is essential for chromosome stability. In budding yeast, the conserved minimal core centromere (~118 bp) wraps around a histone octamer containing a variant of H3 (Cse4, CENP-A). Our findings reveal that the minimal core sequence (CDEI-II-III) is insufficient for proper chromosome segregation. We investigate the contribution of different lengths flanking sequences to the left and right of the 118-bp core CEN8 centromere (CDEIL and CDEIIIR). Placing CEN8-derived centromere constructs on plasmids and in the chromosome, we find that the addition of ~20 bp to the right of CDEIII was necessary for segregation function. Moreover, length of the centromere flanking regions, reflecting the amount of homology between two centromeres on a dicentric chromosome, influenced the repair pathway by which broken chromosomes were healed. We explored the requirements for centromere flanking sequences in strains lacking Yta7p, a chromatin remodeling factor that contributes to deposition of Cse4. Surprisingly, minimal core centromeres exhibited enhanced segregation function in the absence of Yta7p revealing the synergy between flanking centromere sequences and an available pool of Cse4. These results are indicative of facilitated protein dissociation in which excess Cse4 can displace bound Cse4 through multivalent interactions with the centromere DNA sequence. Our data are consistent with models that invoke force as a disrupter of facilitated dissociation, rendering the centromere DNA-protein interaction as a catch-bond; a tension-dependent interaction that strengthens under load. We suggest that the catch-bond between centromere DNA and the CENP-A nucleosome is modulated by an interaction of its flanking DNA (in cis) and chromatin remodelers (in trans) to regulate tension and ensure faithful chromosome segregation.

B515/P0498

The N-terminal Tail of Histone H4 Avoids Acetylation by MOF in the Reconstituted Core Centromeric Nucleosome Complex

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Mitosis is the foundational mechanism of growth through cell divisions. The key role of mitosis is the faithful and equal segregation of an identical set of chromosomes to the daughter cells. Chromosome segregation is facilitated by the centromere, an epigenetically defined locus, upon which the megadalton-sized kinetochore forms to provide attachment to spindle microtubules. The location of the centromere on the chromosome is defined by a histone H3 variant called centromere protein A (CENP-A) that assembles into nucleosomes along with core histones H2A, H2B, and H4. At the base of the kinetochore are the two inner kinetochore components, CENP-C and CENP-N, that directly bind CENP-A nucleosomes. These inner kinetochore components can simultaneously bind CENP-A nucleosomes, generating what we have termed the core centromeric nucleosome complex (CCNC), promoting the stable inheritance of the centromere (Guo et al., 2017, Nat. Commun.). The cryo-electron microscopy structure of the CCNC revealed the peptide backbone portion of the histone H4 tail, including a.a. 12-20, visibly sandwiched between CENP-C and CENP-N (Allu et al., 2019, Curr. Biol.). One possible outcome of this orientation is that it would be inaccessible to enzymes that modify the histone H4 tail. On the other hand, mass spectrometry of post-translationally modified (PTM) H4 co-purifying in nucleosomes with tagged, exogenously expressed CENP-A protein identified many PTMs on the histone H4 tail (Bailey et al., 2016, Mol. Cell. Proteomics). For instance, one of the most abundant combinations of PTMs involved K16ac and K20me2. This suggests that at least some CENP-A nucleosomes in the cell, possibly including those in the context of the CCNC, can undergo modification of the H4 tail. Using a reconstitution system, we test the extent to which the formation of the CCNC can impact the ability of the MOF acetyltransferase to modify one of its known key target residues: K16 on histone H4. Our initial results show that while H4K16 is readily acetylated by MOF in CENP-A nucleosomes in the absence of CENP-C and CENP-N, their assembly into the CCNC leads to a large decrease in H4K16 acetylation. In addition to providing direct evidence that inner kinetochore components can impact the PTM of core histones in centromeric nucleosomes, the system we report will be useful for structure/function analysis of the interactions that are responsible for it.

Cell Division and Cell Cycle Control - Centrosomes

B493/P0475
A KASH-less Msp300 isoform has a novel outer nuclear membrane targeting domain and scaffolds the non-centrosomal microtubule organizing center in fat body cells
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In Drosophila fat body cells, nucleus-associated non-centrosomal microtubule-organizing centers (ncMTOCs) coordinate nuclear positioning and intracellular trafficking. The nesprin homolog Msp300 anchors this perinuclear ncMTOC and recruits the microtubule minus-end stabilizer Patronin and other microtubule regulators. Among the 11 Msp300 isoforms, we show that isoform Msp300-PE is necessary and sufficient for ncMTOC formation, despite lacking a canonical KASH domain that anchors conventional nesprins to the outer nuclear membrane (ONM) through association with a SUN domain protein anchored in the inner nuclear membrane (INM). To dissect its unique function in organizing the ncMTOC, we are investigating Msp300-PE localization in fat body cells and examining the phenotypic consequences of targeted CRISPR truncation mutants, focusing on its role in microtubule organization and nuclear positioning. We demonstrate that the unique C-terminus of Msp300-PE, comprising a putative transmembrane helix followed by a 35-aa domain that resides within the nuclear intermembrane space, is sufficient to target the ONM. This finding identifies a novel ONM-targeting domain at the Msp300-PE C-terminus with no sequence similarity to the canonical KASH

domain. Notably, this short tail contains seven Cys residues that we propose form disulfide bridges with an unknown partner residing at the INM, a hypothesis supported by non-reducing SDS-PAGE. We are employing mass spectrometry and genetic screens to identify the putative INM partner required for Msp300-PE anchoring and ncMTOC function. This study aims to define the molecular mechanisms by which Msp300-PE, together with its unique domains and partners, generates a perinuclear ncMTOC in fat body cells.

B494/P0476

A Novel Mechanism for Centrosome Expulsion Ensures Metabolic Activity in Polyploid Cells

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Programmed polyploidy is often linked to increase cell size to support enhanced metabolism, barrier function or regeneration. In certain polyploid cells, the cytoskeleton is drastically remodeled- most notably by eliminating centrosomes. However, the purpose and mechanisms underlying centrosome elimination have remained unclear. We investigated this question in Drosophila acentrosomal salivary glands (SGs), a physiological polyploid model where cells reach high chromosome content through endoreplication. Using genetic tools, live imaging approaches, super-resolution microscopy combined with tissue clearing and electron microscopy, our study uncovers a novel centrosome elimination pathway in vivo. This process requires non-muscle myosin II (MyoII) activity and the macroautophagic machinery to drive centrosome release into the lumen of the salivary glands via autolysosomal exocytosis. Failure to eliminate centrosomes disrupts the mitochondria network, impairing respiration and ATP production. Our findings reveal a previously unknown mechanism that removes centrosomes through the secretory autophagy pathway to protect mitochondrial function and support the high metabolic demands of polyploid cells.

B497/P0479

A phase separation model for centrosome growth and size regulation

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Centrosomes are the primary microtubule-organizing centres of animal cells and the tight regulation of their size is crucial for accurate chromosome segregation, while oversized centrosomes are closely linked to genome instability and cancer. However, the mechanisms that ensure robust size control remain incompletely understood. We present a theoretical phase separation model that captures both the robust size regulation as well as the sigmoidal growth kinetics observed experimentally. In this model, pericentriolar material (PCM) is recruited autocatalytically at small sizes, driving rapid early growth, but recruitment saturates at larger sizes due to reaction limitations. This self-limiting behaviour establishes a local size equilibrium that depends only on the global concentration of free PCM subunits. In contrast to existing models, the resulting dynamics equalize growth across centrosomes sharing a common cytoplasm without depleting the pool of available subunits. By uniting autocatalytic assembly with reaction saturation, our model offers a simple and robust mechanism for centrosome size control and a general principle for organelle homeostasis.

B490/P0472

From centrosomes to the midbody: dynamic relocalization of optineurin during cell division

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Optineurin (OPTN), a multifunctional autophagy adaptor, is known to localize to a variety of intracellular compartments, including mitochondria, the Golgi apparatus, endosomes, microtubules, and the nucleus, reflecting its involvement in numerous cellular processes. While its roles in autophagy and vesicle trafficking are well established, emerging evidence points toward its potential function in cell cycle regulation and mitosis. Notably, its association with neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) has further intensified research into its diverse functions. Using a combination of wide-field fluorescence, confocal, and structured illumination microscopy (SIM), we found that OPTN localizes to the centrosome, specifically within the pericentriolar matrix, suggesting a direct role of OPTN in centrosome-related events. We discovered these previously unreported localizations, using three distinct human cell lines, HeLa, HEK293, and SH-SY5Y. We probed them for OPTN using centrosomal (Aurora A kinase, Cep170, γ-tubulin, pericentrin, and PCM1), mitotic spindle (β-tubulin), and contractile ring (NMIIB) markers. Observation of the centrosomal duplication process depicted OPTN's selective enrichment at the mother centriole, with a delayed recruitment to the daughter centriole. This asymmetric localization hinted towards a regulated, stage-specific role during centrosome maturation. Tracking synchronized cells through mitosis, we further found that the centrosomal signal of OPTN gradually reduced from both poles as cells progressed through mitosis and completely diminished during cytokinesis. Intriguingly, OPTN dynamically redistributes throughout the cell division processes, transiently associating with the mitotic spindle, migrating to the center of the contractile ring, and finally localizing at the midbody during cytokinesis. These dynamic, phase-specific localizations of OPTN at the right time and right place during cell division emphasize its role in maintaining mitotic fidelity, reinforcing the previous report of mitotic defects in OPTN-depleted cells. Part of this data has recently been published (https://doi.org/10.1002/cm.70015).

B491/P0473

Human Microcephaly Protein RTTN Regulates Ciliogenesis via Cul3-KEAP1-Nek2 Pathway

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Autosomal recessive primary microcephaly (MCPH) is a genetically heterogeneous neurodevelopmental disorder characterized by reduced brain size and intellectual disability, caused by mutations in multiple genes. Among these, mutations in the RTTN gene, which encodes the centrosomal protein rotatin (also known as RTTN), have been associated with lissencephaly, polymicrogyria, periventricular heterotopia, and MCPH. Our previous studies have shown that RTTN is essential for centriole biogenesis and function, including centriole assembly, mitotic progression, and proper spindle orientation. Furthermore, fibroblasts derived from patients carrying RTTN mutations exhibit shortened primary cilia, suggesting a potential role for RTTN in ciliogenesis. However, the molecular mechanism underling RTTN-regulated cilia formation and its link to brain disorders remains largely unexplored. In this study, we uncover a novel mechanism by which RTTN regulates primary ciliary formation and maintains ciliary length. Specifically, RTTN interacts with NIMA-related kinase 2 (Nek2), a known negative regulator of ciliogenesis, and modulates its protein dynamics through the ubiquitin-proteasome system (UPS). Using mass spectrometry and proteomic database analysis, we identified Cullin3

(Cul3), a component of RING-type E3 ubiquitin ligase complexes, as an RTTN-interacting protein involved in Nek2 degradation during ciliogenesis. We further identified Kelch-like-ECH-associated-protein 1 (KEAP1) as the substrate receptor responsible for targeting Nek2 to the Cul3-based E3 ligase. Functional studies revealed that depletion of Cul3 or KEAP1 results in abnormal accumulation of Nek2 and impaired primary cilia formation, phenocoping RTTN deficiency. Conversely, loss of Nek2 rescues the ciliary defects caused by depletion of RTTN, Cul3 or KEAP1, highlighting the critical role of the RTTN-Cul3-KEAP1 axis in regulating ciliogenesis via Nek2 degradation. Importantly, we demonstrate that microcephaly-associated RTTN mutant (A578P) exhibits diminished binding to Cul3 or KEAP1, resulting in Nek2 accumulation and defective ciliogenesis. In conclusion, our findings define a critical role for RTTN in primary ciliary assembly and provide mechanistic insights into how RTTN regulates ciliogenesis.

B492/P0474

Isoform expression and post-transcriptional regulation of centrosomal Plp mRNA

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Centrosome deregulation is associated with developmental disorders such as microcephaly and cancer. Despite their fundamental importance to human health, relatively little is known about the regulation of genes encoding core centrosome components, such as Pericentrin (PCNT), a conserved centrosome scaffold protein. In humans, PCNT gene deregulation results in congenital diseases such as microcephalic osteodysplastic primordial dwarfism type II (MOPD II) and Trisomy 21. Similarly, deregulation of the Drosophila homolog gene, Pericentrin-like protein (Plp), leads to diverse defects, including embryonic lethality, neuron dysfunction, and male sterility. The mechanisms underlying these pleiotropic phenotypes are incompletely understood. Plp is predicted to encode 12 mRNA isoforms, but the mechanisms generating these distinct mRNA species and how their expression is spatiotemporally regulated are completely unknown. Through bioinformatics and qPCR analysis, we uncovered two distinct tissue-specific Plp mRNA isoforms specifically enriched in Drosophila embryos and larval brains. Using genome engineering, we successfully knocked down the larval brain tissue-specific isoform, and we are exploring the necessity and sufficiency of this tissue-specific Plp isoform. In addition, our recently published data reveal that the bulk Plp mRNA is localized to centrosomes in embryos, requiring engaged ribosomes and a robust PCM scaffold. We recently built transgenic flies expressing the embryo tissue-specific isoform and will explore this isoform-specific mRNA localization and the regulatory mechanisms. This work will advance our understanding of the post-transcriptional regulation and function of Plp mRNA isoforms and lend mechanisms insights that may be involved in the etiology of human diseases.

B486/P0468

Mechanisms of Aurora A Recruitment and Activation at Centrosomes

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Progression through mitosis requires tightly regulated action of mitotic kinases within a narrow temporal window. The oncogenic mitotic kinase Aurora A (AurA) is a central mitotic regulator that controls mitotic entry, spindle assembly, and chromosome segregation. AurA is present in several distinct pools associated with different regulators. During mitosis in the C. elegans one-cell embryo, AurA localizes to the pericentriolar material (PCM) matrix, likely in association with the PCM matrix scaffold protein SPD-5 and its associated

protein SPD-2 (orthologs of human CDK5RAP2 and CEP192). AurA also emanates out from centrosomes in a gradient along spindle microtubules, a pattern requiring interaction with its activator TPXL-1, a distant ortholog of vertebrate TPX2. Disrupting TPXL-1's ability to bind AurA leads to a spindle assembly defect, but AurA's PCM localization and roles in mitotic entry and centrosome maturation are unaffected. Loss of the RSA PP2A complex, which localizes to the PCM 1, also leads to a spindle assembly defect that phenocopies disruption of the TPXL-1–AurA interaction. These results suggest that the RSA complex on the PCM matrix promotes generation of active AurA—TPXL-1 complexes that diffuse out of the centrosome to promote spindle assembly. To test this idea, we extended prior work to define AurA and RSA complex binding sites in the PCM matrix scaffold protein SPD-5. Interestingly, the Alphafold3-predicted interface of AurA with SPD-5 overlaps with the predicted interface of RSA-2 with SPD-5. Deletion of a small region of SPD-5 containing the predicted interface residues disrupted both RSA-2 and AurA binding (yeast 2-hybrid), and resulted in a spindle assembly defect that resembles loss of the AurA–TPXL-1 interaction. We are also analyzing the relationship between the RSA complex and TPXL-1, which were shown to interact in a prior proteomic analysis. Based on the results to date, our working model is that SPD-5 recruits both AurA and the RSA PP2A complex. The RSA PP2A complex, in turn, recruits TPXL-1 to locally assemble active AurA–TPXL-1 complexes, potentially in an RSA PP2A activity-dependent manner, that diffuse out from the centrosomes and direct spindle assembly.

B495/P0477

Orb2 is required for neural stem cell centrosome and cell size asymmetries

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Neural stem cells (NSCs) orchestrate repeated rounds of asymmetric cell division to pattern the developing brain with neurons and glia. Each cell cycle, the NSCs differentially segregate cell fate determinants to regenerate one larger, self-renewing stem cell and a smaller daughter cell fated for differentiation. Prior work by our group and others indicates the asymmetric cell division of NSCs is regulated, in part, by the asymmetric activities of the NSC centrosomes. We determined that Drosophila Orb2, a cytoplasmic polyadenylation element binding (CPEB) protein involved in post-transcriptional regulation, supports NSC centrosome asymmetry and brain growth, but the mechanisms behind these phenotypes remain incompletely understood. We found that Orb2 is required for the transient inactivation of the NSC basal centrosome in the third larval instar. Moreover, we discovered steady state protein levels of the master regulator of centrosome activation, Polo kinase, are significantly decreased in orb2 mutant brains. Consistently, live imaging reveals a significant reduction of Polo localization to orb2 NSC centrosomes. We are currently testing whether downstream centrosome maturation effectors compensate for reduced Polo, leading to basal hyperactivation. In addition to impaired centrosome asymmetry, orb2 NSCs are significantly smaller than controls, highlighting a defect in daughter cell size asymmetry. Live imaging shows orb2 specifically regulates daughter cell size asymmetry near the onset of cytokinesis. Our current efforts are examining whether altered distributions of cortical myosin underlie a more symmetric positioning of the cytokinetic furrow. Additionally, we are using MARCM clones to determine if the proliferative capacity of orb2 NSCs is compromised. Taken together, this work supports a model whereby Orb2 contributes to NSC asymmetry required for proper neurodevelopment.

B487/P0469

Reconstituting centriole and centrosome assembly on synthetic beads

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Centrosomes are membraneless organelles composed of a central pair of highly structured centrioles surrounded by an amorphous matrix of pericentriolar material (PCM). Despite comprising several hundred different proteins, centrosomes can assemble rapidly and precisely in time with cell-cycle events. Here we reconstitute centrosome biogenesis on synthetic beads that recruit one of several core centrosome assembly proteins when injected into Drosophila embryos. Remarkably, these beads generate structures that are functionally indistinguishable from centrosomes: they separate from the bead surface, organise PCM and microtubules (MTs), and proceed through multiple rounds of high-fidelity duplication, all in synchrony with the endogenous centrosomes. The beads do this by recruiting PLK4 kinase, the master regulator of centrosome biogenesis, in a spatially and temporally regulated manner that mimics PLK4's recruitment to centrosomes. These findings show that simply recruiting a key protein kinase in the right way is sufficient to drive the biogenesis of a highly complicated organelle.

B489/P0471

SPD-5 and the centriculum exhibit asymmetric disassembly after metaphase in the early c. elegans embryo

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Accurate chromosome segregation and nuclear positioning are essential for proper development and are implicated in disease such as cancers. These processes are controlled by the centrosome, an organelle that nucleates microtubules from its pericentriolar material (PCM). Microtubules are dynamic polymers that partition chromosomes and position nuclei during cell division. In embryonic cells of certain organisms, accumulations of endoplasmic reticulum around centrosomes have been observed. In the 1-cell C. elegans embryo, this membranous structure, termed the centriculum, was shown by our lab to affect centrosome structure and function. So far, the centriculum has only been studied in detail at metaphase; it is unclear what happens to the centriculum once chromosomes have been successfully segregated, the topic of this poster. The two centrosomes and centricula exhibit different behaviors after metaphase. In both centrosomes, a PCM protein, SPD-5, forms a ringlike shape that expands until telophase and ultimately dissociates into puncta. The SPD-5 ring is bounded by the centriculum, which mirrors the shape of SPD-5. On the anterior side, the centriculum is a sphere, while the posterior SPD-5 and centriculum are notably stretched perpendicular to the anterior-posterior axis, forming a disc-like shape. Additionally, the anterior centriculum persists longer, disappearing during the two-cell stage, 7 minutes post-metaphase, while the posterior centriculum disappears earlier: 5 minutes post-metaphase. Likewise, SPD-5 in the posterior centrosome appears to disassemble faster than in the anterior centrosome. Posterior SPD-5 forms puncta during disassembly that fully disappear 7 minutes post-metaphase. Anterior SPD-5 puncta disappear only 9 minutes post-metaphase. We are currently investigating what drives this asymmetry, and whether and how the centriculum affects PCM disassembly. Finally, the method of tracking SPD-5 appears to affect its behavior after metaphase. TagRFP-T::SPD-5 and transgenic GFP::SPD-5 (in the presence of endogenous untagged SPD-5) appear to behave the same as untagged SPD-5 (by immunofluorescence), while several different endogenous GFP::SPD-5 constructs fail to form elongated patterns (either as rings or as puncta). This observation highlights the importance of choosing the right marker to study protein behavior in dynamic processes.

B496/P0478

The DNA damage response pathway is required for multiciliated cell differentiation

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DNA damage can result from external sources or occur during programmed genome rearrangements in processes like immunity or meiosis. To maintain genome integrity, cells activate DNA repair pathways that prevent harmful outcomes such as cancer or immune dysfunction. In this study, we uncover a novel role for DNA damage during the terminal differentiation of multiciliated cells (MCCs). MCCs, which line the airways, reproductive tracts, and brain ventricles, produce hundreds of motile cilia, each anchored by a centriole. Therefore, MCCs must generate hundreds of centrioles during differentiation. Normally, centriole duplication is tightly linked to the S and G2 phases of the cell cycle, raising questions about how MCCs override numerical and temporal restrictions on centriole duplication. We find that differentiating MCCs accumulate extensive double-stranded DNA breaks during centriole amplification, with DNA damage levels correlating to centriole number. DNA damage response (DDR) kinases are essential for supporting centriole biogenesis and ciliogenesis. We also observe that transcriptional activity, required for the expression of centriole and cilia genes, produces RNA-DNA hybrids (R-loops) that co-localize with DNA damage. This suggests that transcription-coupled DNA damage helps MCCs bypass cell cycle rules to permit centriole amplification without triggering full S/G2 phase processes. Our findings indicate that MCCs harness DDR signaling as part of their developmental program, revealing a broader principle in which the canonical cell cycle is adaptively rewired during differentiation.

B488/P0470

Tight Junction Protein ZO-2 Functions as a Scaffold at the Centriole and Mitotic Spindle Poles that Allows the Proper Development of Mitotic Spindles and Cilia

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Several tight junction (TJ) proteins are found in the centrosome, prompting us to investigate whether this also applies to Zonula occludens 2 (ZO-2). Using Western blotting, confocal microscopy, and SIM (structured illumination microscopy), we examined the co-localization of ZO-2 with proteins found in various regions of the centriole. In epithelial MDCK cells, ZO-2 was observed to co-localize with CEP164 in the distal appendage of the mother centriole, at the basal body of primary cilia, as well as in the tails of human and mouse spermatozoa. The absence of ZO-2 altered the cellular content of centriolar proteins CEP164, centriolin, and CEP135, but did not affect centriole morphology studied by TEM. ZO-2 was also found at the mitotic spindle poles, where it acts as a scaffold for KIF14 and the NuMA/pAurora-A/TPX2 complex. Aurora-A, NuMA, and KIF14 co-immunoprecipitated with ZO-2, and pull-down assays revealed direct interactions with NuMA and Aurora-A. At the mitotic spindle poles, silencing ZO-2 causes the accumulation of NuMA condensates while reducing the levels of pAurora and the contents of TPX2 and KIF14. This misregulation of protein accumulation at the spindle pole, combined with the microtubule instability observed in the mitotic spindle, results in a shorter, disoriented spindle. In quiescent cells, the effect of ZO-2 depletion on the levels of several proteins at the ciliary basal body and primary cilium formation was also studied. It was found that ZO-2 depletion led to the

accumulation of pAurora and p-AKT at the ciliary basal body, while the levels of CEP164, the intraflagellar transport protein IFT57, and kinesin KIF14 decreased, inhibiting primary cilia development. IFT57 was further found to co-immunoprecipitate with ZO-2 in quiescent cells. In summary, these results indicate that ZO-2 is not a centrosomal architectural component but rather enhances microtubule stability and functions as a platform for the proper accumulation of proteins at the spindle pole, centriole, and ciliary basal body, thereby supporting the formation of proper mitotic spindles and cilia.

Cell Division and Cell Cycle Control - Chromosome structure and segregation

B522/P0505
Satellite DNA sequence dictates pericentromere heterochromatin formation and function
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Pericentromeres are heterochromatic regions adjacent to centromeres that ensure accurate chromosome segregation. Despite their conserved function, they are composed of rapidly evolving A/T-rich satellite DNA. To test the functional consequences of this rapid sequence evolution, we establish hybrid mouse embryos as a model system to compare divergent satellite arrays from distinct species in a common cytoplasm. We show that variation in satellite sequence impacts heterochromatin formation, recruitment of the Chromosome Passenger Complex (CPC), and interactions with the mitotic spindle. Leveraging recent long-read assemblies of Mus musculus and Mus pahari satellite arrays, we find that differences in satellite sequence between these two species alters their recognition and packaging by Polycomb Repressive Complex 1 (PRC1). Specifically, M. musculus major satellites are enriched for specific A/T-sequences (i.e. AAAT) that the AT-hook of the PRC1 complex preferentially binds whereas M. pahari pi satellites are not, reflecting differences in PRC1 packaging between these two species. PRC1 packaging of a third species pericentromeres (i.e. Mus caroli) led us to identify a novel AAAT-rich satellite DNA (“59 bp satellite”) using a combination of short-read analysis, dCas9 localization and long-read de novo genome assembly. Furthermore, PRC1 heterochromatin modifies pericentromere function by inhibiting recruitment of the CPC, increasing microtubule forces on kinetochores during mitosis. Our results provide a direct link between satellite DNA composition and mitotic chromosome behavior and highlight early embryogenesis as a critical point in development that is sensitive to satellite DNA evolution.

B523/P0506
Transcripts retention by Transcription Termination Factor 2 impairment results in defective chromosome alignment and segregation
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Mitotic chromatin is generally considered incompatible with transcription due to changes in both the transcription machinery and chromosome architecture. However, emerging evidence indicates that a specific mechanism actively ensures transcriptional silencing upon entering mitosis. Despite this understanding, the precise machinery involved and its interaction with chromosome segregation remain largely elusive. Our research, along with that of others, has revealed that the Transcription Termination Factor 2 (TTF2) plays a crucial role in silencing transcription at the onset of mitosis. When TTF2 is depleted, cells exhibit abnormal retention of RNAs on mitotic chromatin; however, the implications of this retention for mitotic progression are

still unclear. In this study, we demonstrate that depletion of TTF2 in HeLa cells leads to a slight delay in mitosis due to improper alignment of chromosomes at the metaphase plate. This misalignment results in asynchronous movements of chromosomes during anaphase. We also observe defects in chromosome segregation, especially characterized by DNA bridges, which stem from inaccurate decatenation of chromosomes in anaphase. Our findings define TTF2's essential role in mitotic transcriptional silencing for proper mitotic fidelity; furthermore, it contributes to our understanding of how abnormal retention of transcripts can affect the function of chromosome structural elements.

Cell Division and Cell Cycle Control - Meiosis

B596/P0577
A Ring-Shaped Chromosome-Associated Protein Complex Promotes Proper Microtubule Organization in C. elegans Oocytes
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Oocytes undergo meiosis, a specialized form of cell division that reduces genetic content by half, mediated by a microtubule-based spindle. Unlike most cells, oocytes assemble their spindles in the absence of microtubule organizers called centrosomes. In C. elegans oocytes, microtubules form lateral bundles alongside homologous chromosome pairs (bivalents) that guide chromosome movements. Moreover, a ring-shaped protein complex (RC) forms at the interface between the two homologs, and this complex is critical for bivalent alignment. We previously found that depletion of the kinase AIR-2Aurora B disrupts RC assembly and causes severe spindle assembly defects, suggesting that the RC may also promote proper microtubule organization. We are therefore investigating the relationship between lateral bundles and the RC to determine if this unique microtubule organization is dependent on the RC. To assess this, we utilized a him-8 mutant, in which the X chromosomes fail to pair, resulting in oocytes containing 5 bivalents with RCs and 2 univalents lacking RCs. We analyzed these mutants on monopolar spindles, to better visualize lateral bundles by eliminating the microtubule overlap zone found in bipolar spindles. This analysis revealed that univalents lacking RCs do not have lateral bundle associations and remain stuck at the monopole. We confirmed this finding using spo-11 mutants, in which double-strand breaks fail to occur, resulting in 12 univalents. Interestingly, only a subset of the univalents recruit RCs in this mutant, and we found that the univalents with RCs had lateral bundles running alongside them, while the univalents without RCs did not. This suggests that RCs promote the formation of lateral microtubule bundles. To further test this hypothesis, we attempted to acutely remove RCs from bivalents and determine if there was an impact on microtubule organization. We therefore acutely depleted AIR-2 using the auxin-inducible degradation system. Upon rapid AIR-2 removal from oocytes, we found the multiple RC components were no longer localized at the midbivalent region, suggesting that the RC was disassembled. Importantly, under these conditions, the spindle began to lose its integrity, suggesting that AIR-2, and potentially other RC components, are required for spindle stability. Altogether, these findings support the view that the RC has a role in generating and maintaining proper spindle architecture in oocytes, revealing important new roles for this protein complex.

B602/P0583
Automated image analysis and applications to the study of meiotic recombination
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Pairing and crossing over between homologous chromosomes is required for their accurate chromosome segregation at the first division of meiosis. Defective crossing over is associated with pregnancy miscarriage, infertility, and congenital disease. Crossing over results from the programmed formation and repair of DNA double-strand breaks via homologous recombination. A major approach to characterizing the steps of meiotic recombination is immunostaining of recombination factors along prophase-I chromosomes. Accurately quantifying recombination foci and chromosome lengths are critical components of this analysis. However, manual approaches are laborious and error prone; and existing automated methods have significant limitations. To address these limitations, we are developing a suite of automated image-analysis tools that streamline and standardize the analysis of immunostained prophase-I chromosomes. Our pipeline incorporates a combination of advanced statistical strategies with unsupervised machine learning to analyze a diversity of recombination markers, enabling rapid and reproducible quantification of focus number, position, and intensity. Importantly, we introduce and validate an innovative chromosome length–measurement method that establishes a completely new approach to quantifying 2D linear objects that is especially well suited to measuring meiotic prophase-I chromosomes. For the analysis of recombination foci, our approach diminishes observer bias by establishing a consistent and objective framework, offering the potential to become a new standard for quantification across studies. Together, these advances make the immuno-cytological analysis of meiotic prophase-I faster, more efficient, and more reliable, paving the way for broader adoption of automated approaches in meiosis research and beyond.

B593/P0574

DDX4 and HDLBP Regulate the Progression of Meiosis through Translational Regulation of Emi2 mRNA in Mouse Oocyte.

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In vertebrates, oocytes store more than ten thousand maternal mRNAs in dormant states during oogenesis. Since the transcription becomes quiescent after initiation of oocyte maturation, proteins crucial for the progression of oocyte maturation are synthesized from mRNAs stored in oocytes. EMI2 protein is synthesized from Emi2 mRNA at the end of meiosis I and is essential for the arrest of meiotic cell cycle at the metaphase of meiosis II (Takei et al., 2021). We have identified TDRD3 protein as a regulator of Emi2 mRNA translation. In TDRD3-deficient oocytes, EMI2 was only slightly synthesized and the meiotic cell cycle was not arrested at metaphase II, and instead the oocytes entered the cleavage stage without fertilization. However, the molecular mechanism by which the translation of Emi2 mRNA is regulated remains unclear. By performing an RNA pull-down assay, we have isolated DDX4 and HDLBP proteins as candidates for Emi2 mRNA-binding proteins. In this study, we aimed to elucidate how these proteins were involved in the translational regulation of Emi2 mRNA. First, we examined the distribution pattern of DDX4, HDLBP, and TDRD3 in mouse oocytes by immunofluorescence. All these proteins formed aggregates in the cytoplasm. We then analyzed the interactions of DDX4 and HDLBP with Emi2 mRNA in vivo by immunoprecipitation followed by RT-PCR. Emi2 mRNA was detected in the precipitations with the anti-DDX4 and anti-HDLBP antibodies. Next, we assessed interaction among these proteins and Emi2 mRNA by in situ hybridization and immunofluorescence. These proteins formed RNA-binding protein complexes (RBP-complexes) including Emi2 mRNA. To assess the functions of DDX4 and HDLBP in the progression of meiosis and Emi2 translation, we knocked down DDX4 and HDLBP in immature oocytes. Both DDX4- and HDLBP-deficient oocytes exhibited abnormal meiosis such as multipolar spindles and parthenogenesis after resumption of meiosis. In addition, the translational activity of Emi2 mRNA decreased in DDX4- and HDLBP-deficient oocytes. Finally, we investigated the structures of the

RBP-complexes in DDX4-deficient oocytes. The TDRD3-Emi2 colocalization was disrupted in DDX4-deficient oocytes. These results suggest that DDX4 and HDLBP regulate the progression of meiosis cooperatively with TDRD3 through the translational regulation of Emi2 mRNA.

B595/P0576
Essential roles of BRCA2 in spermatogenesis and oogenesis
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BRCA2 (FANCD1) is essential for gamete formation and human alleles have been linked to early menopause. However, its functions in fertility remain poorly defined relative to those in somatic cells. BRCA2 plays a central role in homologous recombination, a chromosome-repair process that is defective in many cancers and is essential for meiosis, the specialized cell division that produces eggs and sperm. The major function of BRCA2 is to load the homologous pairing and DNA strand-exchange protein RAD51 onto the ends of broken DNA molecules. To understand BRCA2 functions in fertility, we have exploited a conditional allele of the Brca2 gene in mouse to inactivate its mediator function at different stages of gametogenesis in spermatogonia (stem cells), spermatocytes, and oocytes. Inactivation in spermatogonia reveals an essential function for BRCA2 in the proliferation and differentiation of germline stem cells. When Brca2 is inactivated early in meiosis, assembly of strand-exchange complexes is defective and the early events of meiotic prophase-I fail, including chromosome pairing and synapsis. These observations are consistent with an essential role for BRCA2 in loading RAD51 and DMC1 (the meiosis-specific RAD51 ortholog) onto DNA ends. Later inactivation of BRCA2 mediator function reveals an unanticipated function after initial strand exchange and chromosome synapsis have been achieved. At this stage, BRCA2 mediates the de novo loading of RAD51, but not DMC1, to complete the repair of a subset of ~40 residual DNA breaks. Without BRCA2 mediator function, these breaks persist and become excessively resected. Surprisingly, in spermatocytes, these persistent DNA lesions do not impede the progression of meiosis, and repair is eventually completed during diakinesis/metaphase I. Sperm are produced efficiently but fertility is low suggesting that aberrant DNA repair may cause lethal mutations. Consistently, genome sequencing reveals the formation of de novo mutations, including large deletions, in the progeny of Brca2 mutant fathers. By contrast, in females, Brca2 mutant oocytes undergo apoptosis revealing a sexually dimorphic response to BRCA2 dysfunction and DNA damage arising during meiotic prophase I. Unexpectedly, BRCA2 dysfunction also disturbs the patterning of crossovers – the exchanges between homologous chromosomes that are essential for their accurate segregation. We conclude that BRCA2 functions throughout gametogenesis and mediates distinct modes of DNA repair during the early and late stages of meiosis. Our analysis also reveals sexually dimorphic responses to BRCA2 dysfunction with implications for gametogenesis and germline mutation.

B597/P0578
Examining the Roles of Centrosome Components in Acentrosomal C. elegans Oocyte Meiosis
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Meiosis is a specialized form of cell division used to generate haploid gametes. Interestingly, female (oocyte) meiosis typically occurs in the absence of centrosomes, which are microtubule organizing centers that facilitate spindle assembly in many other cell types. How microtubules nucleate, form a bipolar spindle, and properly segregate chromosomes in this acentrosomal system is still poorly understood. We are probing this question

using C. elegans as a model system, by investigating whether conserved centrosomal components also play roles in oocyte meiosis. One candidate that we are exploring is γ-tubulin, whose function in mitosis is primarily attributed to the nucleation of microtubules. Previous work utilizing RNAi to deplete γ-tubulin suggested that this protein is not essential for spindle assembly or general meiotic progression in C. elegans oocytes1,2. However, we are leveraging the auxin-inducible degradation system, which could potentially provide a more complete level of depletion, to revisit potential roles for γ-tubulin in spindle assembly and maintenance. Our preliminary results indicate a faint localization of γ-tubulin to the meiotic spindle, with depletion of this protein resulting in disorganized, multipolar, and collapsed spindle structures. Strikingly, a subset of oocytes also contained 12 chromosomes instead of the expected 6 bivalents, indicating a polar body extrusion failure during the first meiotic division, and we also observed some oocytes with mispositioned spindles. We then examined SPD-5, another centrosome-associated protein. SPD-5 also appeared to localize faintly to the meiotic spindle, with auxin-induced depletion yielding aberrant spindle structures. Since it has been shown that SPD-5 is required for γ-tubulin accumulation at centrosomes during C. elegans mitosis, and that the SPD-5 homolog CDK5RAP2 interacts with the γ-tubulin ring complex to activate microtubule nucleation3,4, we are currently investigating whether a functional relationship between these proteins exists in C. elegans oocyte meiosis. The relationships between these proteins and other known nucleation factors are also being explored. 1.)McNally et. al. (2006), JCB 2.)Chuang et. al. (2020), Biol Open 3.)Xu et. al. (2024), Dev Cell 4.)Choi et. al. (2010), JCB

B594/P0575

Illuminating actin-driven mechanisms of chromosome segregation in mouse oocyte meiosis

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Accurate chromosome segregation in oocytes is fundamental for the formation and development of healthy embryos. Chromosomes are segregated using a microtubule-based spindle apparatus. We previously discovered that a unique actin-based structure (spindle F-actin) underpins chromosome segregation in oocytes by mediating robust chromosome-microtubule connections. More recently, we revealed that spindle F-actin also prevents premature separation of sister chromatids in eggs of reproductively older females, providing a new explanation for the molecular origins of aneuploidies associated with human infertility and genetic conditions. While these findings have advanced our understanding of chromosome segregation mechanisms in female meiosis, how spindle actin is assembled and exerts its function at the chromosome-microtubule interface remains a mystery. To address this, we are combining advanced microscopy of female meiosis with targeted protein degradation strategies to test candidate spindle actin assembly factors in mouse oocytes. Our emerging studies highlight a potential role for Septins, a conserved family of GTP-binding proteins, in regulating actin organization during oocyte meiosis. We are now building on these data to uncover how spindle actin assembly is controlled by Septins and how this regulation safeguards chromosome segregation in female meiosis.

B599/P0580

Mechanistic diversity in the egg spindle positioning across mammalian species

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Female meiotic cell division is highly asymmetric in animal species, producing a large egg and small polar bodies to preserve maternal storages essential for embryogenesis. To achieve asymmetric division, the meiotic spindle must maintain its cortical localization during meiosis. Previous studies using standard lab mice, Mus musculus, demonstrated that cytoplasmic streaming exerts pushing forces on the spindle to maintain the cortical spindle localization. This is in stark contrast to mitotic spindle positioning, which is regulated by the interactions between the cortex and astral microtubules emanating from the spindle poles. Mechanisms of spindle positioning in other mammalian eggs have not been reported, and therefore, it remains unknown whether the cytoplasmic streaming mechanism is a common strategy across mammalian species. To understand the diversity and conservation of spindle positioning mechanisms in mammalian eggs, we initially focused on Peromyscus mice, which are 25 million years apart from lab mice (note that lab mice and rats are 10 million years apart). We discovered four distinct features in Peromyscus mice from lab mice. First, spindle positioning was independent of cytoplasmic streaming. Second, mitosis-like astral microtubules emanated from the spindle poles. Third, plus-ends of growing astral microtubules reached and stayed at the cortex, indicating astral microtubules anchoring to the cortex. Forth, LGN, which is responsible for the cortex-astral microtubule interactions in mitosis, localized at the cortex overlaying the egg spindle. Collectively, these data suggest that Peromyscus eggs maintain the cortical spindle position by anchoring astral microtubules to the cortex, while lab mice use cytoplasmic streaming forces to do so. We are currently expanding our analyses to include human, bovine, and porcine eggs to determine how pervasive each mechanism is in mammals.

B598/P0579

Microtubule Severing by Katanin Effects Force Generation for Meiotic Chromosome Segregation

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Female meiosis is critical for sexual reproduction. Chromosome missegregation during meiosis is linked with infertility and developmental abnormalities. Chromosome segregation is driven by the meiotic spindle, a dynamic, self-assembling structure composed of microtubules (MT), motor proteins, microtubule-binding proteins, and regulatory factors. Unlike spindles in most animal cells, female meiotic spindles often lack centrosomes, the traditional microtubule organizing centers. Understanding how meiotic spindles form and function without centrosomes is essential. We hypothesize that changes in microtubule length, turnover rates, and organization are pivotal determinants of spindle assembly, function and thus chromosome segregation during female meiosis. To better understand how these changes influence the spindle, we examine the protein katanin. Katanin is a MT severing protein that mechanically cuts the MT. Without katanin, a functional spindle is unable to form. Previous data has suggested that MT severing by katanin is important for promoting the MT remodeling during meiosis ensuring the proper formation and function of the meiotic spindle. Electron tomographic data of meiotic spindles from our lab suggests that katanin prevents MTs in the vicinity of chromosomes from growing too long by selectively cutting those MTs to enhance turnover and possibly to maintain the metaphase spindle. Based on this, we hypothesize that katanin is critical for managing the length of MTs to control the forces that align and segregate chromosomes. As an example, spindles might be composed of longer MTs in the absence of katanin, which could affect how MTs are cross-linked within the spindle network and thus support different polymerization and sliding forces negatively affecting chromosome motion. To examine the role of katanin and the importance of regulating MT length we will use a combination of light microscopy, large-scale electron tomography, and mathematical modeling of the female meiotic C. elegans spindle. This will enable us to quantify changes in MT length, interaction, and arrangement in response to katanin and its effect on force generation and thus spindle structure and function.

B603/P0584

Pathways Driving Sister Kinetochore Co-orientation in Meiosis I

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Errors in chromosome segregation during oocyte meiosis drive aneuploidy, a major cause of infertility and pregnancy loss in humans. Achieving a euploid egg requires two successful meiotic divisions, a process coordinated by kinetochores. During meiosis I, sister chromatid kinetochores orient toward the same spindle pole in a process called co-orientation, while homologous kinetochores orient toward opposite poles to undergo reductional segregation. It is established that co-orientation is regulated by a meiotic kinetochore protein, Meikin/Spo13/Moa1. Here, we explore if chromatin organization underlying the centromere also facilitates co-orientation, in addition to the kinetochore pathway. We exploit species divergence in centromeric satellite organization across five Mus species that significantly impacts centromeric chromatin during meiosis I (El Yakoubi and Akera, 2023). We focused on the differential centromeric localization of Topoisomerase-IIα (TOP2A) across species, which can impact the degree of inter-sister catenation at the centromere. We tested whether the degree of co-orientation is associated with centromeric TOP2A levels by measuring inter-sister kinetochore distance in metaphase I across species. Sister kinetochore distance varied by species, from largely unseparated sisters in M. m. domesticus to highly separated sisters in M. spretus. Across five Mus species, centromeric TOP2A intensity showed a negative correlation with inter-sister kinetochore distance ($R2 = 0.18$, $p = 0.0001$). Our preliminary data suggests that co-orientation of sister kinetochores could also be regulated by a TOP2A-mediated chromatin-based pathway, possibly catenating sister centromeres. Since the centromeric TOP2A level is dictated by major satellite repeat abundance, this study may reveal a new role of centromeric satellites in meiotic segregation through promoting co-orientation.

B600/P0581

Spindle-localized F-actin regulates MTOC organization to ensure meiotic spindle fidelity

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Female gametes are generated through meiosis, a process highly prone to chromosome missegregation that leads to aneuploidy— the primary genetic cause of miscarriage and congenital disorders such as Down syndrome. Faithful chromosome segregation depends on the spindle apparatus, which safeguards genomic integrity during cell division. In contrast to somatic mitotic cells, mouse oocytes lack centriole-containing centrosomes. Therefore, bipolar spindle assembly relies on the self-organization of numerous acentriolar microtubule organizing centers (MTOCs) into two focused poles. Accordingly, following nuclear envelope breakdown, MTOCs undergo two critical steps to assemble a bipolar spindle with focused poles: 1) MTOC clustering (MTOC-MTOC aggregation) and 2) MTOC sorting (i.e., MTOC distribution towards the poles). The traditional view was that MTs, and their associated proteins, are the sole cytoskeletal elements responsible for MTOC organization and spindle pole focusing. However, in mammalian oocytes, F-actin is also localized to the

spindle through entirely unknown mechanisms. To date, no selective tool has been available to perturb spindle-localized F-actin, leaving its biological significance– and whether it contributes to spindle morphogenesis–largely unresolved. Using super-resolution STED microscopy, we identify novel F-actin “cages” that surround MTOCs. We show that the recruitment of F-actin to the spindle and its enrichment at MTOCs is regulated by overlapping functions of the unconventional myosins, Myosin X and Myosin VIIb, which harbor both MT and F-actin binding domains. Depleting Myosin X/Myosin VIIb using siRNAs or perturbing Myosin X function using dominant-negative mutants reduced spindle-localized F-actin and resulted in MTOC clustering and sorting defects, unfocused spindle poles and chromosome missegregation. To further confirm F-actin role with spatial precision, we optimized a novel photoswitchable jasplakinolide tool, Optojasp, to manipulate F-actin dynamics in the oocyte with micrometer resolution upon 405 nm light exposure. Importantly, perturbing F-actin dynamics at either the spindle or the spindle poles phenocopied the MTOC/spindle defects and chromosome segregation errors observed in myosin-perturbed oocytes. Together, our findings establish a previously unrecognized role of spindle-localized F-actin in organizing MTOCs, thereby ensuring the fidelity of meiotic spindle formation and faithful chromosome segregation. This work highlights spindle-localized F-actinas an essential component of spindle integrity in oocyte meiosis, a process notoriously prone to errors.

B601/P0582

Wee1 is required for proper chromosome segregation in C. elegans spermatogenesis

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In the United States, unexplained infertility accounts for approximately 15% of infertility cases. Defects in eggs or sperm production can result in infertility. Meiosis, the specialized type of cell division that produces eggs and sperm, is a complex process that is tightly regulated. This regulation ensures that these gametes are functional. Though similar, female and male meiosis are distinct processes that are differentially regulated. One key difference between the two types of meiosis is the timing of meiotic entry. Oocytes typically arrest during meiosis in a species-specific manner, while spermatocytes proceed through both meiotic divisions. The kinase Wee1 is a critical regulator of M-phase entry. Wee1 inhibits the Cdk1/Cyclin B complex to prevent cells from dividing prematurely. While this regulatory mechanism is required for mitosis, as well as both male and female meiosis, it is clear that Wee1 differentially regulates these processes. In Caenorhabditis elegans spermatogenesis, several gain-of-function (gof) mutations in wee-1.3 cause primary spermatocyte arrest, while oogenesis and mitosis are unaffected, indicating a distinct role for Wee1 in spermatogenesis regulation. The objective of this research is to investigate how Wee1 differentially regulates spermatogenesis compared to oogenesis. To do this we are using auxin-inducible degradation to selectively deplete Wee1 from the C. elegans male germline. High-resolution imaging of both fixed and live samples reveals that Wee1 depletion induces severe chromosome segregation defects during spermatogenesis. Defects include DNA fragmentation and chromatin bridge formation during meiosis I and II. These defects are due in part to errors in spindle assembly, as Wee1 depletion also disrupts centrosome numbers and the organization of spindle microtubules at metaphase I. These phenotypes are distinct from Wee1 depletion during oogenesis. Examining Wee1 localization in the male germline revealed a perinuclear localization pattern throughout meiotic prophase. Once spermatocytes reach metaphase I, Wee1 localization shifts to the centrosomes, suggesting a role for Wee1 in organizing the spindle during male meiosis. Future work will test the hypothesis that Wee1 regulates centriole duplication, determine how Wee1 regulates other components of M-phase entry, and identify spermatogenesis-specific Wee1 interactors.

Cell Division and Cell Cycle Control - Mitosis

B548/P0531
A non-canonical mitotic mechanism in the pre-implantation mouse embryo
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During preimplantation development, a single copy of the genome must be propagated through numerous rounds of mitotic cell division. Mitotic errors, if uncorrected, frequently result in aneuploidy, a condition defined by abnormal chromosome number. Aneuploidy represents a major source of congenital defects. Therefore, elucidating the complete set of mechanisms required for mitotic fidelity has remained a longstanding goal of developmental cell biology. The classical view of mitosis is that the spindle apparatus maintains principal control of chromosome organization. However, the early mouse embryo does not possess centrosomes, the organelles responsible for fast and efficient spindle assembly. Thus, the acentrosomal spindles of early mouse embryos are initially highly disordered, and proper spindle morphology is not achieved until many minutes after nuclear envelope breakdown. Although this fact has been appreciated for over a decade, the field has struggled to resolve the paradox of compromised spindle assembly during a highly consequential developmental period. A growing body of evidence has suggested numerous roles for the actin cytoskeleton in mitotic cell division. However, this notion challenges the microtubule-centric view of mitosis and has been treated with some skepticism. We set out to reevaluate the paradigm of mitosis and examine the roles of actin filaments in achieving faithful segregation of the genetic material. Using live fluorescence microscopy, we resolved a network of actin filaments in the nucleus of early mouse embryos. This network establishes physical interactions with prophase chromosomes through myosin-10, an actin motor localized on mitotic chromatin. Following nuclear envelope breakdown, the network contracts and gathers chromosomes towards the cell center. Notably, network contraction occurs in a motor-independent manner. Rather than relying on nonmuscle myosin II, contractile stress is generated by filament disassembly. This mechanism of chromosome organization during the early phases of mitosis is microtubule-independent and occurs before the mitotic spindle is assembled. Disruptions of actin-based chromosome positioning resulted in increased rates of chromosome missegregation. Furthermore, we observed an additional network of actin filaments, which encloses the metaphase spindle. This network is composed of branched actin filaments nucleated by the Arp2/3 complex. We found that perispindle actin prevents uncontrolled spindle elongation, likely by serving as a physical barrier with inhibits microtubule elongation. Together, our results provide an explanation as to how the early mouse embryo achieves faithful chromosome segregation despite lacking a complete set of mitotic machinery

B537/P0520
A potential role for the ESCRT-III nucleator, Alx1, in mitotic nuclear envelope sealing
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The nuclear envelope (NE) is responsible for maintaining genomic integrity and by compartmentalizing chromatin. In the fission yeast Schizosaccharomyces pombe, a transient mitotic hole in the NE is created to allow insertion of the yeast microtubule organizing complex, the spindle pole body (SPB). We have shown that members of endosomal sorting complexes required for transport (ESCRT) machinery are responsible not only for sealing this NE hole, but for acting as a molecular grommet to maintain mitotic nuclear

compartmentalization. Notably, recruitment of ESCRT-III subunits to this hole depends on the NE-specific ESCRT Cmp7, bypassing the need for canonical ESCRT-I/II machinery. Recent work has suggested that other ESCRT-III initiators, such as Alx1, may be involved in nuclear envelope sealing. We examined the localization of endogenously tagged Alx1-GFP, finding this protein specifically localized to the site of NE SPB extrusion with the ESCRT protein Cmp7. However, NE localization was dependent on Cmp7. We determined the approximate copy number of Alx1 to be between 70 and 80 molecules at a NE sealing site using ratiometric quantification of live-cell microscopy images. To ascertain the extent to which Alx1 acts as a nucleator at the NE, we analyzed the recruitment of GFP-ESCRT subunits in an alx1Δ background. Overall, we hypothesize that Cmp7 and Alx1 act in concert in S. pombe to efficiently recruit downstream ESCRT proteins to a NE sealing site.

B546/P0529

Beyond chromosome congression: resolving the role of the SKA complex in cell cycle regulation of mitosis

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Accurate chromosome segregation during mitosis is dependent on the spindle checkpoint, a surveillance mechanism that prevents premature anaphase onset by inhibiting a ubiquitin ligase termed the Anaphase-Promoting Complex/Cyclosome (APC/C) through assembly of the mitotic checkpoint complex (MCC). Upon proper kinetochore-microtubule attachments, the spindle checkpoint is silenced, and the APC/C triggers anaphase onset. The Spindle and Kinetochore-Associated (SKA) complex, composed of SKA1, SKA2, and SKA3 subunits, localizes to kinetochores following nuclear envelope breakdown. Conventionally the SKA complex was thought to function primarily in promoting chromosome congression through the stabilization of kinetochore-microtubule attachments. However, our past studies suggested that the principal role of the SKA complex is to promote cell cycle progression from metaphase to anaphase and exit from mitosis. To resolve debate regarding the SKA-null phenotype that may have arisen due to partial vs complete RNAi-mediated depletion of SKA complex components, we used a CRISPR/Cas9-engineered HeLa cell line harboring a homozygous degron knock-in at all alleles of the SKA1 gene, allowing for rapid and complete SKA complex depletion. Acute SKA depletion triggered a strong metaphase arrest followed by cohesion fatigue, a process characterized by asynchronous and inappropriate chromatid separation. Chromosome congression was minimally impacted. Additionally, we used an MPS1 inhibitor to drive control and SKA-depleted metaphase cells into anaphase. Anaphase chromosome movements in control and SKA-depleted cells were identical. These findings support the idea that the SKA complex does not mechanically reinforce kinetochore attachment to spindle microtubules and hence does not play a direct role in chromosome movements. Rather our work supports a model in which the primary function of the SKA complex is to control the metaphase-to-anaphase transition through suppression of spindle checkpoint signaling and/or activation of the APC/C. These results offer novel insight into the molecular orchestration of mitotic exit and potential therapeutic targeting of mitotic regulators in cancer.

B544/P0527

Beyond Nuclear Transport: The Y-complex nucleoporin, NUP107, ensures faithful chromosome segregation in mitosis

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The nuclear pore complex (NPC) is a large protein assembly which forms a channel through the nuclear envelope and facilitates nuclear-cytoplasmic transport. The Y-complex consists of SEH1, SEC13, NUP37, NUP43, NUP85, NUP96, NUP107, NUP133 and NUP160; multiple copies of the Y-complex form an interlaced structural scaffold that comprises the outer cytoplasmic and nucleoplasmic rings of the NPC. Upon nuclear envelope breakdown (NEB) in mitosis, Y-complex nucleoporins localize to kinetochores on condensed chromosomes. To elucidate the mitotic function of the Y-complex, we employed an auxin inducible degron system to rapidly degrade individual nucleoporins. After individual Y-complex nucleoporins were depleted, we monitored the behavior of other Y-complex members, changes in mitotic progression and the dynamics of spindle assembly. We observed that the loss of Y-complex nucleoporins influenced the association of other members at kinetochores to varying degrees. Their depletion also elongated the interval between NEB and anaphase onset, with depletion of NUP107 causing both the greatest increase in mitotic timing and the most elevated frequency of chromosome mis-segregation. Notably, the severity of the mitotic phenotype for each Y-complex member correlated with the proportion of NUP107 lost from kinetochores, suggesting that it may have a particularly important role in promoting timely and accurate chromosome segregation. Moreover, spindles formed in NUP107-depleted cells showed less stable kinetochore-microtubule attachments and a reduced localization of the kinesin motor, CENP-E, to unattached kinetochores. Together, these findings suggest that the Y-complex, and NUP107 in particular, is essential for faithful chromosome segregation in mitosis at least partially through recruitment of CENP-E to kinetochores.

B526/P0509

Bubin guides the integrity of the chromosome passenger complex for accurate chromosome segregation

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The chromosomal passenger complex (CPC), essential for kinetochore assembly and microtubule attachment during mitosis, requires precise regulation for accurate chromosome segregation. Here, we identify C17ORF47 (designated Bubin) as a novel kinetochore protein that localizes to centromeres in prometaphase and metaphase. Bubin directly interacts with CPC components (Aurora B, Borealin, Survivin, and INCENP) and is critical for CPC centromeric localization. Depletion of Bubin disrupts spindle pole formation, kinetochore-microtubule attachment, and chromosome segregation, leading to mitotic catastrophe. Intriguingly, while Bubin loss impairs Aurora B centromere localization, INCENP kinetochore targeting persists, aligning with the kinetochore-proximal CPC pool. Conversely, Aurora B or Survivin knockdown displaces Bubin, revealing mutual dependence. Bubin recruitment further requires Bub1, Hec1, and Mps1 kinase activity. Our findings establish Bubin as a key regulator linking CPC dynamics and kinetochore function, providing mechanistic insights into mitotic fidelity.

B539/P0522

Chemical genomic approach to defining novel early mitotic inhibitors

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Mitosis is a highly ordered series of events that results in replication of genetic material and subsequent cellular division. Although many mitotic regulators have already been characterized, there are likely additional unidentified ones that control this intricate process. Mitotic errors may ultimately lead to cell death or an abnormal exit from mitosis. These consequences of mitotic errors emphasize the importance of discovering

new targets to support the development of novel cancer therapies. This study aims to identify early mitotic inhibitors through a chemical genomic approach. A high-throughput chemical screen was developed to arrest cells in G2 of the cell cycle, then release them into kinase inhibitor compounds. The percent of cells in metaphase was quantified for each compound and compared to the negative control. In a primary screen, 81 out of the 3,384 compounds in the kinase inhibitor library were identified as potential hits. This was statistically determined as the compounds that deviated by three standard deviations from the negative control. From these hits, there are multiple compounds that are known to regulate spindle assembly and that target the microtubules, which indicates that the screen is working well to identify early mitotic inhibitors. I am currently working on a secondary screen to verify the hits. In this study, the hits found with unknown roles are interesting targets to investigate further as potential candidates for therapeutic development.

B527/P0510
Cohesin malfunction in Saccharomyces cerevisiae activates Mcd1 degradation to promote cell lethality
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Cornelia de Lange Syndrome and Robert Syndrome are developmental abnormalities that often include intellectual disabilities, hearing loss, microcephaly, phocomelia and abnormalities in the heart and gastrointestinal tract. These phenotypes are caused by mutations in cohesin subunits. Cohesin complexes are critical for sister chromatid cohesion, gene transcription and DNA damage repair. Given the fundamental roles that cohesins play in human development and cancer progression, understanding the mechanisms that regulate cohesin stability is of clinical relevance. Here, we provide evidence for a checkpoint that monitors for cohesin malfunction and targets the cohesin subunit Mcd1 (Scc1/RAD21) for destruction. Our results reveal that Mcd1 is reduced in the absence of cohesin regulators Eco1 and/or Rad61. We further document that this reduction is due to Mcd1 degradation, resulting in part from the activity of E3 ligases. Elevated expression of MCD1 suppresses the temperature-sensitive growth defects in cells that contain cohesin complex subunit mutations (smc1-259, smc3-42, scc3-6) or its regulators (eco1 rad61, eco1-203, scc2-4). These findings, coupled with the recognition that it is wildtype Mcd1 protein that is reduced, rather than a mutated or misfolded version, support a model in which cells respond to altered cohesin function to remove cells that might otherwise contribute to an aneuploid or developmentally altered population.

B549/P0532
Condensation-dependent multivalent interactions of EB1 and CENP-R regulate chromosome oscillations in mitosis
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Mitotic chromosomes oscillate between the spindle poles upon the establishment of bi-orientation, which is essential for chromosome alignment and subsequent synchronous segregation. However, the molecular mechanisms underlying the oscillatory movement remain unclear. Recent studies revealed that phase separation of the end-binding protein 1 (EB1) is essential for eukaryotic cell division. Here, we show that EB1 interacts with CENP-R and that the phase separation-defective EB1 mutant fails to power the chromosome oscillations. Biochemical analyses reveal a co-condensation of EB1 and CENP-R, a subunit of the constitutive centromere-associated network (CCAN). Nuclear magnetic resonance assays reveal that the interaction and co-

condensation are largely mediated by the structured end-binding homology domain of EB1 and the non-structured N-terminal intrinsic disorder region of CENP-R. Chromosome oscillation is perturbed in cells expressing the EB1-binding-defective CENP-R mutant. Thus, phase-separated EB1 binding to CENP-R forms a physical link between inner kinetochore and dynamic spindle microtubule plus-ends to guide accurate chromosome oscillations. In addition, the NDC80 complex plays a key role in connecting the chromosome kinetochore to plus ends of spindle microtubules. Here, we identify an interaction between the NDC80 complex and EB1, mediated primarily by the intrinsically disordered region of N-terminal of Hec1. This interaction establishes a molecular basis for kinetochore–microtubule attachment dynamics, and its biological function in chromosome oscillations during mitosis will be further elucidated. Key words: mitosis; CENP-R; EB1; phase separation; chromosome oscillations

B533/P0516

Contribution of Topoisomerase IIa C-terminal Domain to the Structural Organization of Mitotic Chromosomes for Robust Chromosome Segregation

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Mitotic function of Topoisomerase IIα (TopoIIα) is governed by its C-terminal domain (CTD) that is distinct from the β isoform (TopoIIβ). Original studies swapping CTDs between isoforms showed the TopoIIβ-αCTD chimera supports robust mitosis, suggesting that catalytic core of the isoforms works comparably while their CTDs account for the difference in mitotic function. Both CTDs could bind to chromatin in in vitro pull-down assays, and the Chromatin Tether domain (ChT) within the αCTD is responsible for interacting with chromatin containing methylated histones, which is also distinct from TopoIIβ. By using conditional degradation of endogenous TopoIIα and inducible expression of exogenous TopoIIα, we showed that loss of αChT function leads to formation of chromosome bridges and ultra-fine bridges (UFBs) in anaphase. UFBs arise from incomplete decatenation of the tangled genome, thus recognition of specific chromatins by αChT could direct TopoIIα activity to the tangled genome loci. To further examine the contribution of isoform specific CTD roles in mitosis, we created chimeric mutants of TopoII isoforms with swapped ChT domains to test the contribution of each ChT/CTD to the isoform specific mitotic function of TopoIIa. TopoIIβ-αChT rescued UFBs whereas TopoIIα-βChT did not, suggesting recognition of specific chromatin features via αChT is critical for complete decatenation of tangled loci. This aligns with our previous finding that methylated chromatin promotes UFB resolution. However, chromosome bridges were not rescued by either chimera, indicating the complete αCTD is required for robust chromosome segregation. Fractionation of mitotic chromosomes by salt extraction revealed a complete αCTD is required for proper chromosomal association of TopoIIα. An αChT-deletion mutant (TopoIIα-ΔChT) had a similar defect in chromosome association. Trapping active TopoII on chromatin using a catalytic inhibitor (ICRF-193) caused TopoIIα-ΔChT to be retained on chromosome after salt-extraction, suggesting that decatenation activity of TopoIIα-ΔChT is comparable to wild type and the defect in chromosome association does not originate from a loss of enzyme activity. Proteomic analysis of salt-extracted mitotic chromosomes in TopoIIα mutants revealed changes in the composition of chromosomal structural proteins, including Condensin and centromeric proteins. The results provide novel insight into the αChT-dependent mechanism of TopoIIα interaction with mitotic chromosomes and requirements of a complete αCTD for proper structural organization of mitotic chromosomes for robust chromosome segregation.

B551/P0534
Coordinating cell size with glucose availability via regulation of mitotic activator Cdc25
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Cells maintain a characteristic size to function. Most cells control their size by delaying cell cycle transitions until reaching a threshold size, suggesting they can sense and relay size information to cell cycle machinery. Studies in fission yeast have uncovered core regulators of size control and cell division in eukaryotes. These cells divide at a threshold size due to size-dependent activation of cyclin-dependent kinase (Cdk1), in part via nuclear accumulation of the phosphatase Cdc25. Under nutrient-rich conditions, Cdc25 nuclear accumulation scales with cell volume, helping establish a size threshold for division. However, in nutrient-limited environments such as low glucose, cells divide at a smaller size, raising the question of how Cdc25 regulation adapts to changing glucose conditions. To explore this, we investigated how glucose availability influences Cdc25 levels and localization. Using live-cell fluorescence microscopy and an automated ImageJ/MATLAB analysis pipeline, we quantified nuclear and cytoplasmic levels of Cdc25-mNeonGreen in wild-type cells across a range of glucose concentrations. Surprisingly, we found that cells accumulate more Cdc25 in the nucleus as a function of size when grown in low glucose, suggesting that nutrient cues can override the usual size-dependent scaling of Cdc25. To identify upstream regulators, we screened mutants of the glucose-sensing cAMP/PKA pathway, including pka1, gpa2, git3, and git5, and found that disruption of these genes leads to elevated nuclear Cdc25, implicating this pathway as a negative regulator under glucose-rich conditions. In parallel, we observed that pub1Δ cells, lacking an E3 ubiquitin ligase involved in glucose-dependent Cdc25 degradation, also exhibit elevated Cdc25 levels. Our preliminary data, suggest that Pka1 and Pub1 may function in parallel pathways to restrict Cdc25 accumulation in high glucose environments. Together, our findings reveal that Cdc25 levels are actively modulated by glucose-responsive signaling pathways, allowing cells to adjust mitotic entry and division size according to glucose availability.

B562/P0484
Dual kinetochore binding modes of CENPC controls distinct mitotic roles: timely anaphase onset and chromosome segregation accuracy
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The macromolecular kinetochore ensures accurate chromosome segregation, and how signals from the outer kinetochore are relayed to inner components remains unclear. Here, we investigate CENPC, an intrinsically disordered inner kinetochore protein within the constitutive centromere-associated network (CCAN), by leveraging naturally occurring variants, optogenetic photoinactivation and time-lapse microscopy. Using truncating variants, we uncover that while the dynamic interaction of CENPC to the outer-kinetochore KMN complex regulates anaphase onset, it is stable tethering to chromatin that ensures segregation accuracy. Acute photoinactivation confirms this dual role and reveals a role reversal between CENPC's N- and C-terminal regions, which are dynamic in metaphase and anaphase, respectively. We demonstrate that CDK1- and Aurora-B kinase mediated phospho-regulation facilitates these reversals. Our findings show how CENPC's structural flexibility enables dynamic CCAN states and integrates outer kinetochore changes, ensuring checkpoint satisfaction and faithful chromosome segregation.

B532/P0515

Evaluating the role of inhibitory phosphorylation of the E2 enzyme UBE2H

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Cell division is a highly conserved essential process that is tightly regulated by various mechanisms, particularly post-translational modification pathways such as ubiquitination and phosphorylation. Furthermore, cell-cycle dysregulation may cause genetic instability in diseases such as cancer. In this study, we designed an in vitro screening assay based on Xenopus egg extract to detect functional differences between the ubiquitin pathway during interphase and mitosis. Our findings revealed that the activity of the E2 enzyme UBE2H was strongly reduced during mitosis as a result of mitotic CDK-dependent phosphorylation. UBE2H is known to function together with the multi-subunit CTLH/GID complex E3. Cells expressing non-phosphorylatable UBE2H showed persistent E2 activity during mitosis and exhibited mitotic defects including premature mitotic exit and micronucleus formation, indicating that CDK-dependent UBE2H regulation is important for normal cell division. Mass spectrometry analysis identified several proteins that were reduced in abundance in cells expressing non-phosphorylatable UBE2H, including the recently reported CTLH target hydroxymethylglutaryl-CoA synthase (HMGCS1). Restoration of related pathways could partially compensate for the mitotic defects observed in cells expressing non-phosphorylatable Ube2H. Overall, this investigation enhances our understanding of the interplay between ubiquitination and phosphorylation, in particular highlighting how regulation of E2 activity can be an important point of regulation in ubiquitin-dependent protein degradation.

B540/P0523

Investigating the contributions of chondrocyte spindle biophysics and tissue microenvironment to skeletal dysplasia

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Our understanding of how the cell division process varies across different tissues at the molecular level is extremely limited. Most mitotic proteins are ubiquitously expressed, but dysregulation of these highly coordinated component parts can still lead to dysfunction in a tissue-specific manner. One such example can be found in patients with spondyloepimetaphyseal dysplasia with joint laxity type 2 (SEMDJL2), who present with skeletal tissue-specific defects in development. SEMDJL2-causing point mutations have been identified in the ubiquitous mitotic chromokinesin KIF22. We have shown that SEMDJL2-associated mutations in KIF22 disrupt chromosome segregation in anaphase and result in reduced proliferation, abnormal daughter cell nuclear morphology, and cytokinesis failure. Interestingly, the mutations did not disrupt KIF22 motor function or localization, but rather the mechanisms that regulate KIF22 activity over the course of mitosis, leaving the motor hyperactivated. Persistent KIF22 motor activity during anaphase impairs the separation of chromosomes after alignment, which subsequently results in cytokinesis defects. However, it remains to be determined how these effects cause skeletal tissue-specific defects, since KIF22 is ubiquitously expressed. Spatial constraints in the skeletal growth plate may restrain spindle pole separation in anaphase and impair cytokinesis by preventing daughter cells from pulling away from one another during division. These impairments may be amplified by aberrant polar ejection forces to a point where the cell can no longer divide normally. We predict that the skeletal tissue-specific sensitivity to activating mutations in KIF22 is due to a combination of chondrocyte-specific characteristics that impact the force balance within mitotic spindles and the spatial

constraints imposed by the skeletal growth plate. To test how changes in the balance of forces within the spindle affect chondrocyte sensitivity to KIF22 activity, we induced expression of KIF22 mutants in chondrocytes and knocked down KIF4A or PRC1. Preliminary data show that increasing outward sliding forces during anaphase B through KIF4A knockdown partially rescues chromosome segregation and spindle elongation in chondrocytes expressing KIF22 mutants.Investigating the molecular mechanisms by which mutations in KIF22 selectively disrupt growth of skeletal tissue in SEMDJL2 patients will additionally provide insight into the chondrocyte-specific functional roles of KIF22, the regulatory mechanisms governing the activity of KIF22 during mitosis, and the effects of tissue architecture on cell division

B531/P0514

Lipid transfer by ORP3 is required for the regulation of PI4P and PI(4,5)P2 at the plasma membrane in the cell cycle

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During cell division, dramatic cell reorganizations occur in order to reliably segregate chromosomes and organelles in the two daughter cells. These changes are driven by the action of the tubulin and the actomyosin cytoskeleton. Phosphoinositides (PIs) at the plasma membrane, particularly PI(4,5)P2, are key molecular cues recruiting cytoskeleton adapters at the cell cortex. PIs undergo dynamic rearrangements during mitosis, contributing to regulate the cytoskeleton distribution in mitosis. To date, the only known regulators of PI(4,5)P2 at the plasma membrane in mitosis are kinases and phosphatases. But these regulations are not sufficient to justify PIs pattern in mitosis. We consider a less-studied mechanism: the lipid transport non-vesicular trafficking at membrane contact sites. Lipid transfer proteins are evolutionary conserved, transferring lipids between membrane organelles. Lipid transfer proteins also contribute to the regulation of organelle-specific phospholipids including PIs and to organelle membrane identity and functions. The ORP3 lipid transfer protein functions at contact sites between the plasma membrane, where it binds PI(4,5)P2 and the ER, where it binds transmembrane tether proteins of the VAP family, through an FFAT motif. There, ORP3 transfers PI4P from the plasma membrane to the ER. In order to investigate the contribution of ORP3 function to PI homeostasis at the plasma membrane during cell division, we have generated stable HeLa ORP3-knock-out cell lines, and a collection of rescue cells lines with wild-type ORP3 or ORP3 dead mutants for different functional domains. First, we have studied the contribution of ORP3 to the PI(4,5)P2 and PI4P landscape at the plasma membrane. We show that PI4P transport by ORP3 indirectly regulates PI(4,5)P2 both in interphase and mitosis. In mitosis, defects in ORP3 function (KO or dead mutants) result in abnormal PI(4,5)P2 and actin cytoskeleton distributions, abnormal cell division, PI4P accumulation at the cytoplasmic bridge, and cell division phenotypes, which lead to aneuploidy. In addition, we have found that beyond the canonical FFAT domain of ORP3, the activation of a second non-canonical FFAT domain enhances ORP3 recruitment at the ER in mitosis resulting in “priming” for PI extraction from the plasma at ER-plasma membrane contacts. In conclusion, our work demonstrates that lipid transfer protein by ORP3 contributes to PI(4,5)P2 and PI4P regulation at the plasma membrane in the cell cycle.

B525/P0508

Liver regeneration CRISPR screen identifies E3 ligase NEURL1B regulates hepatocyte mitosis by destabilizing microtubule organizing centers

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Background and aims: Centrosomes serve as the microtubule organizing centers (MTOCs) of most cells. Supernumerary centrosomes can lead to multipolar spindles, errors in chromosome alignment and prolonged mitosis. Hepatocytes naturally harbor supernumerary centrosomes but they can proliferate extensively with bipolar spindles, accurately separating chromosomes. How hepatocytes overcome these challenges to efficiently divide is unclear. Methods: To understand how hepatocytes cope with mitotic challenges, we established a high-quality in vivo CRISPRi screen in regenerating mouse liver. By cross-referencing with gene essentiality data in cell lines, we identified genes specifically required for hepatocyte proliferation. Knockout mice, RNA sequencing and proteomic approaches were used to understand the function of the candidate gene and the mechanism in regulating hepatocytes proliferation. Result: We found E3 ligase NEURL1B is essential for hepatocyte division during liver regeneration and tumorigenesis, yet it is dispensable in other cell types and liver development. This dependence on NEURL1B arises from a hepatocyte-specific pathway of spindle pole organization. NEURL1B localizes to microtubule-organizing centers (MTOCs) and destabilizes them. NUMA1 is a substrate of NEURL1B. Prophase hepatocytes exhibit multiple MTOCs organized by NUMA1. These MTOCs undergo dynamic remodeling to form two spindle poles through clustering, and a rarely described mechanism of centrosome inactivation. The MTOCs destabilization activity of NEURL1B promotes inactivation of extra centrosomes and facilitates mitosis progression. Human single nucleus RNA-seq and cell culture data support a similar role for NEURL1B in human liver regeneration. Conclusion: Our approach of studying mitosis in vivo reveals a unique mechanism used by hepatocytes to manage supernumerary centrosomes and identifies NEURL1B as a critical regulator in this pathway, paving the way to new treatments for liver regeneration and cancer.

B557/P0540

Local Regulation of Cyclin B1-Cdk1 by Plk1 at the Kinetochore and Corona

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Cyclin B1-Cdk1 is a key regulator of mitosis, orchestrating events such as chromosome condensation, spindle assembly, and chromosome segregation through phosphorylation of numerous substrates. Cyclin B1 localizes to unattached kinetochores, contributing to spindle checkpoint signalling. However, the mechanisms underlying its kinetochore recruitment and function remain incompletely understood. The objective of this study was to determine how Plk1 regulates the localization and function of Cyclin B1-Cdk1 at kinetochores during mitosis. Using fixed and live imaging, we demonstrate that inhibition of Plk1 using BI2536 reduces Cyclin B1 localization at unattached kinetochores. This correlates with decreased phosphorylation of BubR1 at serine 670, a Cyclin B1-Cdk1 substrate, suggesting that Plk1 is required for proper Cyclin B1 function at kinetochores. Similar to inhibition of Cdk1, Plk1 inhibition leads to detachment of the entire corona complex from the core kinetochore. Furthermore, tethering Plk1 to kinetochores increases the levels of the corona component ZW10 at both prometaphase and metaphase kinetochores, indicating that corona disassembly is facilitated by loss of Plk1 activity. In conclusion, our findings suggest that Plk1 regulates a kinetochore-associated pool of Cyclin B1, facilitating localized phosphorylation of specific substrates by Cyclin B1-Cdk1. This recruitment may also help maintain corona attachment to the core kinetochore, providing new insights into the regulatory interplay between Plk1 and Cyclin B1 at kinetochores.

B553/P0536
Mechanisms of de novo microtubule nucleation in the spindle midzone
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Previous data suggests the spindle midzone – the region between segregating chromosomes – contributes to chromosome segregation during mitosis, even in the absence of cortical pulling forces. Formation of the midzone occurs through the bundling and crosslinking of antiparallel microtubules by several midzone proteins, but the mechanisms of how it assembles and contributes to chromosome segregation are not yet fully understood. 3D electron tomography of one-cell C. elegans anaphase spindles from our lab suggests that a subset of microtubules is nucleated in the midzone rather than at the centrosomes. The identity of the microtubule nucleator in the midzone is currently unknown; g-tubulin cannot be detected in the midzone and an augmin dependent microtubule nucleation mechanism has not been identified in C. elegans. The microtubule stabilizer Cytoplasmic-Linker Associated Protein (CLASP), a protein critical for proper spindle formation and chromosome segregation, that has been shown to have microtubule nucleation abilities independent of the g-tubulin ring complex, also localizes to the spindle midzone. This suggests that CLASP could drive microtubule nucleation in the spindle midzone. Using spinning disk confocal microscopy, we show that depletion of cls-2, the C. elegans homolog of CLASP, eliminates midzone specific microtubule growth and localization of midzone proteins. The absence of a midzone leads to spindle rupture due to cortical pulling forces. Interestingly, depletion of both cls-2 and pulling forces exhibits a rescue effect, restoring the localization of midzone protein during anaphase, albeit in a disorganized manner; even in the absence of visible microtubule growth in the midzone. These results support the idea that cls-2 nucleates microtubules in the midzone, as well as provide us with more insight on the organization of the midzone.

B552/P0535
Microtubule depolymerization at kinetochores restricts anaphase spindle elongation
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Anaphase chromosome segregation depends on forces exerted by spindle microtubules. Current models propose two mechanisms of force generation: kinetochore microtubules (kMTs) depolymerize to pull chromosomes toward the spindle poles (anaphase A), while antiparallel microtubule sliding in the central spindle further separates the chromosomes by elongating the spindle (anaphase B). Experimental evidence in cells supports the sliding mechanism, but contributions of the depolymerization mechanism remain unclear. Here we show that kMT depolymerization limits spindle elongation rather than moving chromosomes apart. We developed a chemical optogenetic approach to recruit a microtubule depolymerase to kinetochores at anaphase onset, thereby increasing the rate of kMT depolymerization without perturbing earlier stages of mitosis. We find that increased depolymerization slows the velocity at which spindle poles move apart without changing kinetochore separation velocities. Our findings support a model in which kinetochores selectively couple to central spindle microtubules parallel to their kMTs, so that antiparallel sliding drives chromosome segregation while kMT depolymerization pulls poles inward.

B545/P0528

Microtubules in the spindle midzone self-organize into distinct domains during anaphase

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The mitotic spindle is a highly dynamic, yet stable structure that segregates chromosomes during anaphase. The anaphase spindle midzone is the region between the segregating chromosomes, which actively contributes to chromosome segregation by the joint activity of microtubule bundlers and motors. Electron tomographic reconstructions of microtubules in the spindle midzone suggest that the midzone can be divided into specific MT subclasses, i) highly dynamic central MTs (cMTs) with both ends located in the midzone that are most likely nucleated within the midzone, and ii) more stable inter-spindle MTs (isMTs) with one end located between poles and chromosomes and the other in the midzone that originate from the centrosomes. Our preliminary data suggests that these specific sub-classes of MTs could be recognized and associated with specific proteins. In mammalian cells and C. elegans the kinesin-5 family motor protein BMK-1 (EG-5) seems to localize to a region close to the inner surface of the chromosomes, which is mainly populated by isMTs, while the microtubule bundling protein SPD-1 (PRC-1) localizes to the center of the midzone, which is mainly composed of cMTs. Selective binding of these proteins to different MTs subclasses could be either mediated by different MT lifetimes, length, and/or specific MT organization, i.e. parallel or antiparallel alignment. As it is difficult to selectively remove individual subclasses, we quantified the localization of proteins in “broken” spindles (induced by depletion of ZEN-4 or SPD-1 that leads to spindle rupture due to cortical pulling forces) and monopolar spindles (induced by ZYG-1 RNAi which prevents centrosome duplication) to assess functionality of the domains. In both “broken” and monopolar spindles, BMK-1 and SPD-1 localize to a region behind the chromosomes upon anaphase onset. 3D Tomographic reconstructions of monopolar spindles show MTs, which have both ends in the region behind the chromosomes, suggesting that this region is composed of isMTs as well as cMTs. We speculate that these cMTs are de-novo nucleated in this area and further bundled by SPD-1 creating an antiparallel MT overlap even in monopolar spindles. Interestingly, depletion of SPD-1 in monopolar spindles leads to a decrease in the accumulation of BMK-1, suggesting that SPD-1 is required for the distinct localization pattern of BMK-1. To determine how the formation of protein domains in the midzone arises we are using mathematical simulations of monopolar spindles. Preliminary data using this approach suggests that SPD-1 can confine BMK-1 to mostly isMTs and/or to the overlap of cMTs and isMTs. We speculate that BMK-1 could then serve as a brake in spindle elongation by ensuring and stabilizing proper overlap of these two subclasses of MTs.

B560/P0543

Midzone bundles of the mammalian anaphase spindle are mechanically coupled both locally and globally to preserve spindle mechanical integrity

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To segregate chromosomes the anaphase spindle must generate and respond to various forces, coordinating its remodeling across space and time. Our understanding of the mechanics of the mammalian anaphase spindle remains limited due to the difficulty of probing mechanics in cells during this short-lived process. Here, we use microneedle manipulation in live cells to ask if anaphase midzone microtubule bundles function as

independent or mechanically coupled force-generating elements. We directly pull on anaphase midzone bundles at different velocities and over different timescales and show that these bundles are strongly mechanically coupled to each other. Along the spindle's short axis, midzone bundles transmit force locally to each other and are mechanically coupled up to 5µm away for over a minute. In the spindle's long axis, midzone bundles are in turn globally coupled through the spindle pole, requiring all bundles to elongate for spindle elongation to occur. Upon microneedle pulling, we find that their connections from pole to pole are strong enough to stall and even reverse global anaphase spindle elongation, and that this response is PRC1 dependent. We propose that strong coupling of force-generating elements enables the anaphase spindle to respond to mechanical disruptions, resulting in highly robust segregation.

B547/P0530

Molecular basis for the activation of aurora a and plk1 kinases by bora during mitotic entry

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During mitotic entry, cells undergo a profound reorganization essential for chromosome segregation. Biochemically, this reorganization is triggered by mitotic kinases including Aurora Kinase A (AURKA), Polo-like kinase 1 (Plk1), and Cyclin B-Cdk1, which activate in cascade, and the concomitant inactivation of protein phosphatases (PPases). Paramount in the activation of the mitotic kinase cascade is the activation of AURKA, which, like other kinases, is activated by phosphorylation of a conserved residue (Thr288) in the activation segment (T-loop). AURKA can autophosphorylate its T-loop, but the site is rapidly dephosphorylated by counteracting PPases in the G2 phase of the cell cycle. As dephosphorylation maintains AURKA inactive, the crucial question arises as to how AURKA can overcome the repressive effect of PPases to drive mitotic commitment. The evolutionarily conserved, intrinsically disordered protein Bora is critical for initiating the activation of mitotic kinases. Once phosphorylated at Ser112 by Cyclin A-Cdk1 kinase, phospho Bora activates unphosphorylated AURKA, directing it towards Plk1, thus promoting Cyclin B-Cdk1 activation and mitotic entry. However, how structurally phospho-Bora binds unphospho-AURKA to promote Plk1 T-loop phosphorylation is not understood. The key Bora determinants of the reaction remain to be identified. To address this issue, I developed MITOKINAC (MITOtic KINases Activation in E. Coli): a versatile approach that reconstitutes phospho-Bora and AURKA-dependent Plk1 T-loop phosphorylation directly in E. coli, which enabled the screening of a set of 39 Bora mutations and identified key molecular determinants of the reaction. By combining MITOKINAC with structural modelling (AF3) and in vitro assays, we provide evidence that Bora wraps around the N-terminal lobe of AURKA to position its phospho-Ser112 near AURKA's T-loop, mimicking T-loop phosphorylation. Additionally, Bora transiently interacts with the Alpha C helix of the Plk1 kinase domain through a conserved motif, guiding AURKA activity towards the Plk1 T-loop, which is otherwise impervious to phosphorylation by AURKA. We demonstrate the importance of this conserved motif in Bora in vitro and for mitotic entry in Xenopus egg extracts. Phospho-Bora thus selectively activates and localizes AURKA close to Plk1 to trigger its activation and mitotic entry. Our results reveal insights into how cells meet the challenge of spatially and temporally limiting Plk1 activation during mitosis, which could lead to the development of pathway-specific inhibitors.

B558/P0541
Motor Protein Dependent Nuclear Number and Spacing in Subperineurial Glial Cells
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Multinucleated cells arise through distinct mechanisms, and in many cases, the number of nuclei and their distribution throughout the cytoplasm are thought to be critical for cell function. In Drosophila, myofibers form through cell fusion, and our lab has shown that both the number and spacing of nuclei are tightly controlled. In contrast, the Subperinuerial Glia (SPG) of the Drosophila blood-brain barrier become multinucleated through endomitosis rather than fusion, and although their nuclei appear to be evenly distributed, it remains unclear how the number of nuclei and their spacing are regulated. We have found that the regulation of the number of nuclei and nuclear spacing in SPG cells requires the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex, suggesting that cytoskeletal forces influence these processes. Given that several microtubule-based motors are required for both mitosis and nuclear spacing, we examined whether the number of nuclei and the spacing of nuclei in SPG cells are co-dependent or independently regulated by identifying the microtubule motors that regulate each process. We found that SPG-specific depletion of cytoplasmic Dynein drastically reduced animal viability without altering the number of nuclei or the spacing of nuclei, indicating that Dynein plays a critical role in SPG cell function independent of both endomitosis and nuclear spacing. Depletion of kinesin-like protein-1 (MKLP-1) increased the number of nuclei per SPG cell, but did not affect nuclear spacing, suggesting that endomitosis and nuclear spacing are regulated independently. Knockdown of either Kinesin-1 or Kinesin-5 increased the number of nuclei per SPG cell, revealing that both regulate endomitosis. Notably, knockdown of Kinesin-1 resulted in a slight disruption of nuclear spacing, whereas knockdown of the bipolar motor Kinesin-5 resulted in a pronounced disruption of nuclear spacing, with nuclei forming clusters within SPG cells. These results suggest that these motors regulate different aspects of nuclear spacing. Taken together, these data suggest that endomitosis and nuclear spacing are independently regulated by several distinct Kinesins in SPG cells.

B550/P0533
Novel mitotic roles of a membrane protrusion-inducing protein
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Various kinds of membrane protrusions are formed by mammalian cells, which mediate cell adhesion, cell movement, and intercellular cargo transfer. A strong positive correlation has been observed between the formation of membrane protrusions during the stages of cell division. It is essential to identify the missing molecular mechanistic links connecting cellular protrusions and mitosis, as this knowledge would reveal novel means of mitotic regulation. We therefore aimed to investigate common protein(s) that could function as molecular links between membrane protrusion biogenesis and mitosis and co-regulate both processes. Using a combination of siRNA-mediated knockdowns and quantitative high-resolution optical imaging, we describe new mitotic functions of a protein central for membrane protrusion biogenesis. We show the requirement of this protein in multiple steps of mitotic progression and its novel sub-cellular localization at mitotic hotspots, suggesting that this important protein required for membrane protrusion biogenesis has critical mitotic roles. Among the novel mitotic functions of this protein, we focused on elucidating its mechanistic contributions to governing spindle orientation, a key function regulating stem cell fate and early organism development, by

examining its association with classical pathways responsible for spindle orientation. Our investigations reveal key functions of this protein in nucleating microtubules from spindle poles during mitosis and also in governing the distribution of a conserved microtubule cortical capture complex required for anchoring and orienting the spindle inside the cell. Domain dissection as well as mitotic interatomic analysis of this protein reveals unprecedented insights into its novel mitotic functions.

B555/P0538

Proteomics Elucidates Cell-Size Dependent Expression of Cell-Cycle Regulators and Other Proteins

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How cells regulate size is a fundamental, outstanding question in cell biology. One plausible hypothesis is that size-dependent expression of cell-cycle regulator controls the size at which cell-cycle transitions occur. Whi5 and Rb are expressed in a size-dependent manner in G1 and have been proposed to regulate the G1/S size checkpoint in budding yeast and vertebrates, respectively. Likewise, both the Cdc13 cyclin and Cdc25 phosphatase are expressed in a size-dependent manner in fission yeast G2 and have been proposed to regulate the G2/M size checkpoint. However, mutations that disrupt the size-dependent expression of these proteins do not impair cell control arguing that, at the very least, there are additional redundant size-control mechanisms. To investigate other potential size-control mechanisms, we have identified proteins expressed in a size-dependent manner in fission yeast G2. Using state-of-the-art mass spectrometry, we have, for the first time, been able to quantitate low abundance proteins, such as Cdc13 and Cdc25, regularly identifying greater than 80% of the proteins encoded in the fission yeast genome. We find that Cdc13 and Cdc25 are the only known cell-cycle proteins that are expressed in a size-dependent manner. However, we do find about 400 other proteins that are expressed in a size-dependent manner; about 300 superscale and 100 subscale. These proteins are not enriched in any particular function and, with the exception of histones, which are presumed to subscale in all eukaryotes, are not conserved with size-dependent proteins from budding yeast or humans. From these result, we conclude that either there are novel cell-cycle proteins involved in size control among the new size-dependent proteins, or that cells use a mechanism independent of size-dependent expression to regulate cell size. Furthermore, we speculate that, for some proteins, size-dependent concentrations are not tightly constrained by evolution, and that the ~400 size-dependent proteins that we observe may deviate from constant concentration by evolutionary drift. We are examining size-dependence in other fission yeast to test this hypothesis. informenter-marker-1.png" class="informenter-marker-hide" style="" id="informenter-marker-id" title="Right click to shift">

B535/P0518

Quantitative 3D-reconstruction of mitotic spindles in multicellular C. elegans embryos

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The embryo of Caenorhabditis elegans serves as an excellent model system for investigating how spatial constraints influence the structure and organization of the mitotic spindle during embryonic development. Building on the structural analysis of the single-cell embryo (Redemann et al., 2017, Nat Commun 8:15288), we examined the organization of mitotic metaphase spindles in 4-, 8-, 16-, 32-, and 64-cell embryos. Using serial-section electron tomography for three-dimensional reconstruction, this study aims to provide a detailed

characterization of spindle ultrastructure, including the length measurements of all microtubule classes and a comprehensive analysis of centrosome organization. Our results revealed that the total number of microtubules decreases with each embryonic stage, while the proportion of kinetochore microtubules (KMTs) increases. Specifically, the percentage of KMTs increased from 3.19% in the 4-cell stage to 27.36% in the 64-cell stage. Spindle microtubules (SMTs) and astral microtubules (AMTs) also displayed stage-dependent variations, with AMTs representing the most abundant class at all stages but decreasing in relative proportion as development progressed. Furthermore, centrosome size declined as the embryo developed, from an average radius of 1.01 µm at the 4-cell stage to 0.47 µm at the 64-cell stage. We also assessed the association of KMTs with the centrosome, distinguishing between direct and indirect connections (Redemann et al., 2017). Our analysis showed an increase in the proportion of directly connected KMTs throughout embryonic development. Altogether, these results demonstrate that the reduction in cell size during C. elegans development does not simply result in a scaled-down version of the single-cell spindle and that spindle scaling is not uniform across microtubule classes. These findings contribute to a deeper understanding of spindle architecture and its regulation during mitotic cell division.

B538/P0521

Role of the Arp2/3 Complex in Regulating Mitotic Entry and Progression in S. pombe

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Fission yeast undergoes closed mitosis, wherein chromosome segregation occurs inside an intact nucleus. The microtubule organizing center, Spindle Pole Body (SPB) is duplicated in the G2 phase followed by insertion into a fenestra on the nuclear envelope (NE). The SPB then nucleates spindle microtubules at mitotic onset. Here we show that the branched actin network promotes mitotic entry by enabling NE fenestration. Inhibiting the branched actin nucleator Arp2/3 complex with the drug CK666 fail mitotic entry and the few cells that enter mitosis show significant delays. Upon CK666 inhibition, most proteins are correctly recruited to the SPB at the onset of mitosis however they fail to properly recruit the POLO-like kinase Plo1 and the SPB docking protein Cut11, suggesting a failure in SPB insertion. The start of NE fenestration is marked by the formation of a Sad1 ring, a SUN domain protein followed by a Cut11 ring. Super-resolution structured illumination microscopy indicates that CK666-treated cells show impaired Sad1 or Cut11 rings. Furthermore, these cells do not leak NLS-GFP, a hallmark of fenestration failure. In agreement with the role for branched actin in NE fenestration, CK666 treatment after SPB insertion does not show any mitotic entry defects as observed with preprophase-arrested tubulin mutants. In agreement with a role for branched actin in mitotic entry, we observed a component of the Arp2/3 complex Arc5, and actin-binding proteins Fimbrin and Coronin, on the nuclear envelope and SPB within minutes of mitotic onset. Indeed, in the absence of branched actin, cells that do manage to enter mitosis show the aberrant SPB interactions with the actin ring, thus physically hindering proper mitotic progression. This provides further evidence that the SPB during mitosis interacts with the actin cytoskeleton. Our findings demonstrate a novel role for Arp2/3 complex-dependent branched actin in promoting NE fenestration and SPB insertion during mitosis. It has been shown that branched actin is present at the centrosome in higher eukaryotes, and its role still needs to be elucidated. Our work suggests a potential role for branched actin in higher eukaryotes in mitotic entry possibly by promoting nuclear envelop breakdown.

B559/P0542
Sequence features important for mediating transport of pericentrin by the dynein motor
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How dynein recognizes and transports specific cargoes along microtubules remains incompletely understood. We have shown that Pericentrin (PCNT), a large coiled-coil centrosomal protein, is transported to the centrosome via a dynein-mediated process co-translationally. However, how PCNT engages with the dynein complex is unknown. Using time-lapse live-cell imaging and AlphaFold3 structural prediction, we dissected the role of sequence features in PCNT required for dynein-mediated transport. We identified a 133-residue region in human PCNT required for mediating active dynein transport. Surprisingly, replacing this region with synthetic sequences with high coiled-coil-forming propensity partially rescued the dynein-mediated transport of PCNT. Structural modeling suggested that similar mechanisms might have been used by known dynein cargo activators in binding and/or activating dynein transport.

B561/P0544
Single-Molecule Study of CENP-L/N Binding to CENP-A Nucleosomes and Single-Stranded DNA
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Accurate chromosome segregation during mitosis relies on kinetochores linking centromeric chromatin to spindle microtubules. Constitutive centromere-associated network (CCAN) assembly on CENP-A nucleosome and provide a platform for the assembly of outer kinetochore, which binds microtubule directly. Based on the structure of CENP-N/CENP-A nucleosome, CENP-N bind with RG-loop of CENP-A. However, Cryo-EM structures of intact CCAN-CENP-A nucleosome complexes reveal that CCAN wraps linker DNA topologically and does not physically bind CENP-A. Thus, a striking divergence exists between CENP-N/CENP-A nucleosome binding with intact CCAN/CENP-A nucleosome binding, suggesting the conformational transition during the assembly of CCAN on CENP-A nucleosome. To resolve this transition mechanism, single-molecule FRET with CY3/CY5-labeled CENP-A nucleosomes and recombinant CENP-LNC (CENP-$C^{180-545}$) was performed and shows that CENP-LNC binding promotes DNA sliding relative to the histone octamer—an effect absent with CENP-LN alone. Optical tweezers further demonstrate that CENP-LN exhibits stronger affinity for single-stranded DNA (ssDNA) than double-stranded DNA (dsDNA), with binding increasing at high force. Together, these findings provide new insights on conformational regulation of dynamic CCAN-CENP-A interactions.

B554/P0537
Single-particle tracking reveals multi-scale temporal tuning of force generation machinery that governs cortical microtubule interactions during the first zygotic division in C. elegans.
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Dynein is an essential microtubule motor with roles in many mitotic processes, complicating efforts to resolve how dynein is regulated within dynamic in vivo systems. Using total internal reflection fluorescence

microscopy and single-particle tracking, we examined dynein dynamics during asymmetric division of the C. elegans zygote. We applied our published classification scheme to categorize trajectories and probed regulation of cortical force generation. LIN-5, the NuMA-related coiled-coil protein, links dynein to the conserved cortical force generation complex (LIN-5NuMA—GPR-1/2LGN—GαGαi). Previous studies have provided insight into the importance of these components, but how dynein is regulated and how the force generation complex proteins interact at the molecular scale has not been investigated. RNAi depletion of regulators revealed a role for the protein phosphatase PP2A B55/SUR-6: depletion of the regulatory subunit SUR-6 significantly increased dynein trajectories with microtubule-interacting behavior (Non-parametric one-way ANOVA, Dunn's post-hoc, $p = 0.007$). We next tracked the dynein anchor LIN-5. Model-based classification of LIN-5 trajectories produced interacting angular resolutions ($\Phi = [40,45]$) in agreement with dynein ($\Phi = [50,55]$), and predicted a tip-to-tail distance (80–90 nm) that closely matched Cryo-EM/AlphaFold predictions (57nm). LIN-5 interacting trajectories were posteriorly enriched during anaphase (A/P ratio = 0.79), consistent with dynein (A/P ratio = 0.87, Linehan, et al. 2023) and prior fluorescence studies (LIN-5 A/P ratio = 0.88, Park & Rose 2008). SUR-6 depletion did not alter LIN-5 force asymmetry (Welch's t-test, $p = 0.2565$), suggesting direct regulation of dynein rather than regulation via LIN-5. We found evidence that two distinct factors in force generation, dynein tethering and cortical flows, are governed on two different time scales. Binding and unbinding of the force generation complex to the membrane occurs on the μ-second scale and follows our tether model. Meanwhile, particle image velocimetry analysis indicated that second scale cortical flows derived from actomyosin cortical contractility position cortically tethered LIN-5, but not dynein, determining longer-term directional flows and positioning of the force generation complex. Our observations provide novel insight into the connectivity among the cell membrane, actomyosin cortex, force generation complex, and microtubules, and how this coupling is regulated, to position the mitotic spindle during mitosis.

B528/P0511
Subcellular transport of RNA during embryonic mitosis
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RNA localization is a conserved and fundamental mechanism for regulating gene expression, impacting diverse processes in early development. Immediately after fertilization, the embryo undergoes rapid mitosis, requiring tight spatial and temporal regulation to ensure accurate chromosomal segregation, with failure resulting in developmental disorders such as aneuploidy, birth defects, and developmental arrest or death. Previous research has shown enriched localization of microRNA-31 (miR-31) and its target mRNAs at the mitotic spindle. We found miR-31 to regulate local translation of its targets at the spindle. miR-31's targets include Fascin, Rab35, Gelsolin, and β-actin, which encode proteins that modulate the cytoskeleton that is critical for mediating proper chromosomal segregation. Upon decreasing miR-31 levels, or forcing Fascin translation at the cell periphery instead of the spindle, embryos display chromosomal segregation defects, developmental delay, arrest, and death. These results indicate that subcellular transport of miR-31 and its targets to the spindle is critical in early development. We hypothesize that miRNAs and their mRNA targets are transported to the mitotic spindle by RNA-binding proteins (RBPs) in ribonucleoprotein complexes (RNPs). We bioinformatically identified a cytoplasmic polyadenylation element (CPE) within Fascin, Rab35, and Gelsolin transcripts that was previously found to govern transport of Cyclin B in the frog and Aurora B in the sea urchin embryo to the mitotic spindle. Using reporter constructs and site-directed mutagenesis, we found the Fascin CPE is necessary for spindle localization. Knockdown of the CPE-binding protein CPEB, also disrupted Fascin localization. Using Oligonucleotide-directed proximity-interactome mapping (O-MAP), an unbiased method for RNA-targeted proximity biotinylation to pull down proteins near target transcripts, we have identified Caprin1, CPSF5, G3BP2, hnRNP-K, and AnnexinA11, with known roles in RNA transport, granule formation, and

interaction with organelles to be associated with Fascin, Rab35, and Gelsolin mRNAs. We will conduct O-MAP experiments in mammalian cells to identify evolutionarily conserved mechanism of subcellular transport. Overall, these results will contribute to an understanding of the tight control of embryonic mitosis to ensure proper embryogenesis.

B534/P0517

The Bifunctional Nature of Actin Capping Protein in Cell Division

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Force generation by the actomyosin and microtubule-based cytoskeletons is crucial for proper cell division, tissue morphogenesis, and embryonic development. During mitosis, cortical actomyosin determines cell shape and drives shape changes; microtubules build and position the spindle and segregate the chromosomes. While the actomyosin and microtubule organize into distinct cellular structures, they are functionally linked via cross-talking regulation and structural coordination in ways that are not fully understood. To better understand how these force-generating systems are coordinated, we investigated the role of capping protein, a conserved actin-binding heterodimer that limit actin polymerization by capping the barbed end. Capping protein also contributes to the microtubule cytoskeleton as a component of the dynactin complex, where it stabilizes the Arp1 filament backbone and promotes dynein-mediated transport. The Caenorhabditis elegans zygote provides a powerful model to dissect the roles of conserved proteins in the actomyosin and dynein–microtubule cytoskeletons during successive mitotic events. We used RNAi to deplete both subunits of capping protein from the C. elegans zygote. Depletion of capping protein accelerated cytokinetic furrowing, consistent with its known role in shaping actomyosin-driven contractility and network architecture. In addition, capping protein depletion led to previously unreported defects in pronuclear migration, centrosome and bipolar spindle organization, and chromosome segregation, phenotypes reminiscent of dynein loss-of-function. Since dynein is essential for cortical pulling forces that drive spindle positioning and dynamics, our findings suggest that capping proteins influence dynein function, likely via its known structural role in the dynactin complex, a key dynein regulator. These results reveal that capping protein regulates both the actin and microtubule cytoskeletal systems during cell division. Future exploration of how the pool of capping protein heterodimers is distributed between actin and dynactin will reveal how contractility and microtubule dynamics are coordinated during cell division and development.

B529/P0512

The Hec1/Ndc80 Tail Domain Contributes to Kinetochore-Microtubule Attachment Stability Through Direct Contacts with the Microtubule Lattice

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Accurate chromosome segregation in mitosis relies on the dynamic interaction between spindle microtubules and kinetochores -- large protein machines assembled during each cell cycle on sites of centromeric chromatin. The kinetochore associated NDC80 complex links directly to microtubules through the Hec1/Ndc80 subunit, which binds via a high-affinity site in its calponin homology domain. In addition, Hec1/Ndc80 contains an unstructured N-terminal tail domain that regulates the stability of kinetochore-microtubule interactions. While it is well established the NDC80 complex harnesses forces generated by depolymerizing microtubules to drive

chromosome movement, the precise mechanism by which the N-terminal Ndc80/Hec1 tail domain contributes to this process remains unresolved. Current models propose that the tail either directly interacts with the microtubule lattice to provide additional binding sites or promotes cooperative binding to microtubules by clustering adjacent NDC80 complexes. To test these models, we engineered Hec1 tail extension mutants that preserve amino acid composition, charge distribution, and phosphorylation motifs while duplicating putative microtubule-binding regions. We hypothesized that elongated tails would provide additional binding sites with the microtubule lattice. In human cells, expression of an extension mutant containing 12 additional residues ("Hec1-92N") increased inter-kinetochore distance by ~25% compared to wild-type Hec1, suggesting elevated tension across sister kinetochores. Interestingly, 92N-expressing cells maintained Aurora kinase–dependent regulation of kinetochore–microtubule attachments. We further investigated the molecular basis for increased tension across sister kinetochores with purified recombinant, full-length NDC80 complexes for use in total internal reflection fluorescence microscopy (TIRFM)-based microtubule binding assays. Single molecule experiments revealed that NDC80 complexes with Hec1-92N exhibited 2–3-fold higher landing rates and increased dwell times on microtubules relative to wild-type, resulting in significantly increased binding affinity. Importantly, the Hec1-92N NDC80 complexes did not exhibit increased oligomerization or clustering at higher concentrations. These results suggest that direct contact between the Hec1/Ndc80 tail domain and the microtubule lattice increases the binding affinity of NDC80 complexes for spindle microtubules, thus promoting stable kinetochore-microtubule attachment and force transduction at kinetochores.

B542/P0525

The MAST kinase KIN-4 carries out mitotic entry functions of Greatwall in C. elegans

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Mitotic entry is triggered by the Cyclin B-Cdk1 kinase, which phosphorylates numerous substrates. The accumulation of these phosphorylated substrates depends on inhibiting the opposing PP2A-B55 phosphatase. Activated by Cyclin B-Cdk1, the Greatwall kinase (MAST-L, Microtubules Associated Serine Threonine-like) an atypical member of the MAST kinase family, phosphorylates ENSA/ARPP19 (Endosulfine alpha / cAMP regulated phosphoprotein 19), turning them into specific inhibitors of PP2A-B55. The Cyclin B-Cdk1 complex thus neutralizes its opposing phosphatase and enables mitotic entry and progression. However, how the balance between kinase and phosphatase activity is regulated by developmental cues to ensure asynchronous mitotic entry during development is still poorly understood. With its asynchronous and stereotyped cell divisions, the C. elegans embryo is an attractive model for dissecting how the cross-talk between Cyclin B-Cdk1 and PP2A-B55 ensures correct cell cycle length during development. Yet, the mechanisms that control PP2A-B55 activity have remained elusive in C. elegans, as its genome encodes no obvious homolog of the Greatwall kinase. Using a combination of genetic, biochemical, and functional assays, we show that the single worm MAST kinase, KIN-4, fulfills the role of Greatwall in C. elegans. We demonstrate that KIN-4 phosphorylates ENSA-1 on its conserved DSG motif to inhibit PP2A-B55, thereby controlling the timing of mitotic entry. Loss of PP2A-B55 regulation alters the stereotyped asynchrony between the AB and P1 blastomeres, underscoring the importance of this pathway during embryonic development. Remarkably, KIN-4 is constitutively active when expressed in bacteria and can functionally replace Greatwall in Xenopus extracts, indicating both mechanistic divergence and evolutionary conservation within the MAST kinase family. Our findings resolve a long-standing puzzle regarding the apparent absence of a Greatwall-like pathway in C. elegans. This work also

highlights C. elegans as a powerful model to explore the developmental regulation of kinase–phosphatase balance and its implications in cell cycle timing regulation during development.

B530/P0513
The Role of γ-Tubulin Ring Complex in Mitotic Spindle Assembly and Minus-End Stabilization
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The mitotic spindle is a microtubule (MT)-based structure that mediates accurate chromosome segregation during cell division. This dynamic structure relies on the coordination of multiple proteins to ensure correct assembly, stability, and function. The γ-Tubulin Ring Complex (γ-TuRC) plays a pivotal role in nucleating the MTs that comprise the spindle. γ-TuRC is composed of γ-tubulin and one or more copies of the subunits GCP2, GCP3, GCP4, GCP5, and GCP6. It localizes to spindle poles and promotes MT growth from the minus ends during spindle assembly. To better understand the specific contributions of individual γ-TuRC subunits to spindle dynamics, we endogenously tagged the γ-tubulin complex proteins GCP2, GCP4, and GCP6 independently with an Auxin-Inducible Degron (AID), enabling precise and rapid depletion of each protein. Depletion of any single subunit resulted in the loss of γ-TuRC localization at spindle poles and prometaphase arrest when depletion occurred before mitotic entry. In cells with preformed spindles, γ-TuRC depletion triggered rapid spindle collapse, highlighting the role of γ-TuRC in the maintenance of spindle integrity. Furthermore, we found that depletion of Kif2A, a microtubule-depolymerizing kinesin, mitigated spindle collapse after γ-TuRC loss, suggesting that Kif2A activity exacerbates spindle instability in the absence of γ-TuRC. These findings highlight the dual role of γ-TuRC in mitosis: not only acting as a critical nucleation factor for MT assembly but also stabilizing and capping the minus ends of spindle MTs to preserve spindle integrity after its initial formation. In future studies, we aim to investigate the specific mechanisms by which γ-TuRC caps and stabilizes spindle MT minus ends, to deepen our understanding of its role in maintaining spindle stability during cell division.

B556/P0539
Two cancer cell lines utilize Myosin 10 and the kinesin HSET differentially to maintain mitotic spindle bipolarity
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Cancer cells often undergo mitosis possessing more than two centrosomes. To avoid a multipolar mitosis, the consequences of which are typically aneuploidy induced senescence, they must cluster their extra centrosomes to create a pseudo-bipolar spindle. Such supernumerary centrosome clustering (SNCC) requires Myosin 10 (Myo10) and the pole-focusing kinesin HSET. We showed recently that Myo10 promotes SNCC in HeLa cells by promoting retraction fiber-based cell adhesion, and that it further supports spindle bipolarity by preventing the generation of extra spindle poles via pericentriolar material (PCM) fragmentation. Here we quantified the contribution that Myo10 and HSET make individually and together to SNCC and PCM/pole integrity in HeLa cells and in MDA-MB-231 cells, which differ from HeLa in being more dependent on SNCC and less dependent on retraction fiber-based cell adhesion. As expected, knockdown of Myo10 and HSET individually increased the frequency of multipolar spindles in both cell types. Their effects were surprisingly not additive, however. For HeLa and MDA-MB-231 cells undergoing mitosis with more than two centrosomes, the defect in SNCC was almost entirely responsible for their multipolar phenotype following knockdown of

either Myo10 or HSET. For HeLa and MDA-MB-231 cells undergoing mitosis with two centrosomes, PCM/pole fragmentation was the primary cause of multipolar spindles following HSET knockdown. Unlike HeLa, however, MDA-MB-231 cells exhibited very little PCM/pole fragmentation following Myo10 knockdown. This difference may be due to the smaller role that Myo10 plays in retraction fiber-based adhesion in MDA-MB-231. Finally, we show that HSET knockdown disrupts retraction fiber formation and organization, which may explain why the defects in double knockdown cells were not significantly greater than in HSET knockdown cells. These and other results can inform efforts to target these two motor proteins to selectively kill cancer cells by increasing their frequency of multipolar divisions.

B536/P0519

Uncoupling Methyltransferase-Dependent and -Independent Functions of SUV4-20H2

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Mitotic centromeres are transcriptionally active, with the resulting ncRNA transcripts serving to tether and regulate key mitotic proteins. Additionally, the establishment of centromere/pericentromere boundaries, where repressive epigenetic marks are enriched at pericentromeres but absent from the centromere, has been proposed to limit mitotic transcription to the centromere, a feature that would enable centromere/kinetochore enrichment of interacting proteins. Our previous work explored this model, demonstrating that persistent centromere localization of SUV4-20H2 (a histone methyltransferase that catalyzes the repressive mark H4K20me3) is sufficient to repress centromere transcription and corrupt mitotic fidelity. Interestingly, we found this effect to be at least partially independent of methyltransferase activity. To better understand the methylation-dependent and -independent functions of SUV4-20H2 in centromere regulation, we have functionally characterized SUV4-20H2 truncations that include or exclude the SET domain (which confers methyltransferase activity). We find that sustained centromere localization of the full-length protein (cen-SUV4-20H2-GFP) and the C terminus (cen-SUV4-20H2AA281–462 - GFP, which lacks methyltransferase activity) but not the N terminus of SUV4-20H2 (cen-SUV4-20H2AA1–280-GFP, which retains methyltransferase activity) promotes chromosome segregation errors. SUV4-20H2 has been demonstrated to interact with Heterochromatin protein 1 (HP1) via its C terminus. Consistent with this, we find that SUV4-20H2 constructs that retain the C terminus sustain HP1 interactions, even in mitosis when the majority of HP1 is stripped from chromatin. Together, these data suggest that SUV4-20H2 contributes to centromere/pericentromere regulation and mitotic fidelity in two distinct ways: by catalyzing the placement of repressive epigenetic marks at the pericentromeres to limit mitotic transcription and, independently, by recruiting key heterochromatin proteins.

B543/P0526

Uncoupling of spindle elongation and chromosome segregation dynamics during embryonic development

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The development of eukaryotic cells requires accurate coordination of several cellular machineries. A central question in cell biology is how embryos adapt these mechanisms as cell size decreases during development. In this study, high-resolution lattice light sheet fluorescence microscopy and automated image analysis were used

to quantify mitotic spindle and chromosome segregation dynamics from 2-cell to 64-cell stages in Caenorhabditis elegans embryos. Our results show that spindle length robustly scales with cell size in both wild-type and size-perturbed embryos, while chromosome segregation dynamics remain mostly invariant, suggesting that these processes are governed by distinct scaling mechanisms. Integration of femtosecond laser ablation and electron tomography revealed that central spindle microtubules mediate chromosome segregation and remain uncoupled from cell volume through development, whereas spindle elongation relies on cortically anchored motor proteins and astral microtubules, hence it is more dependent on cell size. We incorporated this data into an extended stoichiometric force-balance model, demonstrating that modulating microtubule catastrophe rates reproduces spindle elongation dynamics across developmental stages. This work supplies a mechanical map of the spindle, revealing a crucial uncoupling between spindle elongation and chromosome segregation scaling, important for preserving chromosome segregation fidelity even as cell size changes. Collectively, these results provide a comprehensive understanding of the mechanisms governing spindle elongation and chromosome segregation in C. elegans embryos, shedding light on the regulation of mitosis and how embryonic cells adapt mitotic machinery to reductions in cell size during development. This study advances mechanistic insight into the differential regulation of conserved force-generating modules, further elucidating how embryos maintain division fidelity across varied cell sizes.

B524/P0507

Untimely Fibrous Corona Expansion Driven by MPS1 Overexpression Underlies Chromosomal Instability in Colorectal Cancer

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Chromosomal Instability (CIN) is a prevalent feature of human cancers involved in tumour progression and intimately associated with poor patient prognosis. Yet, the mechanistic cause of CIN remains poorly understood. Here, we narrow this knowledge gap by establishing MPS1 overexpression as a driver of CIN and by uncovering the underlying molecular details. RNA-sequencing of colorectal tumours disclosed a correlation between increased aneuploidy and overexpression of the mitotic checkpoint kinase MPS1. Functional studies in patient-derived colon cancer cells (PCCC) revealed an elevated frequency of merotelic kinetochore-microtubule attachments and chromosome segregation errors (lagging chromosomes) in CIN+ cells expressing higher levels of MPS1. Experimentally inducing MPS1 overexpression in RPE-1 and low-passage MSI cells (CIN-) phenocopied these defects and promoted continuous karyotypic heterogeneity and micronuclei formation in previous near-diploid stable cultures. To determine the mechanism by which MPS1 drives CIN, we screened for phospho-epitopes responsive to MPS1 overexpression. The phosphorylation of ROD Thr13/Ser15, required for fibrous corona assembly, stood out as being uncommonly elevated at metaphase kinetochores of MPS1-overexpressing cells. Accordingly, we observed an abnormal accumulation of SPINDLY, CENP-F and ZW10, suggesting that the fibrous corona was atypically expanded. Strikingly, preventing fibrous corona assembly through inhibition of Spindly farnesylation with FTI-277, or partial inhibition of MPS1 activity with suboptimal concentrations of Cpd-5, efficiently decreased the frequency of merotelic attachments and restored the fidelity of chromosome segregation in both RPE-1 cells overexpressing MPS1 and PCCC CIN+ lines. Our results demonstrate that an untimely expanded fibrous corona, driven by excessive MPS1 activity, provides a structural basis for merotely, chromosome missegregation and CIN. These findings not only advance our

understanding of the mechanisms driving CIN but also underscores the potential significance of targeting MPS1 and the fibrous corona as therapeutic strategies in colorectal cancer.

Cell Division and Cell Cycle Control - Spindle Assembly

B505/P0488
Analysis of HURP and CAPS interactions during mitosis
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Calcyphosine (CAPS) is a calcium-regulated, tubulin-binding protein that is associated with the central microtubules of the mitotic spindle. CAPS appears important for proper spindle formation and chromosome segregation because siRNA knockdown of CAPS in cultured U251-MG and HEK293 cells increases the percentage of cells with uncongressed chromosomes and multipolar spindles. In a high-throughput mass spectrometry pulldown screen performed by Huttlin et al. 2021, CAPS co-precipitated with Hepatoma Upregulated Protein (HURP), a spindle-associated protein with loss-of-function phenotypes similar to those of CAPS (Wong & Fang, 2006; Sillje et al., 2006; Zhang et al., 2018). The ability of these proteins to co-precipitate, their shared localization on the central microtubules of the mitotic spindle, and their similar loss-of-function phenotypes suggest that HURP and CAPS may collaborate in the cell to perform their functions. This research project aims to identify the physical and functional interactions between CAPS and HURP. Using pulldowns from transfected HEK293 cell lysates, we show that CAPS and HURP co-precipitation is dependent on calcium, suggesting that CAPS must be in its calcium-bound conformation in order for it to bind (Dong et al., 2008). AlphaFold4 modeling predicts that CAPS and the tubulin-binding domain of HURP bind to opposite sides of tubulin dimers, suggesting that they may associate through tubulin, rather than directly to one another. We are now using proximity labeling and in vitro binding to refine our model of how CAPS, HURP, and tubulin dimers associate with one another. Collectively, these experiments investigate how CAPS and HURP work together to aid in spindle formation and function.

B499/P0481
Characterizing a Novel Class of Antimitotic Compounds
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Microtubule-stabilizing or -destabilizing agents (e.g., paclitaxel) are effective against rapidly dividing cells, such as cancer cells but often cause dose-limiting neurotoxicity because they directly target tubulin, which is intrinsically linked to neurological side effects. This has prompted interest in developing drugs that disrupt microtubule dynamics through alternative targets that are more specific to mitotic cells. In an unrelated screen, we identified four structurally related small molecules (WUF1–WUF4) that impair the microtubule-organizing center (MTOC) and spindle morphology during prometaphase. Treatment leads to loss of astral microtubules, altered spindle length, chromosome misalignment, and subsequent multinucleation, consistent with disruption of a regulator of spindle assembly. These findings suggest that the compounds target a regulator of the mitotic spindle during mitosis. Using synchronized prometaphase cells and the peptide-centric local stability assay (PELSA)—which detects ligand-induced protection of protein regions from proteolysis—we identified potential MTOC-associated mitotic regulator(s) as candidate targets of WUF compounds. Ongoing

studies aim to validate the putative drug target and to uncover the mechanism of action. We will then evaluate the antitumor potential of these compounds as mitosis-selective chemotherapeutic agent with a reduced risk of neurological liabilities.

B500/P0482

Differential contributions of beta-tubulin isotypes to spindle assembly and chromosome separation dynamics during C. elegans acentrosomal oocyte meiosis

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Assembly of bipolar spindles without centrosomes, a universal feature of oocyte meiosis, involves coordination between microtubules (MTs) composed of several different α- and β-tubulin isotypes, MT associated motors and other MT-interacting proteins (MAPs). Here we use high-resolution immunofluorescence microscopy and live imaging of Caenorhabditis elegans strains with altered β-tubulin isotype composition to investigate the contributions of and interplay between MT composition, motors, and MAPs during oocyte meiotic spindle assembly and chromosome segregation. We found that “TBB-2 only” oocytes (containing only one of two partially-redundant germline-expressed β-tubulin isotypes) exhibit a synthetic genetic interaction with klp-18(ts), a missense mutation causing partial loss-of-function of the plus-end directed kinesin-12 MT cross-linking motor KLP-18, resulting in a high incidence of embryo lethality, X-chromosome missegregation, and monopolar oocyte spindles. Moreover, these synthetic spindle assembly and chromosome segregation defects can be suppressed either by a tbb-2(E439K) mutation that confers partial resistance of MTs to the MT-severing enzyme katanin or by a mei-2(A235T) mutation that decreases katanin activity. We further found that both spindle length and the spatial distribution of katanin along spindles are affected by β-tubulin isotype composition, with “TBB-1 only” oocytes exhibiting longer meiotic spindles and a dearth of katanin at spindle centers relative to poles. Through live imaging, we uncovered hidden contributions of β-tubulin isotypes to spindle morphology and chromosome separation dynamics. We found that TBB-2-only oocyte spindles achieve bipolar metaphase with normal timing despite assembling shorter spindle structures, and they exhibit accelerated spindle elongation and 67% faster chromosome separation velocities during anaphase B. Together, our data support a model in which β-tubulin isotype composition helps to maintain a balance between microtubule crosslinking and severing activities during oocyte meiosis. We further propose that this balance is crucial for establishing spindle bipolarity, maintaining spindle structures, and modulating the dynamics of chromosome separation.

B504/P0487

Effect of kinesin-5 inhibition on k-fiber length and architecture in RPE-1 cells

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In mitosis, the bipolar metaphase spindle is important to ensure proper chromosome segregation. This complex structure arises from an interplay of microtubules and opposing molecular motors in which forces are carefully balanced in a steady-state. This balance enables proper chromosome alignment, mitotic error correction, and kinetochore microtubule (KMT) guidance towards the centrosomes, as described by an active liquid crystal theory. In previous work, we demonstrated that both KMTs and non-KMTs in human mitotic

metaphase spindles are strongly aligned along the same orientation field. The trajectories defined by this field connect the kinetochores to the spindle poles. By perturbing the mitotic force balance through the inhibition of the plus-end directed motor protein kinesin-5 (Eg5) using the small molecule monastrol, the spindle transitions into a monoastral configuration. There, the two centrosomes are in the cell center surrounded by chromosomes. Interestingly, we found that there are two different configurations the kinetochore-fibers (k-fibers) can adapt in the cells: an untwisted, and a twisted configuration. Currently, we are investigating why and when cells adapt these configurations when treated with monastrol. Preliminary evidence suggests that there is a correlation of incubation time and the occurrence of the twisted configuration. Additionally, we show that the length of k-fibers is affected by the angle that they adapt regarding to the center of the cell. We explain this dependency by the formation of a domain-wall, a zone within these monoastral cells that forms around the central part of the cell. There, a “mini-spindle” forms from the two centrosomes. The difference in the microtubule order of alignment in the center of the cell and the surrounding region presumably precludes k-fibers from growing further and attaching to the centrosomes directly. This observation confirms our previous finding that existing microtubules are crucial to guide KMTs towards the centrosome and a disturbance of the nematic order will prevent them from growing further.

B498/P0480

Innovation of a new experimental system to discover the origins of female reproductive age-related egg aneuploidy

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Female reproductive aging is associated with a remarkable increase in the incidence of oocyte aneuploidy, which is a leading cause of pregnancy loss, infertility and genetic disorders. Premature loss of meiotic chromosome cohesion leading to untimely separation of chromosomes is thought to underly high chromosome segregation error rates in oocytes. Cohesion depletion in oocytes occurs gradually over female reproductive lifespan. It is thus conceivable that age-related dysfunction of other meiotic mechanisms contributes to the dramatic rise in oocyte aneuploidy at advanced reproductive ages. To discover proteins whose dysfunction accelerates aneuploidies associated with female reproductive aging, we have innovated a new mouse model where Proteolysis Targeting Chimera (PROTAC)-driven depletion of meiotic chromosome cohesion generates oocyte aneuploidies that are prevalent in reproductively older females. In a distinct approach, we coupled nanobodies with TRIM-Away, a powerful protein degradation tool we routinely use to study protein function in oocytes, to induce in this experimental system female reproductive age-related oocyte aneuploidies. Moreover, we applied high-resolution live imaging to visualize, for the first time, the dynamics of cohesion proteins underpinning meiotic chromosome segregation. Our advanced live imaging assays and 3D tracking of chromosomal dynamics revealed that this versatile system allows generation of age-dependent chromosomal phenotypes in young oocytes within 90 minutes, a process that would typically take 1-2 years in mice or decades in humans. We further demonstrate that this experimental system holds strong promise for future screening of factors that exacerbate oocyte chromosomal abnormalities at advanced reproductive ages. Our proof-of-concept experiments identified that acute disruption of the centromeric protein CENP-A in oocytes containing experimentally reduced cohesion significantly increases the incidence of oocyte aneuploidy. Collectively, our findings establish a high-precision experimental system for manipulating chromosome cohesion, offering unique opportunities to elucidate the molecular origins of oocyte aneuploidy. Ultimately, this approach could facilitate the discovery of therapeutic strategies to counteract chromosomal abnormalities in oocytes, improving outcomes in assisted human reproduction.

B502/P0485

Leveraging polyploid Xenopus for probing microtubule spindle adaptation to increases in chromosome number

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Although polyploidy is a common occurrence in plants, fish, and amphibians, all known extant mammals are diploid. Currently, the mechanisms or factors that stabilize the formation of viable polyploid cells are poorly understood. It stands to reason that cell division could be a major barrier to the formation of neopolyploid organisms. The microtubule spindle facilitates the equal partitioning of chromosomes in mitosis and meiosis, but has already been shown to be error-prone and sensitive to changes in chromosome number. To precisely test how the microtubule spindle may adapt to sudden changes in ploidy, we have utilized the Xenopus egg extract system to form microtubule spindles around nuclei of varying ploidies while maintaining an identical cytoplasmic context. Serendipitously, a wide range of ploidy (2N to 12N) exists within the Xenopus genus; by combining cytoplasmic egg extracts with isolated embryo nuclei, we have generated “mismatched” microtubule spindles with up to a six-fold increase in ploidy relative to their native conditions. Our fixed and live imaging show that bipolar spindle assembly decreases significantly as nuclei ploidy is increased. In addition, any bipolar spindles observed in these extracts display distinct morphological changes that scale with ploidy. Moreover, we have successfully generated egg extracts from dodecaploid Xenopus longipes; these extracts rescued bipolar spindle assembly with polyploid nuclei and were morphologically distinct from spindles generated in Xenopus tropicalis (2N) or Xenopus laevis (4N) extract, regardless of nuclei source. We predicted that specific microtubule-associated proteins (MAPs) were driving these morphology changes and visualized the localization of 10 well-characterized MAPs within these "mismatched" spindles. Further investigation of the protein sequence and levels of these MAPs in X. longipes eggs has revealed altered primary sequences at regulatory domains and/or altered levels compared to X. tropicalis or X. laevis eggs. Taken together, our results suggest that the microtubule spindle can, to some degree, compensate for sudden ploidy increases and have exciting implications for how microtubule spindles can be fine-tuned on an evolutionary timescale to be more or less permissible to polyploidization events.

B507/P0490

Mechanics of Spindles in Human Cells: Towards an Understanding of the Forces that Segregate Chromosomes

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Chromosome congression and segregation are dynamic, mechanical processes whose accurate execution is essential for successful cell division. Chromosome motion is a balance of two factors: external forces and resistance from material properties. Understanding the mechanical mechanisms of chromosome segregation requires accurate measurements of both these factors. There are few such measurements to date, however, due to the challenges of exerting and measuring forces in living cells. We use two approaches in human tissue culture cells: magnetic tweezers and deformable elastic microgels. With the magnetic tweezer, we exert calibrated forces on paramagnetic beads positioned inside spindles. We find that the spindle is viscoelastic, with a spatially varying viscoelasticity, which can largely be explained by spatial variations in microtubule density. With the elastic microgels, we characterize forces exerted by the spindle, both internal and external to the spindle. With these measurements combined we hope to resolve the mechanical mechanisms of chromosome congression and segregation.

B506/P0489

Molecular Characterization of the Putative Spindle Assembly Checkpoint Regulator CDK15

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During mitosis, cells are highly regulated to ensure the proper division of genetic material into two daughter cells. This process involves several protein-protein interactions, and each event is temporally controlled to drive chromosomal segregation forward. The spindle assembly checkpoint (SAC) ensures accurate chromosome segregation by monitoring microtubule–kinetochore attachment and regulating the metaphase-to-anaphase transition. While many mitotic factors have been identified, the mechanisms that regulate SAC function remain incompletely understood. Here, we have characterized the kinase Cdk15 as a potential regulator of the SAC. From preliminary proteomic analyses of CDK15 using proximity-based approaches coupled to mass spectrometry, we identified several SAC-associated interactors of CDK15 which have been validated by in-vitro immunoprecipitation assays. Preliminary immunofluorescence studies show that CDK15 localizes to the nucleus and cytoplasm in interphase and potentially the kinetochores during mitosis. Depletion of CDK15 also resulted in chromosome congression and segregation errors. Collectively, this work will provide insights into the mechanism and regulatory role of CDK15 during cell division as well as form the basis for designing novel therapeutic approaches to address diseases associated with abnormal proliferation.

B503/P0486

PLK4 employs its cryptic polo box to scaffold the formation of pseudo-centrosomes that accelerate acentriolar spindle assembly

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Segregation of replicated chromosomes during mitosis requires rapid assembly of a bipolar microtubule-based spindle. In metazoans, this is accomplished by a pair of centrosomes, composed of a centriolar core surrounded by a pericentriolar matrix, that catalyzes the assembly of spindle microtubules. In human cells that lack centrioles due to chemical inhibition of the centriole duplication kinase PLK4, spindle assembly occurs through the slow self-organization of pericentriolar matrix and takes about twice as long as in cells with centrosomes. Loss of the ubiquitin ligase TRIM37 restores rapid bipolar spindle assembly in PLK4i-treated acentriolar cells by enabling the formation of an array of foci that function as pseudo-centrosomes (PCs). PCs nucleate microtubules and robustly cluster to form two spindle poles. PCs contain proteins proximal to PLK4 in the centrosome including CEP192, CEP152, CEP63 and the centriolar satellite protein PCM1, and are reminiscent of PLK4-containing foci required for naturally occurring acentriolar spindle assembly during the early mitotic divisions of mouse embryos. Using inducible gene knockouts in PLK4i-treated TRIM37Δ cells, we found that PLK4 is the only centrosomal component of 8 tested that was required for PC formation. The pericentriolar matrix scaffold protein CDK5RAP2 was dispensable for both PC formation and function. CEP192, while dispensable for PC formation, was essential for their ability to support spindle assembly. To determine how PLK4 supports PC formation, we replaced endogenous PLK4 with mutants engineered to disrupt known features. This analysis revealed that PC formation does not require PLK4 kinase activity but requires the K/R crater in its cryptic-polo box (CPB). Although point mutations in the CPB K/R crater, which mediates binding to the centriolar PLK4 receptors CEP192 and CEP152, prevented PC formation, single or double depletion of

CEP192 and CEP152 did not. Preliminary results suggest that the intrinsically disordered linker regions, L1 and L2, that flank the CPB on either side are also essential for PC formation. Thus, the PLK4 CPB, its K/R crater, and the disordered linkers that flank the CPB are essential for PC formation independent of the ability of this region to interact with CEP192, which confers microtubule-nucleating function, and CEP152. We speculate that PLK4-scaffolded PCs are analogous to structures that promote acentriolar spindle assembly in specific natural contexts. PCs may also serve as a useful proxy for investigating the CDK5RAP2-independent pathway for spindle microtubule generation that supports robust spindle assembly in human cells.

B508/P0491

Role of Ewing sarcoma proteins in microtubule structure and drug sensitivity

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The driving hallmark of Ewing sarcoma (ES) is the t(11:22) chromosomal translocation, generating two aberrations of the EWS protein: the loss of an EWS allele and expression of EWS/FLI1 fusion protein, denoted as EWS aberrations. Previous work identified EWS and EWS/FLI1 as proteins involved in mitosis and chromosome segregation. We hypothesized that EWS and EWS/FLI1 localize to the mitotic spindle, and EWS knockdown and EWS/FLI1 induce spindle damage and microtubule disruptor sensitivity. To test this hypothesis, we established a DLD-1 colorectal cancer line enabling conditional exogenous EWS/FLI1 expression with doxycycline (Tet-On system), single-allele EWS knockdown with auxin (auxin-inducible degron system), or a combination of both (EWS/FLI1-EWS KD). Immunofluorescence revealed that EWS and EWS/FLI1 localize to mitotic spindle tubulin. We probed cells with an antibody known as MB11 that visualizes microtubule damage and repair sites, demonstrating mitotic spindles of cells with induced EWS/FLI1 expression or EWS knockdown exhibit increased spindle tubulin damage. When the EWS/FLI1-EWS KD cells were treated with nocodazole, the rate of cell death increased compared to control nocodazole-treated cells. Taken together, our data support our hypothesis and suggest that the combination of EWS/FLI1-EWS KD increases microtubule damage sites, sensitizing cells to microtubule disruption by nocodazole. ES cell lines also exhibited a higher level of mitotic spindle tubulin damage and nocodazole sensitivity than mesenchymal stem cells, the suspected cell of ES's origin. To determine if EWS/FLI1-EWS KD induced spindle tubulin damage and nocodazole sensitivity is dependent on EWS activity, we established two DLD-1 colorectal cancer lines. The first enables conditional exogenous wildtype EWS overexpression with doxycycline (Tet-On system), homozygous EWS full knockdown with auxin (auxin-inducible degron system), or combination of both (EWS full knockdown-EWS add back). The second enables conditional exogenous R565A EWS expression (mutant with low Aurora B affinity) with doxycycline (Tet-On system), homozygous EWS full knockdown with auxin (auxin-inducible degron system), or combination of both (EWS full knockdown-R565A EWS add back). MB11 staining revealed that EWS full knockdown increases mitotic spindle tubulin damage and is rescuable by EWS add back, but not R565A EWS add back. These results indicate EWS/FLI1-EWS KD induced spindle tubulin damage is dependent on EWS activity and EWS-Aurora B interaction.

B501/P0483

The Eg5 motor, not spindle bipolarity, is responsible for assembling and maintaining interdigitated, anti-parallel microtubule bundles in metaphase spindles

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During cell division, the spindle is responsible for segregating a single copy of each chromosome to each daughter cell. The spindle is a football-shaped bipolar structure composed of dynamic, polar microtubules that interact with molecular motors to assemble the spindle. In previous serial-section resin-embedded cellular electron tomography reconstructions, we observed that the spindle is composed of relatively short microtubules (2 µm mean length vs 10 µm pole-pole spindle length) and that the majority of these microtubules do not connect to either spindle pole. It is not well understood how interactions between these short microtubules, which are not directly anchored to the spindle poles, and molecular motors assemble a bipolar spindle and segregate chromosomes. A critical limitation is that resin-embedded electron tomography does not achieve high enough resolution to determine the polarity of individual microtubules or localize motors, which is crucial for understanding how motor-microtubule interactions assemble the spindle. To overcome this, we utilized FIB-milled, cellular cryo-electron tomography to reconstruct thin cross-sections of the spindle at near-atomic resolution and measure the polarity of individual microtubules. We found that the spindle is composed of dense bundles where ~50% of microtubules are anti-parallel with their neighbors. To understand whether these anti-parallel bundles were formed indirectly by branching nucleation from the spindle poles or directly by interactions with molecular motors, we reconstructed microtubule bundles in monopolar spindles formed either by inhibiting centriole duplication or by inhibiting the Eg5 motor. We found that centriole-depleted monopolar spindles, in which the Eg5 motor is still active, maintained anti-parallel, interdigitating microtubules while Eg5-inhibited monopolar spindles did not. This suggests that Eg5, rather than bipolarity, is required for the formation and/or maintenance of anti-parallel bundles. Additionally, we observed in live-cell fluorescence microscopy that the motor-active centriole-depleted monopolar spindles often self-organized a second spindle pole after initial monopole formation, eventually leading to the formation of a bipolar spindle that entered anaphase while the Eg5-inhibited monopolar spindles remained arrested as monopoles. Taken together, our cryoET reconstructions and light microscopy indicate that the Eg5 motor is responsible for anti-parallel microtubule bundle formation and that these anti-parallel bundles are required to maintain spindle bipolarity. This supports a bottom-up, self-organized picture of spindle assembly, where motor-driven interactions assemble microtubule bundles, which in turn organize the spindle geometry.

Cell Metabolism - Energy metabolism and bioenergetics

B323/P0901
A Novel Seahorse XF Assay for Measuring Mitochondrial Function in Matrigel-Embedded Organoids
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Mitochondria are essential organelles that drive bioenergetic metabolism and support a wide range of cellular processes, including proliferation, differentiation, activation, and cell death. Real-time measurement of the oxygen consumption rate (OCR) is a widely used method for assessing mitochondrial function in cultured cells. However, most previous studies have focused on two-dimensional cell cultures or self-organizing spheroids, limiting physiological relevance. This study presents a novel protocol for measuring cellular respiration in organoids cultured within Matrigel using the Seahorse XF Flex Analyzer. A newly designed 24-well XF Flex Organoid Microplate enables OCR measurements from organoids derived from in vitro cultured cells, established organoid lines, and tissue fragments, under well-validated conditions. The feasibility of this platform was demonstrated through multi-day cultures of tumor organoids and normal hepatic organoids, derived from cancer cell lines and mouse hepatic progenitor fragments, respectively. Key metabolic parameters—including basal respiration, maximal respiration, and spare respiratory capacity—were

successfully quantified. To address well-to-well variability caused by differences in organoid size and number, OCR data were normalized using microscopic image analysis and luminescence-based viability assays. This was facilitated by the microplate's design, which is highly compatible with both microscopy and plate readers. In summary, this new assay platform offers a reliable and reproducible method for evaluating mitochondrial function and metabolic phenotypes in organoids that require matrix scaffolds for growth and differentiation.

B324/P0902

An adult clock regulator links circadian rhythms to pancreatic ß-cell maturation

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Circadian clocks align metabolism with daily energy cycles, but how they program the functional maturation of differentiated cells is poorly understood. In this study, we show that DEC1, a clock transcription factor induced in adult islet β cells, coordinates their glucose responsiveness by synchronizing energetic and secretory rhythms. DEC1 expression distinguishes mature from immature β cells across species, cycling daily in adult mouse and human islets. Using the glucose tolerance testing, indirect calorimetry, static and dynamic glucose-stimulated insulin secretion (GSIS), and live metabolic assays, It is found that loss of DEC1, in either whole-body or β-cell–specific mice, impairs GSIS, causing persistent glucose intolerance and insulin deficiency despite normal islet architecture and intact Clock/Bmal1 genes. Mechanistically, DEC1 binds and synchronizes the rhythmic transcription of genes controlling glucose import, mitochondrial dynamics, respiration, and vesicle exocytosis. Its absence desynchronizes these programs, uncoupling insulin secretion from glucose metabolism and yielding a fetal-like phenotype with lowered glucose threshold and blunted oxidative capacity. Bulk and single-cell RNA-seq, ATAC-seq, and single-cell proteomics revealed suppression of mature β-cell states, rewiring of metabolic and secretory networks, and disrupted intercellular signaling. Proximity-labeling studies identified metabolic and secretory effectors as DEC1 interactors, while human islet DEC1 knockdown recapitulated GSIS defects. Time-of-day CUT&RUN confirmed rhythmic DEC1 binding to metabolic and secretory gene promoters. Finally, pharmacologic enhancement of glycolytic and mitochondrial flux restored GSIS to or above wild-type levels. Together, our results indicate that DEC1 integrates circadian timing with β-cell metabolic specialization, synchronizing bioenergetics and insulin secretion to establish mature function. These findings reveal DEC1 as a key hierarchical regulator of β-cell maturation and suggest that entraining circadian metabolism may improve the functional maturation of stem cell–derived islets for diabetes therapy.

B327/P0905

AVID Mouse: A Genetically Encoded Tool for Real-Time Multiscale ATP Imaging and Spatial Systems Metabolism in Living Mice

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ATP is the universal energy currency, but spatiotemporal changes in ATP levels across organs and cell types in vivo have remained difficult to capture. We developed the AVID (ATP Visualization In vivo Directly) mouse, a genetically encoded FRET-based biosensor model that enables real-time, multiscale ATP imaging across whole-body, organ, and cellular levels. AVID mice ubiquitously express the GO-ATeam2 sensor from the ROSA26 locus and exhibit normal development and energy expenditure. Using this model, we visualized physiological ATP distribution in over 10 abdominal and thoracic organs simultaneously. High-resolution two-photon microscopy

revealed sub-organ ATP gradients, including within hippocampal neurons and intestinal crypt–villus structures. Importantly, millisecond-resolved imaging captured rapid ATP depletion and recovery in skeletal muscle during contraction, confirming the system's high temporal resolution. To assess pathophysiological applications, we imaged ATP dynamics after myocardial infarction. Unexpectedly, we observed rapid, systemic ATP reductions not only in the heart but also in the liver, intestine, and kidney. Notably, ATP depletion in the intestine occurred within 15 minutes post-infarction, preceding changes in the liver, suggesting early organ-organ metabolic communication. Spatially resolved two-photon imaging further revealed localized ATP depletion near the central vein of the liver, a zone prone to hypoxia. Mass spectrometry imaging showed concurrent accumulation of kynurenic acid in this area, which was undetectable by bulk metabolomics. This highlights the AVID mouse's ability to identify early, region-specific metabolic remodeling invisible to conventional methods. In conclusion, the AVID mouse establishes a versatile and quantitative platform for spatial systems metabolism. It enables discovery of localized, transient metabolic shifts across physiological and disease contexts, including heart failure and neurodegeneration, with broad utility for in vivo metabolism research, drug development, and systems biology.

B359/P0937
DEC1 Regulates -Human ß-cell Functional Maturation and Circadian Rhythm
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Stem cell-derived islet (SC-islet) organoids offer hope for cell replacement therapy in diabetes, but their immature function remains a challenge. Mature islet function requires the β-cell circadian clock, yet how the clock regulates maturation is unclear. Here, we show that a circadian transcription factor specific to maturing SC-β cells, DEC1, regulates insulin responsiveness to glucose. SC-islet organoids form normally from DEC1-ablated human pluripotent stem cells, but their insulin release capacity and glucose threshold fail to increase during in vitro culture and upon transplant. This deficit reflects downregulation of maturity-linked effectors of glucose utilization and insulin exocytosis, blunting glycolytic and oxidative metabolism, and is rescued by increasing metabolic flux. Moreover, DEC1 is needed to boost SC-islet maturity by synchronizing circadian glucose-responsive insulin secretion rhythms and clock machinery. Thus, DEC1 links circadian control to human β-cell maturation, highlighting its vitality to foster fully functional SC-islets.

B326/P0904
Discovery of a novel hexose transporter gene in the saccharomyces cerevisiae "unknome"
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Since the sequencing of the Saccharomyces cerevisiae (budding yeast) genome in 1996 and the human genome in 2003, significant advances have been made in understanding the functions of many genes. However, a substantial number of genes, collectively termed the "unknome," remain functionally uncharacterized. This knowledge gap represents a critical research opportunity, as many of these genes may play important roles in fundamental cellular processes and could be conserved across species. This project focuses on a previously uncharacterized open reading frame (ORF) from the yeast unknome that our bioinformatic analysis suggests is involved in glucose utilization: YFL040W. We selected this candidate based on in silico predictions from tools that identify potential structural homologs. We also conducted preliminary experiments that together suggest a possible role as a hexose transporter. Glucose ($C_6H_{12}O_6$) serves as the

primary energy source for cellular functions across all living organisms, including humans and S. cerevisiae. Due to the impermeable nature of the lipid bilayer that forms cell membranes, glucose cannot simply diffuse into cells. Instead, it requires specialized transport proteins integrated into the membrane to facilitate its transport. These glucose transporters are essential gatekeepers that control access to this energy source. In yeast, hexose transporters (HXT) exist, which function through three pathways mediated by glucose availability and with differing glucose affinities (e.g., high, low, or other). These transporters are crucial for the cellular uptake of hexoses, which serve as carbohydrate glycogen stores, metabolic intermediates, and signaling molecules in the cell. In humans, comparable functions are performed by the GLUcose Transporter (GLUT) and Sodium-GLucose co-Transporter (SGLT) genes. The conservation of these transport mechanisms across species highlights the fundamental importance of glucose uptake in cellular energetics. Preliminary growth assays performed with varying glucose concentrations (0%, 0.01%, 0.05%) confirmed that deleting YFL040W conferred significant growth defects compared to wild-type cells, particularly under low glucose conditions, supporting its involvement in glucose metabolism. Studies using 2-Deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-D-glucose (2-NBDG), a fluorescent glucose analog, also showed a noticeable difference in uptake between YFL040W in comparison to when no 2-NBDG is applied. Altogether, our work will establish a framework that can be adapted for studying unknown genes, contribute to a deeper understanding of conserved biological processes, and potentially identify new pathways relevant to human health and disease.

B325/P0903

Dissecting Novel Mechanisms of Mitochondrial Hyperpolarization in Response to Phosphate Depletion

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Mitochondrial membrane potential (MMP) is a vital indicator of cellular health. While decreased MMP is a hallmark of aging, cardiovascular disease, and neurodegeneration, elevated MMP is linked to increased lifespan in model organisms. We recently showed that depleting inorganic phosphate (Pi) induces a robust MMP increase in mammalian cells.This parallels our findings in yeast, where Pi depletion increases MMP via an OxPhos-independent mechanism, suggesting a conserved Pi-sensing response. However, the mammalian signaling components that execute this response to Pi remain unknown. This represents a critical gap in our understanding of Pi homeostasis and MMP regulation. Therefore, our goal is to elucidate the signaling pathways and molecular players that regulate MMP in response to Pi depletion in mammalian cells. We hypothesized that Pi depletion activates a signaling pathway to activate OxPhos-independent mechanisms to increase MMP. To test this hypothesis, we cultured A375 cells in Pi-depleted media, which induced a 47% MMP increase despite a 90% reduction in total ATP. Supporting an OxPhos-independent mechanism, these cells showed a ~33% reduction in oxygen consumption, and the MMP increase was not prevented by the ATP synthase inhibitor oligomycin, suggesting that ATP hydrolysis by the F_1F_o-ATPase might not be the source of the increase in potential. Additionally, to identify the molecular regulators, we performed a CRISPR screen targeting both mitochondrial genes and the human kinome. This unbiased approach revealed that depletion of cyclin-dependent kinases 1 and 2 (CDK1 and CDK2) was sufficient to increase MMP. Consistent with this finding, we observed that Pi depletion itself causes a decrease in CDK1 and CDK2 at the mRNA (8- and 3-fold) and protein (~90%) levels, providing a direct link between the Pi abundance, these key kinases and MMP regulation. These findings uncover a novel, non-canonical pathway for MMP regulation mediated by CDK1 and CDK2 in response to Pi availability. Future work will focus on validating the necessity and sufficiency of CDK1 and CDK2 in this process. We will dissect their downstream mitochondrial targets using unbiased phosphoproteomics and proximity labeling to identify key regulators. Ultimately, understanding this Pi-CDK1 and CDK2 signaling axis could provide new strategies for therapeutically augmenting mitochondrial function in conditions associated with mitochondrial dysfunction, such as cardiovascular disease and aging.

B328/P0906
Identification of Pdgfrl-associated adipose progenitor populations linked to thermogenic remodeling in aging and obesity
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Obesity is a key driver of type 2 diabetes (T2D) through insulin resistance and adipose tissue dysfunction. White adipose tissue (WAT) and brown adipose tissue (BAT) play critical roles in systemic metabolic regulation. During obesity, chronic caloric excess initially promotes WAT expansion through hyperplasia (new adipocyte formation) and activation of browning/beiging, a thermogenic reprogramming process that dissipates stored energy as heat. Contributing to metabolic balance, BAT specializes in non-shivering thermogenesis by oxidizing fatty acids and glucose to improve lipid handling and insulin sensitivity. However, when adipose tissue surpasses its healthy remodeling capacity, fibrotic and inflammatory signaling disrupts adipogenesis and thermogenic reprogramming, resulting in adipocyte hypertrophy and tissue dysfunction. This maladaptive response produces a hypoxic and inflamed environment that exacerbates metabolic decline and ectopic lipid deposition. The mechanisms governing this balance remain poorly understood. This study identifies cell-type - specific changes in WAT and BAT during aging and obesity-induced prediabetes that impair beige adipogenesis and alter adipocyte responses. Single-cell RNA sequencing revealed Pdgfrl as one of the top differentially expressed genes in aged mouse inguinal WAT. A distinct adipose progenitor population characterized by Pdgfrl expression was also identified, and its abundance decreases with obesity in epididymal WAT. Pdgfrl encodes a predicted secreted protein and known tumor suppressor that is enriched in the stromal vascular fraction (SVF) of both mouse and human BAT. The objective of this study is to determine the role of Pdgfrl in modulating pathways that regulate mesenchymal stromal cell and adipocyte activity as a potential therapeutic target for maintaining metabolic homeostasis and adipose tissue health during aging and obesity.

B329/P0907
Optical Single-Cell Respirometry
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Respiration rate can be readily measured in whole organisms and in bulk cell culture. In contrast, techniques for measuring respiration rate in single cells remain limited. I present initial progress on a method to make optical single-cell measurements of oxygen consumption in tissue culture cells, yeast, and bacteria using phosphorescence lifetime imaging. In this method, cells are trapped in oxygen-impermeable wells micropatterned on a glass coverslip, allowing correlative high-resolution fluorescence imaging with other reporters. I discuss future plans to use this system to address questions of metabolic population heterogeneity and cell-cycle dependent variation in respiration.

B322/P0900

Overexpressing Creatine Transporter 2 increases survival in a heart failure mouse model

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Heart failure (HF) is a common condition that affects approximately 6.7 million adults in the United States alone. Although extensively studied, the mechanisms underlying cardiac metabolism in HF patients remain incomplete. Creatine is essential for maintaining proper cardiac function, as the creatine-phosphocreatine buffer allows for sufficient energy storage and heart contraction. In the endogenous synthesis pathway, L-arginine:glycine amidinotransferase (AGAT) catalyzes the formation of guanidinoacetate (GAA) from arginine and glycine. Guanidinoacetate N-methyltransferase (GAMT) then converts GAA to creatine. From previous studies within the lab, reduced creatine levels and increased GAA levels are observed in HF patients. Previously we have also shown that Methionine adenosyltransferase 2A cardiac knockout (Mat2a cKO) mice exhibit heart failure with decreased creatine and an accumulation of GAA. In this study, we aimed to rescue heart failure symptoms in Mat2a cKO mice by restoring creatine in cardiomyocytes via overexpression of creatine transporters Crt1 or Crt2. We designed a cardiac specific myofibril-AAV (myo-AAV) to overexpress Crt1 or Crt2. Western blots and qPCR then confirmed the myo-AAV's specificity and potency. Crt1 OE did not alter cardiac function or creatine levels. In contrast, Crt2 overexpression mice exhibit improved survival and ejection fraction. Interestingly, metabolomics results showed decreased creatine levels upon Crt2 OE, indicating a creatine-independent survival mechanism. Additionally, given that Crt2 has a GAA efflux role in the kidney, that in human heart failure samples and Mat2a cKO hearts GAA levels are increased, and that GAA accumulation is associated with unfavorable cardiometabolic risk, we hypothesized that the cardioprotective mechanism of Crt2 overexpression is due to a release of GAA buildup. We indeed observed Crt2 OE decreased GAA levels that were accumulated in the hearts of Mat2a cKO mice. Overall, these findings show cardiac specific Crt2 overexpression can mitigate heart failure, likely by reducing GAA levels. Our future studies aim to further elucidate the mechanism behind this cardio-protection and to evaluate Crt2 function in various conditions of creatine and nutrient availability using in vivo heavy isotope tracing.

B321/P0899

Purinosome fuels mitochondrial DNA and RNA synthesis to ameliorate myocardial ischemic injury

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Purinosomes represent cytoplasmic condensates that compartmentalize de novo purine synthesis (DNPS) pathway enzymes to enhance the pathway flux. However, the pathophysiological functions of purinosome remain unexplored. We previously found that ASB11-mediated ubiquitination of DNPS enzyme PAICS drives purinosome assembly. Here, we report that purinosomes are constitutively formed in cardiomyocytes and myoblasts through ASB11 high expression. In these mitochondria-rich cells, purinosome formation supplies sufficient purine deoxyribonucleotides and ribonucleotides for mitochondrial DNA and RNA synthesis, thereby maintaining OXPHOS biogenesis, mitochondrial dynamics, and redox homeostasis. The selective impact of purinosome on mitochondria is mediated by a purinosome-mitochondria tethering via Spartin, whose loss-of-function mutations cause Troyer syndrome with muscle defects. Importantly, ASB11 is downregulated in the hearts of cardiomyopathy patients. In a mouse model of ischemic injury, myocardial induction of purinosome

or purine supplementation mitigates cardiac dysfunction and infarction. Our study identifies the importance of purinosome in maintaining mitochondrial functionality and preventing myocardial injury.

B330/P0908
Spatial Patterning of Glycolysis in a Single Cell
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Cells constantly consume energy to drive a myriad of processes necessary for life. In most eukaryotic cells, glycolysis and OXPHOS are the two main energy producing pathways. While the biochemistry of central metabolism is well understood, the spatiotemporal dynamics of glycolysis in a single cell remain largely unknown. Here, we study meiosis in oocytes of the bat star, Patiria miniata, to address this question. We monitor fructose 1-6 bisphosphate(FBP), a key glycolytic metabolite using a recently developed sensor HYlight, which gives a fluorescent signal reporting on the concentration of FBP. Surprisingly, we observe that there is a significant increase in the signal at the boundaries of the cell, increasing as the cell undergoes meiosis. We show that this increase is highly correlated with actin polymerization, as evidenced by the flattening in the signal upon pharmacological perturbations that target actin polymerization. Moreover, we show that this increase in FBP concentration is not dependent on mitochondrial localization. We're currently investigating the consequences of this spatial patterning on metabolic flux. Overall, this work provides evidence of a feedback between the actin activity and the energy metabolism inside a cell.

Cell Metabolism - Lipid and Membrane Metabolism

B338/P0916
Fluxes of cholesteryl esters between lipid droplets and the endoplasmic reticulum
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The metabolism of the main cellular storage lipids, triacylglycerols (TG) and cholesteryl esters (CE), is controlled by maintaining a balance between their deposition in lipid droplets (LD) and their mobilization through ester hydrolysis. Dysfunctions in these fundamental regulatory processes lead to severe diseases such as dyslipidemias, lipodystrophies, and lipid storage diseases. We have shown that the endoplasmic reticulum (ER) membrane protein seipin controls the biogenesis of both TG and CE LDs. Whether seipin participates in the mobilization of neutral lipids from LDs in lipolytic conditions to facilitate cellular energy production or membrane synthesis, is not well understood. Moreover, the roles of specific hydrolytic enzymes in liberating cholesterol from LDs remain unclear. We combined imaging and biochemical analyses to assess CE mobilization and redistribution from LDs under lipolytic conditions. We also utilized the degron system to acutely remove seipin from existing LDs during lipid hydrolysis. We show that the ER membrane protein neutral cholesteryl ester hydrolase 1 (NCEH1) is a major CE hydrolase in A431 cells and in primary human macrophages and that inhibition of CE hydrolysis in the ER prevents CE from leaving LDs. Additionally, our results suggest that CE and TG hydrolysis are coordinated and that seipin helps CE stored in LDs reach the ER.

B342/P0920
How Ceramides Cripple beta-Cell Energy Flow: Identifying Molecular Targets by Multi-Omic Integration of Proteome Integral Solubility Alteration (PISA) and Metabolomics
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Ceramides are bioactive sphingolipids implicated in lipotoxicity and Type 2 Diabetes. Although they have long been associated with β-cell dysfunction, recent studies have produced conflicting findings, highlighting the unresolved nature of ceramide biology in this cell type. In particular, the metabolic actions of ceramides in β-cells remain poorly defined. We previously showed that excess Cer(18:1;O2/16:0) in rodent islets impairs insulin secretion. Treatment of INS-1 832/13 cells with the short-chain analog Cer(18:1;O2/2:0) revealed that the defect was attributable to impaired glycolysis and aerobic respiration—reducing ATP production, lowering the ATP-to-O_2 ratio, increasing electron leak, and decreasing glucose carbon flow into pyruvate and tricarboxylic acid (TCA) intermediates. To define the underlying mechanisms, we applied Proteome Integral Solubility Alteration (PISA) thermal proteomic profiling to ceramide-treated INS-1 cells. PISA identified 363 proteins with significant ceramide-induced solubility shifts, enriched in pathways regulating lipid metabolism, fatty acid elongation, pyruvate metabolism, mitochondrial organization and respiration, and iron–sulfur cluster biogenesis. Untargeted GC- and LC–MS metabolomics revealed accumulation of glycolytic and TCA intermediates, increased pentose phosphate, polyol, and glycerogenesis metabolites, and redox imbalance as indicated by elevated NADH/NAD^+ and NADPH/$NADP^+$ ratios. Integration of these datasets in MetaboAnalyst 6.0 suggested that ceramides divert glucose-derived carbons into the pentose phosphate pathway and other biosynthetic routes, while impairing mitochondrial structure and function. Together, these findings suggest that ceramides compromise β-cell energy metabolism by rewiring carbon flux, limiting oxidative phosphorylation, and reducing insulin secretion—highlighting potential molecular targets for diabetes therapeutics.

B332/P0910
Identifying the role of Snx13 in cholesterol metabolism
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Snx13 is a mammalian triorganelle tether localized at the membrane contact sites (MCS) between the endoplasmic reticulum (ER), lysosomes, and lipid droplets (LD), all three of which are involved in cholesterol metabolism. This protein has been implicated in heart failure, underscoring the importance of studying its functions. Snx13 belongs to the SNX-RGS family of proteins, which are conserved from yeast to mammals. Studies on other members of this protein family, Mdm1 in yeast and Snx14 in mammalian cells, have previously identified definitive roles of those proteins in intracellular lipid metabolism. Previous work from another research group suggested that Snx13 regulates intracellular cholesterol distribution. Additionally, a high-confidence AlphaFold model of Snx13 predicted the formation of a tunnel-like hydrophobic cavity, a hallmark of lipid-transfer proteins. Nonetheless, the functional role of Snx13 in lipid metabolism remains unknown. Our proximity labeling-based proteomics of Snx13 identified proteins with roles in intracellular cholesterol synthesis, uptake, and transfer. In addition, proteins that are required for normal lysosomal function and quality control were also identified in our proteomics hits. These findings suggest the potential

existence of a novel role of Snx13 in regulating intracellular cholesterol metabolism and lysosomal dynamics, warranting further experimental verification.

B340/P0918
Implications of the rhomboid protease RHBDL4 in cellular glucose regulation
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Rhomboid intramembrane proteases are an evolutionarily conserved family of proteins indicating their involvement in fundamental cellular processes. Our lab is interested in the rhomboid protease RHBDL4 since we found that it cleaves a central protein involved in Alzheimer's disease, the amyloid precursor protein (APP). When cultivating mouse embryonic fibroblasts deficient for RHBDL4 (kindly provided by Dr. Matthew Freeman), we observed that they present with a defect in the expression of glucose transporter 1 (GLUT1) as compared to wild type cells. To reveal whether this difference is physiologically relevant, the systemic glucose metabolism of RHBDL4-knockout mice (also kindly provided by Dr. Matthew Freeman) was assessed but found comparable to that of wild type mice. However, the GLUT1 defect is physiologically relevant in cerebral endothelial cells of RHBDL4-knockout mice as they show a GLUT1-defect, resulting in decreased cerebral glucose uptake as compared to wild type. Cerebral glucose uptake could be rescued by the exogenous expression of RHBDL4 in cerebral endothelial cells using adeno-associated viruses. Further, we found that the GLUT1 defect stems from a non-catalytic RHBDL4 function that is likely carried through protein interactions in the VCP-binding domain and/or the ubiquitin-interacting motif, rather than from its proteolytic activity. Finally, we identified a single nucleotide polymorphism in the RHBDL4 gene that significantly associated with cerebral glucose uptake in humans. We conclude that RHBDL4 carries non-catalytic functions with a wider impact on cellular pathways resulting in a downregulation of GLUT1 proteins.

B341/P0919
In vitro disruption of human CYP8B1 to study bile acid metabolism
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Bile acids are liver-synthesized molecules essential for lipid absorption and metabolic signaling, and their synthesis is a key route for eliminating excess cholesterol. In the classical pathway, cholesterol 7α-hydroxylase (CYP7A1) catalyzes the first and rate-limiting step, while sterol 12α-hydroxylase (CYP8B1) is necessary for cholic acid production, which in turn serves as a feedback regulator that suppresses bile acid synthesis when levels are high. The objective of this study was to disrupt CYP8B1 in human embryonic kidney 293T (HEK293T) cells using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 gene editing to establish an in vitro model for studying bile acid pathway regulation. Guide RNAs targeting human CYP8B1 were designed, cloned into CRISPR/Cas9 plasmids, and validated by restriction digest and Sanger sequencing before large-scale plasmid preparation. HEK293T cells were co-transfected with the validated CYP8B1-targeting plasmids and a CYP8B1 expression construct. Transfection efficiency was confirmed by visualization of green fluorescent protein (GFP) fluorescence, total protein was quantified by bicinchoninic acid (BCA) assay, and CYP8B1 protein levels were determined by Western blot analysis. CRISPR/Cas9-treated samples exhibited markedly reduced or absent CYP8B1 protein compared with non-targeting controls, confirming successful gene disruption. These

findings demonstrate that CRISPR/Cas9-mediated disruption of CYP8B1 can efficiently generate a human cell model suitable for investigating bile acid biosynthesis and lipid homeostasis. This in vitro system provides a foundation for future studies in hepatocyte-derived cell lines to quantify bile acid composition, feedback regulation, and mechanisms linking cholesterol metabolism to metabolic disease.

B346/P0924

Inter-organelle proteomic analysis of mitochondria-lipid droplet contacts by high-precision microscopy-guided photo-biotinylation

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Mitochondria-lipid droplet (LD) interactions play a critical role in lipid metabolism and the progression of metabolic diseases such as non-alcoholic fatty liver disease (NAFLD). However, the dynamic nature of these interactions has hindered the identification of novel protein constituents and their functional roles. Here, we employed optoproteomics, an advanced technology integrates microscopy-guided automated photo-biotinylation, pulldown and mass spectrometry, to isolate and identify proteins localized at mitochondria-LD contacts in oleic acid (OA)-treated HepG2 cells, an in vitro model for studying fatty liver disease. Two-photon illumination was directed to the locations of contacts, which were obtained by calculating the intersection of TOM20 and PLIN2 signals, to trigger photo-induced biotinylation at a high precision. We found the proteome of the mitochondria-LD contact, including both well-characterized lipid-associated proteins and previously unrecognized candidates. Among the 373 common proteins identified across replicates, several novel candidates with no prior association to lipid metabolism were found and 5 of them were selected for validation. Immunofluorescence staining confirmed their localization at mitochondria-LD contact sites, with more pronounced association in OA-treated cells. Notably, suppression of one candidate, FHL3, led to reduced LD size, elongated mitochondrial morphology, and diminished mitochondria-LD interactions, suggesting its role in regulating fatty acid β-oxidation. Together, optoproteomics was able to identify novel functional proteins at the mitochondria-LD contacts, shedding light on potential therapeutic targets for related lipid metabolic disorders.

B339/P0917

Investigating the role of CLCC1 in hepatic steatosis in vivo

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Hepatocytes are unique in their capacity to both store and secrete lipids as either cytoplasmic lipid droplets or as very low density lipoproteins. The mechanism of this lipid flux point is not well understood and is often dysregulated in disease conditions such as hepatic steatosis. We previously identified CLCC1 as a novel regulator of lipid droplet accumulation in hepatocytes through a series of genetic screens under various metabolic stress conditions. In cell models, CLCC1 knockout results in a massive neutral lipid increase and nuclear pore defects. In this project, we characterize a liver specific CLCC1 knockout mouse model and investigate how CLCC1 loss impacts lipid metabolism and physiology in vivo. We examine liver tissue and blood plasma at ages ranging between 1 to 18 months to track the progression of physiological changes over time. We discover that our liver specific CLCC1 knockout mouse model results in a dramatic increase of aberrant

lipoproteins trapped within the endoplasmic reticulum of hepatocytes at younger time points. When examining the blood plasma levels, there is a significant reduction in secreted lipoproteins. Physiologically, as the mice age, the lipid burden in the liver reduces which coincides with the development of liver fibrosis and macrophage invasion. We performed bulk RNA-seq to assess differential gene expression in this disease relevant model. In addition to signatures of immune activation, we found alterations in the metabolism of lipogenesis and mitochondrial function. Further investigation is required to explore the failed early nuclear pore complexes in tissue. Our findings provide important insight into the mechanisms by which a heavily lipid burdened liver progresses over time as well as the physiological effects of having reduced lipoprotein secretion.

B333/P0911

Manipulation of Host Cholesterol by SARS-CoV-2

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Cholesterol is essential for cellular structure and function, and its levels and distribution are tightly regulated by multiple pathways. Lysosomes contribute by degrading lipids, releasing free cholesterol, and distributing it to other cellular compartments. Here, we show that SARS-CoV-2 disrupts lysosomal cholesterol export through the viral protein ORF3a. ORF3a is localized on lysosomes and binds the Homotypic Fusion and Protein Sorting (HOPS) subunit VPS39. Disrupting this interaction restores cholesterol trafficking. Using high-content imaging, lipidomics, and proteomics, we identified two pathways impaired by the ORF3a–VPS39 interaction. First, ORF3a caused increased secretion and mislocalization of the lysosomal cholesterol transporter NPC2. NPC2 trafficking relies on the protein sorting receptor CI-MPR for lysosomal delivery. ORF3a expression led to endosomal/lysosomal retention of CI-MPR and the VPS35 subunit of protein retrieval complex retromer. Deletion of another retromer subunit VPS29 reproduced the cholesterol and CI-MPR defects, indicating that impaired protein endosome-to–trans-Golgi transport resulted in disturbed cholesterol export. Second, levels of the lysosomal phospholipid bis(monoacylglycerol)phosphates (BMPs), which facilitate cholesterol egress, were reduced. Although BMP biosynthetic enzymes were unaffected, lipidomics and proteomics revealed altered metabolism of BMP precursors, mitochondrial phosphatidylglycerols (PGs), and reduced mitochondrial proteins that associated with lysosomes. Electron microscopy revealed that ORF3a–VPS39 interaction decreased lysosome–mitochondrion membrane contact sites (MCS) with exclusion of autophagy or mitochondrion-derived vesicles by RNAi as routes for PG transfer. Furthermore, VPS39 is known for mediating the MCS formation in yeast, and depletion of VPS39 had decreased MCS and BMP levels. These findings identify VPS39 as a regulator of NPC2 trafficking and BMP biosynthesis and reveal that ORF3a disrupts both pathways to block cholesterol egress.

B343/P0921

Mechanisms of SARS-CoV-2 ORF3A alternation of BMP

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Altered plasma cholesterol levels have been observed in COVID-19 patients, suggesting that SARS-CoV-2 disrupts host cholesterol homeostasis. Lysosomes are central to this process, as they process dietary cholesterol and distribute free cholesterol throughout the cell. Cholesterol export from lysosomes is mediated by the transport proteins NPC1 and NPC2, in coordination with the phospholipid bis(monoacylglycerol)phosphate (BMP). We found that SARS-CoV-2 infection causes cholesterol accumulation in lysosomes without defects in NPC1 function. We therefore hypothesize that the virus interferes with cholesterol egress by disrupting NPC2 and BMP activity. The viral protein ORF3a contributes to this phenotype by binding the HOPS complex, which normally targets NPC2 to lysosomes. In cells stably expressing ORF3a, NPC2 was mislocalized; this defect was rescued by a point mutation in ORF3a that prevents HOPS binding. Additionally, SARS-CoV-2 infection and ORF3a expression reduced BMP levels, despite normal localization of BMP-synthesizing enzymes. Mechanistically, ORF3a-HOPS interaction disrupted mitochondria–lysosome contact sites, impairing the delivery of BMP precursor lipids to lysosomes. Together, these findings suggest that SARS-CoV-2 promotes lysosomal cholesterol sequestration by mislocalizing NPC2 and reducing BMP levels via ORF3a. Elucidating this mechanism provides new insight into how SARS-CoV-2 alters host lipid metabolism and may reveal therapeutic targets for COVID-19.

B337/P0915
Novel Mechanisms Regulating Fatty Acid Elongation in S. cerevisiae
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Although most of the enzymes and transporters involved in fatty acid metabolism have been identified, major questions remain regarding how fatty acid metabolic pathways are spatially coordinated and regulated within the cellular space. The overarching goal of my research is to understand how the diverse species of fatty acids are derived and maintained, and what mechanisms determine their metabolic fates and functions. My work investigates a novel mechanism by which cells regulate fatty acid elongation by strategically sequestering fatty elongation enzymes in spatially defined cellular microdomains. My recent data shows that the trans-2-enoyl-CoA reductase Tsc13, which catalyzes the final step of the elongation cycle, is enriched at the nucleus-vacuole junction (NVJ) in response to glucose depletion; initial liquid chromatography mass spectrometry (LC-MS) data also suggests a perturbation in the prevalence of very-long chain fatty acids (VLCFAs), the products of fatty acid elongation. I hypothesize that the sequestration of Tsc13 at the NVJ regulates its ability to interact with elongation enzymes, thus altering VLCFA profile and metabolism into downstream lipids such as ceramides.

B344/P0922
Quantification of endothelial fatty acid uptake using fluorescent fatty acid analogs
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Endothelial cells (ECs) play a central role in regulating fatty acid (FA) transport from the bloodstream into metabolic tissues, yet tools to quantify EC FA uptake in a reliable, scalable manner remain limited. Here, we present a rapid, quantitative, and cost-effective assay to measure FA uptake in ECs using fluorescent FA analogs (BODIPY-C12 and BODIPY-C16), which allow investigation of chain length-specific uptake dynamics in a 96-well plate format. The protocol incorporates positive (3-hydroxyisobutyrate, lactate) and negative (niclosamide) controls and is validated in both primary (HUVECs) and immortalized (EA.hy926) EC lines. The assay detects time- and dose-dependent FA uptake, with results normalized to cell number using Hoechst nuclear staining

and corrected for background using appropriate controls. It can be adapted to a variety of cell types, imaging modalities, and experimental conditions, including live-cell imaging and pulse-chase formats. Compared to traditional lipid staining or radiolabeled tracers, this method offers improved safety, speed, and versatility while capturing dynamic FA uptake in live cells. This assay provides a robust platform for studying the regulation of endothelial lipid transport and its contribution to metabolic disease. This work has been published recently in the Journal of Visualized Experiments (JoVE): Ibrahim, A., Choudhury, T., Kim, B. Quantification of Endothelial Fatty Acid Uptake using Fluorescent Fatty Acid Analogs. J. Vis. Exp. (222), e68859, doi:10.3791/68859 (2025).

B347/P0925

Rab32/38-Positive Lysosome-Related Organelles Mediate Lipid Droplet Degradation in Hepatocytes

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Rab proteins, members of the small GTPase family, play critical roles in vesicle trafficking. Among them, Rab32 and Rab38 are conserved homologs known for their involvement in melanosome formation and the biogenesis of lysosome-related organelles (LROs). However, how Rab32 and Rab38 regulate lipid metabolism remains unclear. In this study, we found that Rab32 and Rab38 double knockout (DKO) male mice exhibited a significant age-dependent increase in body weight compared to wild-type (WT) males, and their livers appeared darker. Given the central role of the liver in lipid metabolism, we analyzed AML12 hepatocytes and observed Rab32 and Rab38 forming ring-like structures that colocalized with Lamp1. Rab38 played a dominant role in the formation of large Rab32/38-positive LROs, whose size varied with cell confluency. To investigate whether Rab32/38-positive LROs are involved in lipid droplet (LD) degradation, we inhibited lysosomal acid lipase with Orlistat. This treatment delayed lipid degradation, suggesting that LD breakdown occurs inside Rab32/38-positive LROs. Combined treatment with Orlistat and Bafilomycin A1 further revealed invagination-like internal membranes within large LROs. Using an mCherry-FYVE probe, we detected PI3P-positive ring-like signals within or adjacent to LRO membranes. After Orlistat treatment, LDs were frequently observed inside or near these PI3P-positive structures, suggesting that PI3P contributes to the formation of internal membranes within LROs. Furthermore, Rab32/38 double knockdown (DKD) AML12 cells showed greater lipid accumulation compared to WT controls. Upon Orlistat treatment, accumulated LDs were mainly localized within lysosomes in control cells. In contrast, LDs rarely accumulated further in lysosomes upon Orlistat treatment in Rab38 knockdown or DKD cells, indicating that the loss of Rab38, particularly in combination with Rab32, impairs the ability of LROs to engulf LDs. Together, our findings demonstrate that Rab32/38-positive LROs provide the structural platform for lipid droplet degradation in hepatocytes, with Rab38 playing a dominant role in lysosomal engulfment.

B334/P0912

Sterol composition regulates mitochondrial fission fate through dynamic control of membrane integrity

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Mitochondrial fission is a dynamic process required for maintenance of cellular health. While crucial fission components, such as the dynamin related GTPase Dnm1/Drp1 and its adaptors, have been characterized, roles for mitochondrial lipids in regulating fission remain mysterious. Here we conducted a microscopy-based screen of lipid mutants in budding yeast (S. cerevisiae) and found a surprising role for sterols in maintaining fission.

Specific mutants in the ergosterol (the major fungal sterol) biosynthetic pathway drove the formation of characteristic fission-defective netted mitochondrial morphologies that resembled structures observed upon deletion of dnm1, or its adaptors. Netted mitochondrial topologies retained Dnm1 localization and were restricted to young cells, which exhibited reduced DNM1 expression. Analogous perturbation of sterol biosynthesis in mammalian cells drove the formation of donut-like topologies that resembled Drp1-inhibited mitochondrial structures. Liposomes representing mitochondrial lipidomes with aberrant sterol compositions exhibited altered Dnm1/Drp1 GTPase activity, suggesting sterol requirements for Dnm1/Drp1 function. Overall, our study identifies sterols as novel lipid components necessary for maintaining mitochondrial fission.

B335/P0913
Stochastic Gene Expression and Metabolism at the Single-cell Level
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Phenotypic variation among clonal cell populations arises from stochasticity in gene expression. Studies in Escherichia coli have demonstrated that both intrinsic and extrinsic noise significantly contribute to this variability. The former originates from the biochemical processes of gene expression, while the latter arises from fluctuations in other cellular components. Consequently, de-novo lipid production dynamics of Yarrowia lipolytica at the single-cell level, even within a time-invariant microfluidic environment, exhibit sporadic rather than monotonic behavior over time. To investigate this further, we introduce the concept of bioprocessing noise and trace its origins to epigenetic mechanisms. Our analysis reveals that the culture microenvironment amplifies phenotypic diversity in lipid production across individual cells.

B331/P0909
Structural and mechanistic insights into lipid droplet biogenesis by the seipin-LDAF1 complex
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Lipid droplets (LD) are essential organelles that originate from the endoplasmic reticulum (ER), but how they form has remained a long-standing mystery. We investigated the LD-assembly complex (LDAC), made of the proteins seipin and LDAF1, which is critical for this process. Using reconstitution assays, structural biology, and molecular dynamics simulations, we show that the LDAC alone can geenrate LDs from a simple membrane. Our studies reveal that LDAF1 sits inside the cage-like structure formed by seipin, together forming a unique protein assembly that spans the membrane. This architecture creates a chamber within the ER that restricts phospholipids but allows triacylglycerols (TGs) to accumulate. Inside this space, TGs interact with each other and with LDAF1, forming an oil-like core that kickstarts LD formation. These findings uncover how the LDAC acts as a chamber to catalyze LD birth, shedding light on a fundamental aspect of cell biology.

B336/P0914
TorsinA ATPase Activity is Essential for ApoB-mediated VLDL Secretion from the ER of Hepatocytes

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TorsinA is a member of the AAA+ ATPase family located in the endoplasmic reticulum (ER). A single glutamic acid deletion (delE) in torsinA causes DYT1 dystonia in humans. Our previous research has demonstrated that torsinA plays a significant role in apolipoprotein (apoB)-mediated very low-density lipoprotein (VLDL) secretion in hepatocytes. Mice with depletion of torsinA from hepatocytes (referred to as A-CKO mice) exhibit a marked accumulation of lipids within hepatocytes (steatosis) due to defects in VLDL secretion. The main objective of the current study is to determine whether ATPase activity of torsinA is necessary for VLDL secretion and whether the torsinA delE mutant affects the process. To address the questions, we used adenoviral and adeno-associated viral vector-mediated gene delivery to express wild-type or mutant torsinA variants in A-CKO mice. We demonstrated that the striking steatosis and decreased VLDL secretion in hepatocytes from A-CKO mice were normalized upon the re-expression of wild-type torsinA or delE DYT1 dystonia variant. However, expression of a torsinA ATPase hydrolysis-defective variant (EQ mutant) failed to reverse steatosis or rescue the VLDL secretion defect, demonstrating that torsinA ATPase activity is essential for the process. Confocal microscopy of isolated hepatocytes revealed that accumulated abnormal lipids in torsinA-depleted hepatocytes did not colocalize with ADRP, a lipid droplet-associated protein in the cytosol. Upon the re-expression of wild-type or delE torsinA, lipid droplets were colocalized with ADRP. In contrast, most of the lipid droplets in hepatocytes expressing EQ torsinA were not colocalized with ADRP, similar to hepatocytes lacking torsinA. Electron microscopic images of liver sections from these mice suggested that the excess lipids were located within the ER of hepatocytes. These results indicate that torsinA ATPase activity is necessary for the proper formation of lipid droplets in hepatocytes. Genome-wide transcriptomic analysis showed that the sterol biosynthesis pathway was the most significantly dysregulated in livers of A-CKO mice expressing EQ torsinA, compared to those expressing wild-type or delE torsinA. Spatial lipidomic analysis revealed a marked increase in neutral lipids and a fatty acyl chain-dependent dysregulation of phospholipid species in livers of A-CKO mice expressing EQ torsinA, compared to those expressing wild-type or delE torsinA. These results demonstrate that the ATPase activity of torsinA is essential for VLDL secretion from the ER and normal formation of lipid droplets in hepatocytes.

Cell Metabolism - Metabolic Pathway Crosstalk

B361/P0939

Deciphering the role of polyamine metabolism in heart failure

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Heart failure (HF) is a leading cause of death worldwide. Despite therapeutic advances, the five-year mortality rate of HF remains at ~50%. Novel treatment strategies targeting alternative pathways are urgently needed to improve patient outcomes. One class of promising targets in HF is polyamines. Polyamines are small, polycationic metabolites that regulate key cellular processes. Their intracellular levels are tightly controlled by a balance of import, endogenous biosynthesis, and catabolism. Multiple lines of evidence suggest that spermidine, one of the major polyamine species, may be cardioprotective. In humans, higher dietary spermidine intake has been associated with reduced cardiovascular disease risk. In aging mice, spermidine supplementation mitigated age-related declines in cardiac function, while in a rat model of hypertension-induced HF, it lowered blood pressure and delayed the onset of HF. Despite these promising findings, the

mechanisms through which polyamines and their metabolism contribute to cardiac physiology remain unclear. In targeted metabolomic profiling of human hearts explanted from patients with end-stage dilated cardiomyopathy (a type of HF), compared to control discarded Gift-of-Life donor hearts, we found a substantial elevation of polyamine levels, specifically putrescine and spermidine. Furthermore, through bulk RNAseq, we observed transcriptional downregulation of spermidine/spermine N1-acetyltransferase 1 (SAT1), the rate-limiting enzyme for polyamine catabolism. These findings suggest that altered polyamine metabolism may play a previously unrecognized role in HF. To further mechanistically investigate this metabolic shift, we are using in vivo isotope tracing and metabolomics to quantitatively map whole-body polyamine metabolism in conscious mice under both physiologic and HF conditions. In parallel, we are manipulating Sat1 expression in vivo to assess how altering polyamine catabolism impacts cardiac function in the setting of HF. Given that SAT1 consumes acetyl-CoA to acetylate polyamines in the first step of the catabolic pathway, we hypothesize that increased polyamine catabolism exacerbates HF by depleting cellular acetyl-CoA pools, which may have broader impacts on cardiac energetics and epigenetic regulation. Conversely, we hypothesize that suppression of this pathway confers cardioprotection by preserving acetyl-CoA. Together, these studies aim to quantify the dynamics of systemic polyamine metabolism and elucidate the role of its alteration in HF pathophysiology.

B362/P0940

Defining a regulatory switch between sugar and lipid metabolism in intestinal stem cells

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What we eat has a profound impact on health and susceptibility to disease. Dietary nutrients directly alter metabolism to influence intestinal stem cell (ISC) behavior, yet the extent of regulatory control over the metabolic-transcriptional axis is not well defined. We focused on the metabolic hub, mitochondria, to elucidate mechanisms of cellular control over nutrient utilization that impacts ISC function. Our scientific premise is based on our demonstration that adult mice fed a High-fat Western Diet (HFD) acquire a phenotypic signature marked by increased ISC numbers, proliferation, capacity to regenerate, and ability to give rise to adenomas. These properties are dependent upon activation of PPAR-delta or PPAR-alpha, nuclear receptors orchestrating multiple aspects of cellular lipid metabolism. The reliance on a PPAR transcriptional axis underscores that lipid mediators play an exquisite role in HFD-exposed stem cells and tumor initiation along the intestinal tract and that mitochondrial metabolism is central to this axis. To further expand on the mitochondria signaling that shapes ISC behavior, we used the MITO-tag mouse to rapidly isolate metabolites explicitly from ISC mitochondria in mice on a control, 24hr-fasted, and HFD. We identified amino sugar metabolism enriched between conditions, with UDP-GlcNAc more abundant in control and fasted ISCs over HFD ISCs. UDP-GlcNAc is the end product of the hexosamine biosynthesis pathway (HBP) that integrates carbohydrate, fatty acid, nucleotide, and amino acid metabolism. UDP-GlcNAc serves as the substrate for O-GlcNAcylation, a post-translational modification adding O-GlcNAc to Ser/Thr residues by O-GlcNAc transferase (OGT). Using murine intestinal organoids, we find that pharmacologic inhibition of OGT results in a rapid and robust increase in stem cell frequency, proliferation, regeneration and PPAR target proteins, hallmarks of the HFD-induced ISC phenotype. Mechanistically, these features are dependent on the PPARs as genetic loss of Ppar-d/a blocks the ISC effects of OGT pharmacological inhibition. We posit that a reduction in O-GlcNAcylation but not complete inhibition, recapitulates the HFD phenotype, and that the intricate balance between lipid and glucose utilization within ISCs is inherent to O-GlcNAcylation regulation. Our ongoing studies test this regulatory control exerted on PPAR-mediated transcription that may serve as a key metabolic switch between glucose and lipid metabolism.

B364/P0942

Hepatic regeneration via vascular modulation: Phosphatase of regenerating liver-1/hepatocyte growth factor axis as a key regenerative mechanism in Thioacetamide-injured rat model

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The vascular system is essential for maintaining organ function by transporting nutrients and oxygen for cell survival. Hepatocyte growth factor (HGF) signaling is critical pathway in hepatic function, vascular stabilization and maturation. Recently, it is known that phosphatase of regenerating liver-1 (PRL-1) plays a key role during the early liver regeneration. In our previous reports, we found that PRL-1 overexpressed placenta-derived mesenchymal stem cells (PD-MSCsPRL-1) improve liver function through epithelial-mesenchymal transition (EMT) based on antifibrotic effect. However, the correlation between liver regeneration and vascular function remains unclear. Therefore, we examined whether PD-MSCsPRL-1 could improve liver tissue regeneration and function via vascular regeneration by regulating HGF. HGF secretion in PD-MSCsPRL-1 cultured medium was significantly higher than that of PD-MSCs (*$p<0.05$). The expression of HGF receptor as a mesenchymal-epithelial transition factor (c-Met), in PD-MSCsPRL-1 transplanted groups significantly increased than other groups (*$p<0.05$). Also, the diameter and the abnormal shape of the portal vein in liver tissues of non-transplantation (NTx) were significantly increased compared to normal, whereas it significantly decreased in PD-MSCs transplantation (Tx) groups compared to NTx group (*$p<0.05$). Moreover, vascular permeability significantly decreased in human umbilical vein endothelial cells co-cultured with PD-MSCsPRL-1 compared to other groups (*$p<0.05$). To further investigate the effect of PRL-1 on HGF signaling, we treated WB-F344 cells with Tivantinib, a c-Met inhibitor. The expression of phosphor-c-Met in tivantinib-treated group was significantly decreased compared to control, and it was increased when co-cultivated with PD-MSCs and PD-MSCsPRL-1 (*$p<0.05$). Therefore, increased HGF by PD-MSCsPRL-1 ameliorated liver regeneration by enhancing vascular function. These findings will help understand the therapeutic mechanism of functionally enhanced MSCs and provide strategies for cell therapy in liver regenerative medicine. This research was supported by National Research Foundation (NRF) grant funded by the Korea Government (RS-2022-00070304) and Tech Incubator Program for startup Korea (TIPS) (RS-2023-00303130).

B363/P0941

Irisin regulates exercise mimetic effect on insulin signalling in hippocampal cells in vitro

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Exercise is requisite for the proper functioning of the body, triggering the release of numerous exerkines into circulation. One such exerkine is Irisin, a PGC1α dependent myokine. PGC1α is a transcriptional co-activator that mediates effects of exercise on muscle, brain, and other organs. Exercise-induced PGC1α activates Fndc5/Irisin gene expression, which further enhances BDNF, an indispensable neurotrophic factor in brain. Studies in skeletal muscles have identified Irisin to enhance insulin signalling and glucose homeostasis, implying that irisin can possibly improve brain health via insulin signalling. To this end, in mouse hippocampal cells, we found that exogenous irisin enhances expression of brain exercise markers such as BDNF, PGC1α and also activates insulin sensitive molecules like AKT and AS160, suggesting a definitive role in neuronal insulin signalling. Therefore, we opine that Irisin could be therapeutically used to ameliorate brain insulin resistance and associated neurological complications.

Cell Metabolism - Metabolic stress, redox regulation and adaptation

B354/P0932
Effects of Alcohol and Nicotine E-Cigarette Co-Exposure on Biochemical Changes in Developing Mice
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Fetal Alcohol Spectrum Disorders arise from prenatal ethanol exposure, often compounded by concurrent nicotine use, both of which can significantly impact fetal metabolism. Despite the high prevalence of co-exposure, little research has examined how these substances interact to alter lipid metabolism and hepatic drug-metabolizing enzymes. This study investigates the effects of combined prenatal ethanol and nicotine exposure on triglyceride levels and liver enzyme expression in neonatal mice, compared to exposure to each drug alone. Pregnant dams were exposed to vaporized ethanol and nicotine during a developmental window equivalent to the human third trimester, modeling real-world maternal substance use. Offspring serum triglyceride concentrations were quantified using a colorimetric assay to assess metabolic disruptions, while western blot analysis determined changes in alcohol dehydrogenase expression in liver tissue. The inflammatory markers TNF-α and serum amyloid A were determined by enzyme-linked immunosorbent assays. No significant differences in triglyceride levels, liver enzyme expression, or inflammatory markers were found among offspring from the different exposure groups, suggesting that prenatal co-exposure to ethanol and nicotine does not synergistically alter these specific metabolic pathways in neonatal mice. This study provides a foundational understanding of the lack of synergy on these specific markers, warranting future research to investigate other potential mechanisms and outcomes of combined prenatal substance use.

B355/P0933
Iodoacetic acid induces metabolic and oxidative damage in porcine uterine epithelial cells and oviduct epithelial cells
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Iodoacetic acid (IAA), a disinfection byproduct present in drinking water, has been detected in the female reproductive tract. Although its reproductive toxicity has been suggested, the underlying mechanisms remain largely unknown. In this study, we demonstrate that IAA induces glycation stress, driving cytotoxicity in primary porcine uterine and oviduct epithelial cells. Mechanistically, IAA acts as an alkylating agent that inhibits GAPDH activity and promotes advanced glycation end products (AGEs) accumulation, leading to metabolic imbalance and oxidative stress. These alterations result in mitochondrial dysfunction, calcium dysregulation, and activation of the NF-κB and p38 MAPK signaling pathways. Consequently, cells undergo G1/S arrest, impaired migration, and apoptosis. Collectively, our findings identify glycation stress as a key initiating event in IAA-induced reproductive epithelial injury and provide new mechanistic insights into its cytotoxicity.

B356/P0934
Magnetic modulation of reactive oxygen species production in cells
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Reactive Oxygen Species (ROS) are involved in cellular homeostasis. Excessive ROS disrupts important cellular processes such as protein synthesis and secretion, decreasing the yield of biologics secreted by cells used for pharmaceutical manufacturing. Antioxidants have been applied to reduce harmful ROS generation with mixed results. Therefore, a more effective approach to counter ROS is needed to boost secretory activities. Here, we explore whether this goal can be achieved by applying external magnetic fields. Magnetic fields in the order of milliTesla are known to alter the energy landscape of radical pairs (RPs) in excited flavoproteins, which are responsible for producing various ROS molecules. It was reported that singlet RPs decaying to the ground state result in hydrogen peroxide (H_2O_2), while triplet RPs decaying to the ground state result in superoxide ($O_2{}^{*-}$), both of which are ROS affecting the secretory machinery. One can manipulate the spin state of the RPs using magnetic fields. Yet simply applying strong magnetic fields or removing geomagnetic fields to achieve a 0 Tesla condition will not minimize the total ROS, as the former might favor $O_2{}^{*-}$ production and the latter H_2O_2. Here, we experimentally identify the optimal field strength resulting in minimal ROS production. We used NIST-Chinese Hamster Ovary (NISTCHO) cells, which are engineered to secrete monoclonal antibodies, as the model system. We applied 10 mT, 20 mT, and 50 mT magnetic fields to NISTCHO cells.The ROS level in single cells was quantified using fluorescent ROS dyes after exposure to the magnetic fields for 24 and 72 hours. We found the ROS level in cells was reduced by ~65% and ~87% after 24 hours of exposure to the 10-mT and 20-mT fields, and by ~77% and increased by ~46% after 72 hours of exposure. Moreover, the ROS level was reduced by ~34% and increased by ~7% after 24 and 72 hours of exposure to the 50-mT field. These results confirmed our assumptions that magnetic fields can modulate ROS production, but not in a monotonic manner. Our findings underscore the potential of using magnetic fields as a novel and economic approach to enhance the yield of biologics by decreasing ROS interferes with the secretory pathways.

B358/P0936

Nutrient-Dependent Nuclear Translocation of PKA Catalytic Subunit in ARPE-19 Cells

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Protein kinase A (PKA) serves as a central mediator of cyclic adenosine 3’,5’ monophosphate (cAMP) signaling and regulates gene expression through spatially controlled phosphorylation events. The PKA holoenzyme is a protein complex consisting of two regulatory subunits (R) and two catalytic units (C). Several studies have established the importance of cAMP and PKA signaling in maintaining photoreceptor health in mouse retinal degeneration models, as well as mitochondria fission and fusion in photoreactive zones in the retina. However, the mechanism by which nutrient availability influences PKA localization and nuclear signaling in human retinal pigment epithelial (RPE) cells, as well as the accompanying transcriptional response, remains unclear. This study investigates nutrient-dependent localization dynamics of the PKA catalytic alpha subunit (Cα) in human ARPE-19 cells using a combination of immunohistochemistry, fluorescence staining, and confocal microscopy. Under glucose-rich conditions, the Cα subunit localized primarily to the cytoplasm. In contrast, cells cultured in glucose-deprived medium (B27) displayed nuclear accumulation of Cα, consistent with activation of the cAMP/PKA pathway under nutrient stress. Time-course analysis revealed distinct nuclear distribution patterns: Cα first accumulated in euchromatin regions after 3 days in B27, followed by enrichment in heterochromatin domains after 6 days. Ongoing analyses include nuclear fractionation and phosphoproteomic profiling to identify nuclear substrates of PKA under nutrient stress. These observations suggest that nuclear translocation of PKA under nutrient deprivation may dynamically influence chromatin organization and transcriptional

programs. Qualitative imaging analysis supports a model in which nutrient stress drives PKA nuclear translocation to modulate chromatin accessibility and transcriptional responses.

B357/P0935
Phthalates in E-Cigarettes and their impact on male reproductive tissues
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Endocrine-disrupting phthalates have been associated with declines in fertility, yet the underlying biochemical mechanisms affecting male reproductive tissues remain poorly understood. Vaping exposes the user's lung epithelial tissues and pulmonary circulation to varying concentrations of phthalates from both the e-liquid and the vaping devices themselves. In this project, our aim is to assess the biochemical impact of phthalates derived from e-cigarettes on male reproductive tissues using rat and cell culture models. Our hypothesis is that phthalates, particularly dibutyl phthalate, negatively impact male reproductive tissues through inhibiting spermatogenesis and testosterone synthesis, along with elevated inflammation signaling cascades. To examine the mechanisms, we exposed several rats and primary derived testicular Sertoli cells to vaporized phthalates and measured protein expression changes through western blot analysis and cell viability assays. Our preliminary findings indicate that several phthalates exhibit cytotoxic effects on primary-derived rat testicular Sertoli cells. The protein expression of enzyme CYP11A1, a key enzyme involved in testosterone synthesis, did not display statically significant differences with and without dibutyl phthalate. Future investigations will extend exposure durations and concentrations of phthalates to rats, as well as exploring the changing expression patterns of other testosterone synthesis pathways.

B353/P0931
Protective effects of Vitex Agnus-Castus against oxidative stress-induced damage in pre-osteoblastic cells
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In postmenopausal osteoporosis caused by estrogen deficiency, bone-forming cells are subjected to oxidative stress due to elevated reactive oxygen species (ROS). Their increase disrupts redox balance, damages macromolecules, and impairs cellular viability, ultimately compromising bone homeostasis. Vitex agnus-castus (VAC), a medicinal plant rich in flavonoids structurally similar to endogenous estrogen, has been hypothesized to interact with estrogen receptors and provide protective effects against oxidative stress–induced cellular damage. The objective of this study was to investigate VAC's potential on bone-forming cells. For this purpose, MC3T3-E1 pre-osteoblastic cells were cultured on glass coverslips, seeded at 1×10^4 cells/well and supplemented with VAC at a concentration of 1 μg/mL for 72 hours. Experimental analyses included (a) immunofluorescence detection of FOXO1, NRF2, and SOD3 proteins, (b) scanning electron microscopy (SEM) for assessment of cell shape and integrity, (c) ROS quantification and (d) mitochondrial visualization using MitoTracker. Oxidative stress markedly increased FOXO1 expression in untreated cells (C + OS), whereas VAC treatment reduced labeling to the level comparable of non-stressed controls. Cellular alterations such as cytoskeletal disorganization and actin-rich protrusions were evident under oxidative stress but were attenuated by VAC, although stressed VAC-treated cells displayed reduced number compared to non-stressed groups. NRF2 labeling remained similar across groups, with a slight increase in VAC-treated cells. SOD3 expression was detected in VAC-treated stressed cells, suggesting an induced antioxidant defense. ROS

detection revealed high levels in all stressed groups, indicating that VAC did not directly reduce ROS generation. Mitochondrial analysis showed a decrease in number and altered morphology under stress; VAC partially preserved mitochondrial abundance and cell size but did not fully restore structure. SEM confirmed VAC's non-cytotoxicity based on morphology criteria and partial protection against stress-induced structural damage. In conclusion, VAC did not directly suppress ROS production but partially preserved cytoskeletal organization and mitochondrial integrity under oxidative stress, while inducing SOD3 expression, indicating an indirect antioxidant effect and potential to maintain osteoblasts function in postmenopausal osteoporosis.

Cell Metabolism - Nutrient sensing pathways

B352/P0930
Deciphering the Mechanisms of Cytoplasmic Protein Degradation under Nitrogen-limitation in E. coli
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In Escherichia coli, cytoplasmic protein degradation is thought to be primarily mediated by four ATP-dependent proteases: ClpP, Lon, HslV, and FtsH. Recent work from our lab demonstrated that nitrogen limitation triggers extensive cytoplasmic protein degradation. Strikingly, cytoplasmic protein degradation persists even in the absence of the non-essential ATP-dependent proteases ClpP, Lon, and HslV, and appears to be independent of the essential protease FtsH, suggesting the presence of an uncharacterized proteolytic pathway. This study investigates the molecular mechanisms and regulatory pathways responsible for this enhanced protein turnover. We established in vitro degradation assays using casein, which show that lysates from the triple protease knockout strain (ΔclpP Δlon ΔhslV) exhibit lower proteolytic activity than wild type under nitrogen limitation. Additionally, measurement of β-galactosidase activity upon IPTG addition as an estimate for new protein synthesis showed that wild-type strain displays higher protein synthesis under nitrogen limitation compared to carbon or phosphorus limitation. The triple knockout strain, however, exhibited reduced protein synthesis relative to wild type under nitrogen limitation. Interestingly, deletions of clpX (the ClpP-associated chaperone) and relA had minimal effects on protein synthesis, whereas deletions of the upstream nitrogen regulators glnG and glnL resulted in elevated protein synthesis compared to wild type. Thus our preliminary results point towards a complex interplay between nutrient sensing, protein synthesis, and degradation in bacterial adaptation to nutrient limitation.

B348/P0926
Determining the role of PRDM16 in enteroendocrine cell differentiation and gut nutrient sensing
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The intestinal epithelium consists of specialized cell populations essential for nutrient absorption and the secretion of mucous, hormones, and antimicrobial factors. A small population of hormone-producing enteroendocrine cells (EECs), which comprise only ~1% of the intestinal epithelium, are crucial for maintaining endocrine homeostasis. Serotonin-producing enterochromaffin cells regulate gastrointestinal motility, insulin release, and inflammation, while non-enterochromaffin cells secrete hormones that regulate glucose metabolism, appetite, and satiety. Moreover, while EECs are present throughout the stomach and small and large intestines, the types and functions of EECs differ depending on their location along the digestive tract. However, there is still a limited understanding of how the differentiation, regional distribution, and function of these cells are controlled. Recent work identified selective expression of the transcription factor PRDM16

along the EEC differentiation trajectory, with enriched expression in the proximal small intestine. Given PRDM16 regional expression in the small intestine and its known role regulator as a of fatty acid oxidation, PRDM16 represents a critical link between intestinal epithelial differentiation and systemic metabolic responses. Using NeuroD1-Cre mice, we deleted Prdm16 specifically in the EEC lineage. Our preliminary data shows that Prdm16 deletion decreases leads to increased lipid accumulation in the proximal small intestine (duodenum and jejunum specifically) following dietary fat challenge. Functionally, knockout mice exhibit trends toward improved glucose clearance during oral glucose tolerance testing. Together, these findings suggest that PRDM16 regulates both the differentiation trajectory of EECs and their metabolic adaptation to dietary lipid and glucose intake.

B349/P0927

Impact of a-arrestins on cellular metabolism and organelle function

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To adapt to changes in nutrients or stressors, cells reorganize their membrane proteome through vesicle-mediated protein trafficking. α-Arrestins are a critical family of protein trafficking adaptors that are essential for the internalization of amino acid transporters (AATs), thereby restricting amino acid uptake from the environment. When α-arrestins are absent, AATs become aberrantly retained at the plasma membrane, potentially leading to excessive amino acid accumulation within the cell. While the regulation of AATs by α-arrestins is well-documented, the physiological role of α-arrestins in preventing cytosolic amino acid overload remains largely unknown. In S. cerevisiae, we explore the loss of the α-arrestin Art1 as a model for the dysregulation of amino acid levels. Using metabolomics, we find that cells lacking the α-arrestin Art1, a known regulator of the arginine and lysine transporters, have elevated intracellular arginine and lysine. We find that this excess of arginine and lysine in art1Δ cells leads to fragmented mitochondria with reduced membrane potential and impaired cellular respiration, evidenced by their inability to grow on glycerol as a carbon source. The defective mitochondrial function is coupled with a vacuolar defect, as vacuoles become enlarged and hyper-acidified in the absence of Art1. Our findings indicate that the vacuolar proteome and lipid composition are compromised in the absence of Art1, and this vacuole dysfunction is directly due to excess amino acids. Mitochondria are the major site of arginine degradation while over 90% of basic amino acids in the cell are stored in vacuoles; these two facets of amino acid management likely underlie the cause of the amino acid-induced dysfunction in these organelles in art1Δ cells. Using RNAseq analysis, we have defined key pathways that are upregulated in cells lacking Art1 and demonstrate their role in managing amino acid imbalance. We find that in cells lacking Art1, the arginine degradation pathway is highly upregulated and is essential for art1Δ cell survival. In contrast, both the PHO regulon and polyphosphate synthesis pathway, which have been implicated in the storage of basic amino acids in vacuoles, are still highly upregulated in art1Δcells, but are surprisingly not essential for their survival. In conclusion, our study shows that the heightened uptake of amino acids in cells lacking Art1 leads to vacuolar and mitochondrial dysfunction. These results clarify the essential relationship between cellular metabolism and organelle function, enhancing our understanding of how cells manage excess amino acids. Notably, arginine is a critical nutrient for the growth of mammalian cells and is frequently elevated in cancers, underscoring the significance of our findings in human health.

B351/P0929
Impact of Excess Amino Acids on Metabolism and Phosphate Regulation
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The accumulation of excess amino acids in a cell causes cytotoxicity. α-Arrestins regulate the endocytosis of amino acid transporters (AATs) from the plasma membrane (PM); in the absence of α-arrestins, AATs remain at the PM and allow excess amino acid uptake. Our previous work found that cells lacking α-arrestin Art1 in Saccharomyces cerevisiae 1) aberrantly retain Can1 and Lyp1, the AATs for arginine and lysine, at the PM, 2) have increased cellular arginine and lysine, and 3) this increased arginine and lysine cause mitochondrial fragmentation, reduce mitochondrial membrane potential, and lead to enlarged, hyper-acidified vacuoles. What cellular pathways help manage this arginine and lysine overload? Our RNAseq analysis of cells lacking Art1 revealed that the arginine degradation pathway and phosphate (PHO) regulon are both transcriptionally upregulated, suggesting that these pathways may be important for managing excess amino acids. The arginine degradation pathway clearly plays a well-defined role in managing excess arginine by converting it into other metabolites. However, the connection between amino acid excess and the PHO regulon is less apparent; Elevated polyphosphate synthesis helps store basic amino acids in the vacuole, which would help mitigate excess cytosolic arginine and lysine in the art1Δ cells. However, while both the arginine degradation and phosphate regulon pathways are increased in expression, the importance of these two pathways in excess arginine and lysine management remains unclear. To assess the relative importance of these pathways, double mutant cells lacking Art1 and key genes in these pathways were generated. Tetrad dissections, serial dilution assays, and quantitative fluorescence confocal microscopy revealed that the arginine degradation pathway is essential for art1Δ cell survival. In contrast, loss of the PHO regulon had little impact on art1Δ cell phenotypes, indicating that this pathway, though highly upregulated transcriptionally, is not as functionally significant in managing arginine and lysine excess. Understanding these systems will help elucidate how α-arrestins regulate metabolite balance to control cell physiology. All the pathways considered in these studies are conserved from yeast to man, making our findings relevant to the dysfunction of the kidney, cardiovascular, and central nervous systems, as seen in hyperargininemia, a disease of arginine excess.

B350/P0928
Synergistic Role of Amino Acids in Enhancing mTOR Activation Through Lysosome Positioning
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Lysosome positioning, or lysosome cellular distribution, is critical for lysosomal functions in response to both extracellular and intracellular cues. Amino acids, as essential nutrients, have been shown to promote lysosome movement toward the cell periphery. Peripheral lysosomes are involved in processes such as lysosomal exocytosis, cell migration, and metabolic signaling functions that are particularly important for cancer cell motility and growth. However, the specific types of amino acids that regulate lysosome positioning, their underlying mechanisms, and their connection to amino acid-regulated metabolic signaling remain poorly understood. In this study, we developed a high-content imaging system for unbiased, quantitative analysis of lysosome positioning. We examined the 15 amino acids present in cell culture media and found that 10 promoted lysosome redistribution toward the cell periphery to varying extents, with aromatic amino acids showing the strongest effect. This redistribution was mediated by promoting outward transport through BORC-

kinesin1/3 axis and simultaneously reducing inward transport via inhibiting the recruitment of Rab7 and JIP4 onto lysosomes. To identify the upstream amino acid transporters, we individually knocked down seven members of the Solute Carrier (SLC) family with established roles in amino acid transport and localization to either the plasma membrane or lysosomes. Lysosome positioning assays revealed that SLC3A2, SLC7A11, and SLC7A1 are required for aromatic amino acid induced lysosome dispersion. When examining the effects of amino acids on mTOR activation, a central regulator of cell metabolism, we found that the aromatic amino acids, such as phenylalanine did not activate mTOR on their own. However, combining phenylalanine with arginine, which activates mTOR without affecting lysosome positioning, synergistically enhanced mTOR activity. This synergy was lost when lysosomes failed to localize to the cell periphery, as observed in kinesin 1/3 knockout (KO) cells. Furthermore, breast cancer cells exhibited heightened sensitivity to phenylalanine-induced lysosome dispersal compared to noncancerous breast cells. Expression of an engineered lysosome membrane protein LIMP2 that is fused with kinesin 3 motor protein KIF1B relocated lysosomes to the cell periphery and significantly enhanced arginine-induced mTOR activation in a breast cancer cell line. In summary, amino acid-regulated lysosome positioning and mTOR signaling depend on distinct sets of amino acids. Combining lysosome-dispersing amino acids with mTOR-activating amino acids synergistically enhances mTOR activation. This synergistical effect relies on lysosomal cell-peripheral distribution and may play a role in cancer cell activities.

Cell Stress and Quality Control - Autophagy

B317/P0895
Adaptive Increase in BNIP3 Rescues Mitophagy in a PINK1-/- Model of Parkinson's disease
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Defective autophagy and impaired mitochondrial maintenance are pathological hallmarks of neurodegenerative diseases, such as Parkinson’s disease (PD). Familial mutations causative for PD in PINK1 or Parkin are predicted to disrupt mitophagy, the selective autophagic removal of damaged mitochondria. However, PINK1 knock out mouse models display mild phenotypes and lack the pronounced motor deficits associated with disease progression in humans. To understand how the loss of PINK1 affects the engulfment of mitochondria under normal conditions and following mitochondrial damage, we performed biochemical fractionation of autophagosome and proteomic profiling from control or PINK1-/- mouse brain, in addition to monitoring rates of autophagy and mitophagy by immunblotting and live imaging in control and PINK1-/- primary neurons. At baseline, we found few changes in the amount of mitochondrial proteins within the autophagosomes and the maintenance of FIS1 in autophagosomes, a marker of mitophagy. Rather, we observed that basal rates of autophagic degradation were impaired, and clearance of mitochondria following acute damage was delayed but not prevented in PINK1-/- neurons. In search of mechanisms to support the continued clearance of damaged mitochondria without PINK1, we found higher levels of the selective mitophagy receptor BNIP3 in PINK1-/- derived autophagosomes and increased expression of BNIP3 at both the transcript and protein level in PINK1-/- brains and neurons. We performed shRNA-mediated depletion of BNIP3 in primary neurons and found that it completed ablated autophagic turnover of damaged mitochondria in the absence of PINK1, suggesting BNIP3 is required for mitophagy in the absence of PINK1. Thus, an adaptive mitophagy pathway mediated by BNIP3 compensates for the loss of PINK1. Using publicly available RNA sequencing data, we found that decreased levels of Bnip3 correlate with disease status in PD patients. Thus, we hypothesize that BNIP3 supports mitophagy in neurons, but as either BNIP3 levels decline or mitochondrial damage accelerates, it becomes insufficient to maintain neuronal homeostasis, leading to neurodegeneration.

Our results help to explain the lack of phenotype in this mouse model of PD, and indicate both selectively vulnerabilities that contribute to disease progression, as well as potential avenues for therapeutic intervention.

B318/P0896

An exocyst subcomplex is essential for autophagosome biogenesis in Saccharomyces cerevisiae

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Autophagy is an evolutionarily conserved intracellular degradative pathway that is found to be dysregulated in several pathological conditions, such as neurodegenerative diseases, mitochondrial diseases, intracellular infections, and cancer. Macroautophagy, one of the key autophagy pathways, begins with the emergence of a cup-shaped phagophore that progressively surrounds the cargo, sealing it within a double-membrane vesicle called the autophagosome. Autophagosomes then fuse with lysosomes/vacuoles and undergo degradation. The by-products are then recycled back to the cytosol, where they serve as nutrients. Thus, the autophagy pathway is crucial in maintaining cellular homeostasis. Despite decades of research, many fundamental questions remain unresolved in the field. One of the major open questions is how the autophagosomal membrane expands. To understand the mechanism of autophagosomal membrane expansion, a yeast genetic screen was carried out in our laboratory. Several vesicular trafficking genes were screened for their involvement in autophagy using loss-of-function mutants. The screen identified a tethering complex exocyst to be involved in autophagy. The exocyst complex, an octameric tethering complex, has been widely studied for its function in anchoring secretory vesicles to the plasma membrane during secretion. Our findings reveal that the exocyst complex plays a crucial role in autophagosomal membrane expansion and in preserving the integrity of the autophagosome biogenesis site, a biomolecular condensate. Interestingly, not all subunits of the complex are essential for autophagy, suggesting the presence of an autophagy-specific exocyst complex. Currently, biochemical studies are underway to identify interactors of the complex during autophagy.

B319/P0897

Changes to the liver proteome and lysosomal targetome in long-lived PTEN overexpressing mice

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Chaperone-mediated autophagy (CMA) is the most selective form of autophagy, which targets individual proteins bearing KFERQ-like motifs for lysosomal degradation. CMA negatively regulates rate-limiting enzymes in several energy utilization and anabolic pathways, including glycolysis, cytoplasmic acetyl-CoA generation, de novo lipogenesis, and translation. However, CMA also degrades proteins whose overaccumulation contributes to age-related diseases, including Parkinson's disease, Alzheimer's disease, fatty liver diseases, and most cancers. Previous work from our lab has shown that PTEN is both a regulator and a substrate of CMA. Overexpression (OE) of PTEN enhances resilience to cancer, reduces adiposity, improves insulin sensitivity, and extends lifespan in mice. Our current goal is to characterize the effects of PTEN OE on the liver proteome and lysosomal targetome, via quantitative proteomics. Our results show that PTEN OE induces more pronounced changes in the male liver proteome (651 proteins upregulated and 666 downregulated) compared to the female liver (371 and 353, respectively), including significant downregulation ($p < 0.01$) of proteins involved in the acetyl-CoA shuttle. A STRING pathway enrichment analysis showed that, in both male and female livers, the "lysosome" is the most significantly upregulated KEGG term ($p < 0.0001$). Comparative analysis with other

longevity models in mice, such as dietary restriction and drug interventions (canagliflozin and acarbose), revealed similar proteomic changes, suggesting shared molecular mechanisms. Acetyl-CoA proteins, ACACA, ACACB, and FASN, were significantly downregulated ($p < 0.05$) in all treatments; and the lysosome pathway was the second most upregulated ($p < 0.0001$) in all treatments. To assess the effects of PTEN OE on protein targeting to the lysosomes for degradation (lysosomal targetome), mice were treated with leupeptin to block protein degradation, and lysosomes were then isolated from liver tissue. PTEN OE mice exhibited increased protein uptake compared to controls, including acetyl-CoA related proteins such as FASN, ENO1, ACLY, ACACA, and ACACB. Changes were more significant in female mice than in males. To identify the lysosome targetome, we cross-referenced the significantly accumulated proteins in the lysosome with significantly downregulated proteins of the liver. As well as the 20% enriched lysosome proteins and decrease in liver. We identified proteins spanning diverse cellular functions, including components of the proteasome. Together, these findings highlight PTEN broad influence on cellular metabolism and protein turnover, serving as the basis for our ongoing and future work to characterize the role of selective lysosomal proteolysis in shaping the proteome.

B310/P0888

Deciphering the Ubiquitin Code: Protein Degradation, Autophagy and Protein Trafficking

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The functional consequences of protein polyubiquitination are dictated by the type of ubiquitin chain assembled on the substrate. Of the eight known linkage types, lysine 48 (K48)-linked polyubiquitinated proteins are destined to the proteasome for degradation, whereas lysine 63 (K63)-linked chains predominantly modulate intracellular protein trafficking. K63 poly-ubiquitination is also responsible for removing damaged mitochondria (mitophagy) and promoting mitobiogenesis. In recent years, the ubiquitin–proteasome system (UPS) has emerged as a powerful avenue for therapeutic intervention, giving rise to strategies like PROTACs (Proteolysis Targeting Chimeras) and molecular glues (MGs), which co-opt E3 ligases to drive targeted protein degradation. Mass spec proteomics approaches are cumbersome, and chain selective antibodies don't work well. High-throughput approach for measuring endogenous target ubiquitination with linkage specificity has remained elusive. To address this gap, we employed linkage-selective TUBEs (Tandem Ubiquitin Binding Entities) with sub-nanomolar affinity for distinct polyubiquitin architectures in microtiter-plate format. Focusing on RIPK2, a pivotal mediator of inflammatory pathways, we used L18-MDP to trigger K63-linked ubiquitination and a RIPK2-directed PROTAC (RIPK degrader-2) to promote K48-linked ubiquitination. Our results show that chain-specific TUBEs can clearly distinguish between these ubiquitination patterns, revealing context-dependent, linkage-specific modification of endogenous RIPK2. Drugs that promote mitophagy are also shown to promote K63 poly-ubiquitination of mitochondrial proteins. The broader application of chain selective poly-ubiquitin TUBE technology in discovery of PROTACs and MG drugs and uncovering the mechanism that operate mitophagy and autophagy will be described. Gross, P.H., et al., Accelerating PROTAC drug discovery: Establishing a relationship between ubiquitination and target protein degradation. Biochem Biophys Res Commun, 2022. 628: p. 68-75. Ali S.M et al., "Unraveling chain specific ubiquitination in cells using tandem ubiquitin binding entities." Scientific Reports 15, no. 1 (2025): 22961. https://doi.org/10.1038/s41598-025-07242-9.

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Impaired autophagy leads to mitotic errors and chromosomal instability.

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Micronuclei are DNA fragments left behind after anaphase as a result of the failure to incorporate lagging chromosomes or acentric fragments into the nucleus. These disruptive fragments can signify genomic instability, or even tumorigenesis, and have the potential to contribute to further chromosomal anomalies, as well as stimulate innate inflammatory pathways. Autophagy, an integral process for cellular homeostasis, involves the degradation and recycling of waste materials within the cell facilitated by the autophagosome. This is an incredibly important pathway for the cell to dispose of proteins, organelles, and other cytosolic waste whereby the autophagosome takes in debris for enzymatic degradation in the lysosome. Evidence suggests that under normal cellular conditions, chromosomal instabilities (CIN) such as micronuclei may be disposed of via autophagy. However, other studies have shown that DNA repair processes are inhibited when autophagy becomes disrupted. We sought to determine which mechanistic pathways that contribute to chromosomal instability were interrupted upon the loss of autophagy. Using a combination of pharmacological and genetic manipulation in several mammalian cell lines with different levels of ploidy, we confirmed that impaired autophagy contributes to CIN, inducing micronuclei formation and other aberrant chromosomal phenotypes such as bi- and multi-nucleation. Loss of ATG9A and FIP200, essential upstream autophagy proteins, results in mitotic abnormalities that contribute to CIN. In addition, disruption of autophagy in ATG9A knockout cells led to the mitotic kinase Tank binding kinase 1 (TBK1) being overactivated and mislocalized. We observed punctae formations comprised of activated TBK1 that colocalized in the cytosol with autophagy-associated protein p62. The generation of these punctae interfered with TBK1's ability to function, both at the centrosome and in mitosis. When pharmacologically inhibiting TBK1 activity in the absence of autophagy, many of the CIN phenotypes were reverted. Thus, our results indicate that inhibition of autophagy leads to chromosomal instability in several ways, namely via irregular cell division, as a result of dysfunctional TBK1.

B313/P0891

Neuronal autophagy is differentially regulated in distinct neuron types during aging

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Neuronal autophagy is essential for cellular homeostasis, particularly in neurons, which must remain functional throughout an organism's lifespan. Although critical for neuronal health, its role during aging remains poorly understood. Our lab found that neuronal autophagy declines and becomes dysfunctional with age in cultured mouse dorsal root ganglion neurons (DRGNs). However, detailed age-related analyses of neuronal autophagy have only been conducted in DRGNs, which are relatively homogeneous in morphology and neurotransmitter identity. It remains unclear whether these autophagic changes generalize to other neuron types. To address this gap, I asked: Do distinct neuron types, defined by their morphology and neurotransmitter identity, display unique spatial patterns of autophagy that change during aging? To investigate this, I have used C. elegans to study the cellular and molecular bases of autophagy across the lifespan in vivo. Using spinning disk confocal microscopy, I quantified autophagosomes labeled with the canonical autophagosome marker LGG-1 (LC3) in

two neuron types during aging. A serotonergic neuron, called NSM, displaying a primarily axonal structure, presented an increased number of autophagosomes during aging. In contrast, CEP, a dopaminergic neuron with a predominantly dendritic structure, showed a decrease. These results suggest that different neuron types have distinct autophagic mechanisms, such as differences in autophagosome biogenesis and lysosomal fusion capabilities that change with age. Notably, by late aging, CEP dopaminergic neurons exhibited signs of degradation, while NSM neurons remained intact. This suggests that reduced autophagy may contribute to neurodegeneration, while enhanced autophagy may provide neuroprotection. In future studies, I will examine the temporal dynamics of autophagy and measure neuronal activity across aging. Additionally, I will examine other neurons in C. elegans with different morphologies and neurotransmitter identities to compare trends during aging. I will also test whether overexpressing early autophagy components can rescue aging-associated neurodegeneration in dopaminergic neurons. Understanding how autophagy is regulated in distinct neurons may reveal the role of autophagy in neurodegeneration during aging in specific neuron types and brain regions. Ultimately, this can be applied to novel, targeted strategies to treat age-related neurodegenerative diseases in a more efficient manner.

B314/P0892

Novel interaction between SLC7A14 and ATG8 proteins suggest SLC7A14's role in linking amino acid homeostasis and autophagy

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Autophagy is a homeostatic process that supports cellular health by degrading and recycling damaged macromolecules and organelles. Retinal cells consistently experience high metabolic demands which makes autophagy essential in maintaining their overall integrity and function. Disruption of autophagy has been implicated in blinding disorders, including retinitis pigmentosa, cataracts, and glaucoma. However, the molecular mechanisms by which impaired autophagy leads to the development and progression of disease and subsequent visual dysfunction is incompletely understood. SLC7A14 encodes a proposed lysosomal cationic amino acid transporter, and autosomal recessive mutations have been linked to retinitis pigmentosa. However, the precise biological role of SLC7A14 in retinal health is unknown. To fill this gap in knowledge, we generated an SLC7A14 knockout mouse model and found that SLC7A14 has a functional role within retinal interneurons and contributes to visual processing. Next, we investigated the subcellular localization of SLC7A14 using live- and fixed-cell microscopy of cultured cells. We observed that SLC7A14 localizes within a range of endolysosomal compartments, rather than exclusively lysosomes. Consistent with this finding, SLC7A14 showed poor colocalization with the lysosomal marker LAMP1 in retinal cross sections. We performed yeast two-hybrid screens to identify novel SLC7A14 binding partners. Several autophagy-related family 8 (ATG8) proteins, including LC3A, GABARAP, and GABARAPL1, were identified, suggesting a role in autophagy regulation. Bioinformatic analysis revealed an LC3-interacting region (LIR) motif within an unstructured cytoplasmic loop and binding assays using recombinant protein confirmed the interaction. Our results suggest that SLC7A14 acts as a regulator of vesicular trafficking and autophagy via ATG8-mediated interactions. Interestingly, disease-associated mutations were predicted to increase cytoplasmic presentation of LIR motifs, suggesting a mechanism in which aberrant recruitment of autophagy machinery by SLC7A14 may contribute to disease progression. Testing this prediction is an area of active research. Together, our work identifies a novel mechanism by which SLC7A14 regulates autophagy, and highlights ATG8 interactions as a potential target for understanding and treating visual disorders driven by impaired autophagy.

B311/P0889

Nuclear autophagy safeguards genomic integrity and drives stem cell-mediated fusion upon DNA damage in skeletal myofibers

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In all cell types, DNA instability is typically resolved by a DNA damage response (DDR). Myofibers are multinucleated skeletal muscle cells formed during development by the fusion of proliferating myoblasts, derived from muscle stem cells, or satellite cells (SCs). Postnatally, SCs remain essential: they can activate, proliferate into myoblasts, and fuse with pre-existing myofibers to increase myonuclear number and muscle mass in response to defined stimuli. In post-mitotic myofibers, DNA repair mainly relies on the error-prone non-homologous end joining (NHEJ) pathway, active across the cell cycle. Nevertheless, myonuclei are remarkably resistant to cell death, showing minimal nuclear turnover in adult life. Our data suggests the existence of an additional protective mechanism allowing myotubes myofibers to cope with DDR, such as NHEJ. Autophagy is a process by which cells degrade and recycle proteins or molecular debris to maintain intracellular homeostasis. We show that myonuclear genomic repair is impaired when autophagy is blocked suggesting that autophagy preserves genomic integrity in myonuclei, supporting long-term myofibers survival following DDR. To further investigate this mechanism, we used an AAV-based strategy to induce DNA damage specifically in myonuclei of the tibialis anterior muscle in a controlled manner. Surprisingly, this resulted in an increase in proliferative activated SCs and in newly fused myonuclei. Moreover, we observed in vivo autophagy activation, with an increase of Atg7 (autophagy marker) protein levels in muscle following DNA damage. These findings suggest a link between autophagy-induced DDR, SC activation in vivo and DNA compensation via myoblast fusion. According to this data, autophagy facilitates the release of factors promoting SCs activation and subsequent fusion to myofibers. This process would support nuclear accretion, compensating for the DNA loss associated with autophagy-driven DDR, thus maintaining healthy muscle homeostasis.

B315/P0893

PP2A and CDK16 regulate WIPI2B phosphorylation and neuronal autophagy

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With today's aging population, the incidence of age-related diseases, such as neurodegenerative diseases (NDDs), is increasing. As such, there is a growing need to understand the cellular mechanisms contributing to neuronal aging. A hallmark of aging is the aggregation of misfolded proteins. Autophagy is a highly conserved degradation pathway that neurons utilize to clear protein aggregates and is essential for maintaining neuronal health. However, autophagy function decreases with age, and dysregulated autophagy is implicated in many NDDs. Previously, we restored autophagy function in aged primary neurons by overexpressing WIPI2B, a key autophagy component. Interestingly, this restoration was contingent upon the phosphorylation state of WIPI2B. These data suggest that WIPI2B phosphorylation regulates its function in neuronal autophagy. Thus, identifying the kinase and phosphatase that regulate WIPI2B phosphorylation will provide an avenue to modulate WIPI2B function and control rates of neuronal autophagy. To identify these WIPI2B regulators, we utilized Caenorhabditis elegans as a screening system. The autophagy pathway is highly conserved between

mammals and C. elegans and the WIPI2B ortholog in C. elegans (CeWIPI2B) also has a conserved phosphorylation site. Using a simple, visual readout of neuronal autophagy function, we determined that phosphorylation of CeWIPI2B regulates neuronal autophagy in vivo. With this same phenotype, we screened for regulators of neuronal autophagy and identified PP2A and CDK16 as candidates. Epistatic analysis suggested that PP2A, CDK16, and CeWIPI2B function in the same pathway to modulate neuronal autophagy. Given the genetic evidence, we verified PP2A and CDK16 enzymatic activity on mammalian WIPI2B through in vitro phosphorylation assays and showed localization of PP2A and CDK16 to autophagosomes in cultured primary neurons. Altogether, these data support PP2A and CDK16 as regulators of WIPI2B phosphorylation and neuronal autophagy. Ultimately, a better understanding of how neurons regulate autophagy will uncover novel targets for modulating neuronal autophagy, providing potential therapeutic strategies for NDDs.

B309/P0887

Progranulin supports basal autophagic flux but is dispensable for ER stress-induced autophagy in HT22 cells

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Autophagy is a conserved degradation and recycling process essential for cell development, function, and survival. While it serves as an anti-inflammatory mechanism, impaired autophagy contributes to inflammatory and neurodegenerative diseases. Progranulin (PGRN) is a multifunctional glycoprotein implicated in neuroprotection and lysosomal function, but its role in stress-induced autophagy remains unclear. This study aimed to investigate the regulation of how cellular stress regulates autophagy by cellular stress in neuronal HT22 cells and to explore clarify the contribution of PGRN to this process. We first examined whether basal expression of PGRN influences autophagic flux. siRNA-mediated knockdown of PGRN resulted in reduced LC3-II/I and elevated p62, indicating that basal PGRN is required to maintain autophagic flux. Because endoplasmic reticulum (ER) stress induced by tunicamycin (TM) increased both autophagic flux and PGRN expression, we next asked whether ER stress-induced PGRN contributes to ER stress-dependent autophagy. Upon ER stress, BiP upregulation confirmed successful stress induction and siRNA effectively reduced PGRN expression. Despite these manipulations, LC3-II accumulation elicited by ER stress was not significantly altered by PGRN knockdown, indicating that ER stress-induced PGRN does not play a prominent role in driving ER stress–dependent autophagic activity. Taken together, these results support two key conclusions. First, basal PGRN is required to sustain autophagic flux under resting condition. Second, although ER stress can elevate PGRN expression, such ER stress-dependent increases in PGRN do not, by themselves, produce a substantial changes in autophagic flux. Rather, ER stress-induced PGRN may function primarily as a buffering factor that helps further deterioration of autophagy capacity, instead of actively imposing large shifts in ER stress-evoked flux. These findings provide new insights into the context-dependent roles of PGRN in autophagy regulation and their relevance to neurodegenerative disease mechanisms.

B316/P0894

Screening of the Factors for Autophagy Termination in Long-Term Starvation

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In our previous study, Tag1 is identified as a vital gene regulating autophagy termination in Saccharomyces cerevisiae. The termination occurs between 10-12 hours, which is also observed in mammal cells recently in our laboratory. By using HaloTag7 in HeLa cell, the autophagic flux was measured and characterized with a termination occurring around 9-12 hours. To identify genes crucial for the termination in mammal cells, a genome-wide screening was conducted. A combination of lentiCRISPR knockout library with FACS (fluorescence-activated cell sorting) is sufficient to meet requirements of a high-throughput loss-of-function assay. The sequencing data reveals enrichment of 18 genes knocked out in sorted cells with increased signal of autophagosome even after 24 hours starvation, which suggests the genes are in control of autophagy termination. Further screenings refine the range and finally identify a gene PCDH17, a gene controlling expression of protocadherin 17. We will report ongoing analysis of how it is involved in autophagy termination.

Cell Stress and Quality Control - Cellular stress signaling pathways

B267/P0845
A direct role for a mitochondrial targeting sequence in signalling stress
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Mitochondrial protein import is required for maintaining organellar function. Perturbations in this process are associated with various physiological and disease conditions. Several stress responses, including the mitoCPR, combat damage caused by mitochondrial protein import defects. However, how this defect is sensed remains largely unknown. Here, we reveal that the conserved mitochondrial Hsp70 co-chaperone, Mge1, acts as a stress messenger in budding yeast. During mitochondrial stress, unimported Mge1 entered the nucleus and triggered the transcription of mitoCPR target genes. This was mediated by Mge1's interaction with the transcription factor Pdr3 on DNA regulatory elements. Mge1's mitochondrial targeting sequence was both sufficient and essential for mitoCPR induction, demonstrating that in addition to their roles in mitochondrial protein import, targeting sequences can also function as signaling molecules. As protein import defects are a common consequence of various types of mitochondrial damage, these findings suggest a novel function for Mge1's targeting sequence as an indicator of mitochondrial health.

B264/P0842
Alternative splicing of Eif2ak3 as a new potential regulator of PERK function in response to neuronal ER stress
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One of the main hallmarks of most neurodegenerative disorders is the presence of protein aggregates. On a molecular level, these misfolded proteins induce ER stress, leading to activation of the unfolded protein response (UPR), a homeostasis pathway aimed at restoring cellular proteostasis. PERK is one of the three ER-resident kinases which comprise the UPR and, in response to ER stress, it becomes active leading to downregulation of cap-dependent translation via phosphorylation of eukaryotic imitation factor 2 alpha. PERK activity regulation at the protein level is well characterized, however, little is known about regulation of EIF2AK3, its coding gene, at the RNA level. Our lab has described that certain Eif2ak3 genetic variants have an effect on PERK function regulation. Moreover, different genomic databases including Ensembl, UCSC and Vastdb, include different Eif2ak3 transcript variants, even though, to our knowledge, none of them have been

validated or studied. Therefore, this project aims at investigating the role of Eif2ak3 alternative splicing in PERK regulation during ER stress in primary neurons. Our results identify and validate expression of previously unknown Eif2ak3 mRNA variants in rat neuronal cultures. Expression levels of all Eif2ak3 transcripts significantly increase in response to ER stress, but not to other neuronal stressors. Sequence analysis predicts that these Eif2ak3 mRNA variants lead to the activation of the nonsense-mediated decay (NMD) pathway due to the presence of premature stop codons. Previous studies have shown evidence supporting the existence of a mutual inhibitory regulation between the UPR and NMD pathways. Therefore, we propose that the new mRNA transcripts participate in the crosstalk between these two pathways and might represent an unexplored mechanism with implications in most neurodegenerative disorders which present with UPR activation.

B265/P0843

An allele-specific ISR inhibitor, mISRIB, is a cognitive enhancer

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In eukaryotes, a major strategy to cope with cellular stressors is to reprogram mRNA translation through activation of the Integrated Stress Response (ISR). The ISR is well-established as a central regulator of cellular homeostasis, controlling translation initiation by reducing the levels of functional ternary complex (TC). In addition to this homeostatic function, the ISR has also emerged as a primary way to regulate mnemonic processes. Indeed, converging pharmacological and genetic evidence across multiple model organisms has shown that repression of the ISR in the brain enhances long-term memory consolidation, and conversely, overactivation blocks memory formation. The ISR's role in cognitive health is further strengthened through its implication in age-related cognitive decline, traumatic brain injury, Down syndrome, and several neurodegenerative diseases. The discovery of ISRIB, a non-toxic small-molecule inhibitor of the ISR and cognitive enhancer in rodents, has propelled a detailed molecular and functional dissection of the pathway in recent years. However, precise knowledge of which specific cells and circuits drive the ISRIB effect, in the brain or elsewhere, is lacking. More broadly, it is becoming increasingly clear that cellular identity and developmental stage selectively influence the mechanistic and functional details of the ISR. Here, we present a molecular tool for direct temporal and cell-specific ISR inhibition. To create a protein-ligand pair that is functionally orthogonal to ISRIB and its molecular target, eIF2B, we engineered a single point mutation in the ISRIB-binding pocket of eIF2B (dubbed eIF2Bmut) to accommodate a previously inactive ISRIB analog (mISRIB). eIF2Bmut does not affect normal eIF2B or ISR function in vitro nor in cellula, but renders cells sensitive to ISR inhibition with mISRIB. Conversely, mISRIB recapitulates ISRIB's mechanism of action, but in a manner that is selective for eIF2Bmut and not wild type eIF2B. In the rodent brain, the mISRIB :: eIF2Bmut pair functionally mimics ISRIB's mnemonic effects in multiple mouse models, providing further evidence for ISRIB's on-target function, and reinforcing ISR modulation as a compelling avenue for therapeutic development. Since eIF2Bmut can be expressed cell-specifically, and mISRIB injected can be timed, this chemical-genetic tool will enable precise dissection of ISR function in the brain and beyond and will help contribute to our understanding of how memories are formed.

B268/P0846

Coordination of Mitochondrial Stress Responses in Human Cells

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The DELE1-HRI axis of the integrated stress response (ISRmt) is activated by most mitochondrial stress conditions, yet how the ISRmt regulates mitochondrial homeostasis remains unclear. Here, we investigated its role in regulating mitophagy, a key mitochondrial quality control mechanism. Our findings show that the ISR suppresses PINK1-dependent mitophagy under many mitochondrial stress conditions by maintaining mitochondrial presequence protein import, independently of ATF4 activation. Mitochondrial presequence protein import efficiency is tightly linked to the rate of protein synthesis. Without ISR, increased protein synthesis overwhelms the mitochondrial import machineries, reducing import efficiency. This impairment can be mitigated by pharmacological attenuation of protein synthesis, such as with mTOR or general translation inhibitors. Under severe depolarizing stress, mitochondrial import is heavily impaired even with active ISR, leading to significant PINK1 accumulation. In contrast, mild mitochondrial stress allows more efficient protein import in the presence of ISR, resulting in lower mitophagy. Without ISR, mitochondrial protein import becomes significantly compromised, causing PINK1 accumulation to reach the threshold level necessary to trigger mitophagy. These findings reveal a novel link between ISR-regulated protein synthesis, mitochondrial protein import, and mitophagy, offering potential therapeutic targets for diseases associated with mitochondrial dysfunction.

B270/P0848

Epidermal Growth Factor vs Oligo-215: Calcium Signaling in PC12 Cells After Laser-Induced Shockwave

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This study compares EGF and Oligo-215's effects on calcium dynamics in PC12 cells subjected to injury via Laser-Induced Shockwave (LIS). EGF promotes cell regeneration, while Oligopeptide-215 (Oligo 215) has anti-inflammatory and healing properties, though its signaling mechanisms are less understood. PC12 cells were loaded with Fluo-4 AM(calcium indicator), ethidium dimer III (dead cell marker) and treated with the following conditions: 110 ppm Oligo and 110 ng/mL EGF or solvent controls. Live-cell imaging was performed with the ET-GFP channel every 3 seconds for 30 minutes; LIS was applied 3 minutes after imaging began. ImageJ was used for image sorting and processing and Matlab was used to extract calcium changes. Dead cells were identified via positive ethidium dimer III staining and excluded from analysis. Post-treatment calcium transients were calculated by F/F0, where F0 is the mean pixel intensity before treatment. One-way ANOVA followed by post hoc multiple comparisons showed no significant difference between Oligo-215 + SW and EGF + SW in peak responses, indicating comparable shockwave-evoked calcium signaling under both conditions. These findings suggest that Oligo-215 may not affect the injury evoked calcium increase. Future studies will determine whether this peptide has an effect on cell survival and more downstream calcium signalling. LIS has been previously demonstrated to trigger an immediate calcium increase however the recovery to pre-LIS levels has not been investigated in the presence of Oligo-215.

B263/P0841

Eukaryotic translation initiation factor 3D regulates stress granule assembly via its RNA binding domain

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Stress granules are cytoplasmic mRNA-protein complexes that form by liquid-liquid phase separation in response to a variety of stresses. Their assembly is contingent upon the inhibition of mRNA translation. Depending on the type of stress and severity, they can promote stress resistance or act to reduce cellular fitness and they are implicated in ageing and related pathologies, including neurodegeneration. Components of the eukaryotic translation initiation factor 3 (eIF3) complex are found in stress granules and some are proposed to promote their assembly. Here we identify several eIF3 complex factors that are required for robust in vivo assembly of heat-induced stress granules in the nematode worm C. elegans. One of these is eIF3d, a non-core eIF3 complex factor with distinct roles in translation initiation. We found that eIF3d is recruited to stress granules in human cells in response to a range of stresses, however, it's importance for stress granule assembly is stress selective. The RNA-binding domain of eIF3d was essential and sufficient for its recruitment to stress granules. The deletion of this domain blocked stress granule formation in response to arsenite-induced stress and decreased cell survival. Furthermore, the exogenous expression of the RNA-binding domain rescued stress granule assembly in eIF3d-depleted cells. This study demonstrates that eIF3d is a critical factor in promoting stress granule assembly and does not simply enter stress granules as a passive consequence of the inhibition of translation initiation.

B277/P0855

Exposure to Environmental Fine Dust Promotes Stress Pathway Activation and Macropinocytic Alterations in p53-Deficient Cells

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Fine dust, or particulate matter (PM), is a pervasive environmental pollutant with profound implications for human health. Due to their small size, particles such as PM2.5 (particles ≤2.5 µm) can penetrate deep into the respiratory tract, translocate into the bloodstream, and evade innate defense mechanisms, thereby initiating cascades of oxidative stress, inflammation, and genotoxic damage, all of which are key contributors to tumorigenesis. In this study, we investigated the molecular and cellular responses to PM2.5 exposure in an in vitro model using wild-type (WT) cells and p53-deficient cells with accumulated genetic alteration. Cells were subjected to short-term and chronic exposure, followed by assessments of viability, morphology, different pathway activation levels and gene expression. PM exposure reduced cell viability and increased cytotoxicity in a concentration-dependent manner. Effects were more pronounced in p53-deficient cells. After exposure to PM2.5, p53-deficient cells exhibited a distinct phenotype characterized by prominent intracellular macropinosomes, which were absent in WT cells, as observed via advanced imaging techniques. Luciferase-reporter assays demonstrated that PM treatment activated stress-responsive pathways, including oxidative stress (ARE) and xenobiotic response (XRE). In WT cells, this was accompanied by p53 pathway activation. The macropinosomes, present in p53-deficient cells after PM exposure, may have a functional advantage: PM-treated p53-deficient cells exhibited higher survival under nutrient-deprived conditions compared to WT cells. This is a phenotype associated with enhanced survival under stress conditions, similar to mechanisms reported in early tumorigenesis. Antioxidant treatment with N-acetylcysteine partly suppressed ARE activation, supporting oxidative stress as a central driver of the response. Transcriptomic profiling after chronic PM exposure revealed broad reprogramming, including regulators such as SMAD3, Gli, ELF2 (NERF), and AP4, which are pathways linked to proliferation, transcription, differentiation and immune signaling. These findings demonstrate that p53 status influences both stress pathway activation and cellular uptake mechanisms, such as macropinocytosis, in response to environmental particulate matter. Overall, our results suggest that fine dust exposure, in the context of impaired tumor suppressor function, may drive cellular processes relevant to early stages of cancer development.

B274/P0852
Heat stress-induced regulation of myokines and extracellular vesicles in skeletal muscle
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Skeletal muscle is recognized as an endocrine organ that releases bioactive factors, including myokines and extracellular vesicles (EVs). These secreted molecules play critical roles in inter-organ communication, regulating systemic metabolism, inflammation, and homeostasis. While exercise-induced changes in the skeletal muscle secretome have been widely studied, their regulation under other environmental stressors remains unclear. The present study aimed to elucidate the effects of heat stress on skeletal muscle–derived myokines and EVs. In vitro, C2C12 myotubes were cultured in Dulbecco's Modified Eagle Medium (DMEM) under low glucose (1.0 g/L glucose) or high glucose (4.5 g/L glucose) conditions for 24 h. High glucose markedly increased the expression and secretion of the inflammatory chemokines C-C motif chemokine ligand 5 (CCL5) and C-X-C motif chemokine ligand 10 (CXCL10). However, exposure to acute heat stress (42℃ for 3 h) significantly attenuated this glucose-induced induction. In vivo, mice fed either a standard chow diet or a lard-based high-fat diet were subjected to heat stress (45℃ for 1 h followed by 23 h recovery). Consistent with the in vitro results, skeletal muscle from heat-stressed mice displayed reduced CCL5 and CXCL10 expression compared with controls. These findings indicate that heat stress counteracts glucose- and diet-induced pro-inflammatory signaling in skeletal muscle. We next focused on another class of secreted factors, EVs, and performed a global analysis of the impact of heat stress on the miRNA profile encapsulated within skeletal muscle–derived EVs. RNA sequencing (RNA-seq) revealed significant alterations in multiple EV-encapsulated miRNAs in response to heat stress, suggesting that thermal stress not only modulates cytokine secretion but also dynamically reshapes the regulatory information carried by EV cargo. Collectively, these findings demonstrate that skeletal muscle responds to heat stress through dual mechanisms involving both myokines and EV-encapsulated miRNAs, with important implications for understanding skeletal muscle regulation of inflammatory signaling under environmental and nutritional stressors.

B278/P0856
Interplay of protein dynamics and modifications regulate temporal cell response
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Cells are subjected to a vast range of cellular stresses (input) and subsequently adjust its response (output). Dynamical changes in protein levels are important for relaying these signals in a variety of signaling networks, with information being conveyed through the frequency, amplitude, duration, or other features of the temporal signal. Another form of signaling can occur through protein post-translational modifications (PTMs), whereby single or multiple of modifications on a protein consequently impact its function, localization, or activity. How the dynamics of protein levels and PTMs are integrated however remains poorly understood. The mammalian stress sensing protein p53, known to be post-translationally modified at over 100 different sites, is also a transcription factor whose levels oscillate in response to stress signals. It therefore represents a paradigm for studying the integration of cellular information between complex protein dynamics and combinatorial post-translational modifications. We employed a state-of-the-art mass spectrometry method (I2MS), which enabled us, for the first time, to measure the complete combinations of PTMs on individual intact human p53 proteins over time. By combining different mass spectrometry methods using mathematics, we devised a method to decipher the combinations of PTMs on a single protein. Our analysis enabled us to

scope the landscape of p53 PTMs which was surprisingly relatively restricted compared to the theoretically vast number of combinations (2^100 possibilities). Using a complementary candidate-based approach, we ascertained that specific acetylations in the C-terminus of p53 were especially dynamic, being reduced in the second pulse of p53 compared to the first. By manipulating acetylation states via expression of non-acetylatable p53 mutants or use of inhibitors targeting acetylation enzymes, we determined that p53 acetylation causally influenced downstream gene expression, with higher acetylation levels corresponding to expression of senescence and apoptosis genes. Furthermore, using time-series RNA-Seq, we established that the differentially modified p53 pulses caused a temporal separation in p53 transcriptional outputs and functional stress-response programs. The earlier pulses of acetylated p53 instigated immune cell modulating signals whereas latter unacetylated p53 pulses preferentially induced DNA repair and metabolic alterations. These results highlight the importance of dynamical changes in both protein levels and PTMs in cellular signal transduction and information processing, a phenomenon that may have implications in other similar temporally regulated biological systems.

B273/P0851

Pharmacological Ascorbate Abrogates Autophagy Initiation in Pancreatic Cancer Cells by Disrupting Vps34 Complex Activation

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Pharmacological ascorbate (high-dose ascorbate) shows selective cytotoxicity against some cancer cells, yet its mode of action remains unclear. Autophagy is a critical pro-survival pathway in pancreatic ductal adenocarcinoma (PDAC), a cancer type known for its metabolic plasticity and resistance to therapy. Here, we investigated the acute effects of high-dose ascorbate on autophagy regulation in PDAC cells harboring KRAS and TP53 double mutation. To overcome the subjective limitations of traditional autophagy assays, we established a high-content screening (HCS) platform using cells infected with adenoviruses containing the mCherry-GFP-LC3 reporter. By analyzing the number and ratio of autophagosome (yellow) and autolysosome (red) puncta while excluding non-expressing or overexpressing cells, our method provides objective and quantitative autophagy flux data without requiring reporter expressing stable cell lines. This robust platform was used to interrogate the effects of high-dose ascorbate across multiple cell lines. Our HCS analysis in PDAC cell lines (MIA Paca-2, SNU410) and a normal pancreatic epithelial cell line (H6c7) demonstrated that high-dose ascorbate (4 mM for 2h) rapidly and potently inhibits starvation-induced autophagy specifically in pancreatic cancer cells. This effect was dependent on intense oxidative stress, as it was fully rescued by the antioxidant N-acetylcysteine (NAC) in both MIA Paca-2 and SNU410 cells. Further investigation revealed that high-dose ascorbate blocks autophagy at the initiation stage. This was corroborated by live-cell imaging of a phosphatidylinositol 3-phosphate (PI3P) reporter (mCherry-WDFY) in MIA Paca-2 cells, which showed an ascorbate-induced decrease in the production of PI3P, the lipid product of the Vps34 kinase complex essential for autophagosome nucleation. Our study identifies a novel anti-cancer mechanism of pharmacological ascorbate, where acute oxidative stress blocks a critical survival pathway in PDAC by inhibiting autophagy at its earliest step. By disrupting the activation of the Vps34 complex, ascorbate effectively shuts down the cell's ability to initiate a protective autophagic response to stress. This vulnerability in the autophagy initiation machinery by high-dose ascorbate may represent a promising strategy for pancreatic cancer therapy.

B279/P0857
Pre-clinical model of dysregulated FicD AMPylation causes diabetes by disrupting pancreatic endocrine homeostasis.
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The proper balance for the synthesis, folding, modification and degradation of proteins, also known as protein homeostasis, is vital to cellular health and function. The unfolded protein response (UPR) is activated when the mechanisms maintaining protein homeostasis in the endoplasmic reticulum (ER) become overwhelmed. Prolonged or strong UPR responses can result in elevated cellular inflammation, damage, and even death. Previously, we discovered that the bi-functional enzyme FicD can modulate the UPR response via post-translational modification of BiP by AMPylation during homeostasis and deAMPylation during stress. Human FicD with an arginine-to-serine mutation disrupts FicD deAMPylation activity resulting in severe neonatal diabetes. To investigate the importance of FicD-mediated AMPylation in a mammalian system, we generated a conditional null allele of FicD and a human disease associated mutant allele FicDR371S in mice. We observed elevated BiP AMPylation levels across multiple tissues and signature markers for diabetes including glucose intolerance and reduced serum insulin levels. While the pancreas of mFicDR371S mice appeared normal at birth, adult mFicDR371S mice displayed disturbed pancreatic islet organization that progressed with age. Interestingly, immunohistochemical staining of FicDR371S islets reveal striking similarities in islet disorganization to that of type 2 diabetic mouse models in which chronic overstimulation leads to β-cell dysfunction. Thus, mFicDR371S mice provide an novel preclinical mouse model for the study of UPR associated diabetes and demonstrate the essentiality of FicD for tissue resilience.

B280/P0858
Spray modification shapes the spatial proteomic architecture
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How local post-translational modifications (PTMs) shape the spatially localized proteome remains largely unexplored. Traditional lock-and-key models emphasize strict motif recognition, yet many PTM writers act with unexpectedly broad specificity that cannot be explained by sequence rules alone. Progress in this area has been limited because conventional proteomic methods catalog global modification sites but cannot disentangle intrinsic spray activity from secondary effects of overexpression, stress, or chemical perturbation. To overcome these limitations, we introduced a versatile GFP–GBP recruitment platform that tethers diverse PTM enzymes—including acetyltransferases, biotin ligases, ubiquitin and SUMO conjugating enzymes, and ADP-ribosyl transferases—to a non-physiological substrate. In this enforced-proximity context, robust GFP modification serves as a stringent indicator of spray activity. Comprehensive proteomic dissection of acetyl spray architecture revealed a spatial signature characterized by compartment-restricted distribution, relaxed sequence selectivity, and enrichment at solvent-exposed, disordered regions, notably within nuclear localization motifs. Guided by these features, we further demonstrate that acetyl spray disrupts nuclear import and drives cytoplasmic accumulation of proteins, thereby remodeling the spatial proteome. Our results establish spray-PTMs as an inherent enzymatic property that organizes localized proteome, uncovering hidden substrates and revealing how proximity-driven modification reshapes protein localization and cellular function.

B275/P0853

Temperature Specific Regulation of NDR Kinase Orb6 by MAP kinase Sty1 to Promote Heat Stress Resilience

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Cellular response to environmental fluctuations, such as increased temperature, is crucial in promoting cell survival and plays an increasingly recognized role in cancer biology. Important cellular functions altered by heat stress are cell polarization and protein translation. Previous studies have shown that heat stress alters the dynamics of Cdc42, a key regulator of cell polarization in eukaryotes, and promotes RNP granule formation, reprogramming protein translation. The biological mechanisms underlying these vast changes are only partially known. Here, we report that conserved NDR kinase Orb6, a homologue of mammalian STK38, responds to heat stress and regulates heat stress resilience by modulating Cdc42 dynamics and promoting stress granule assembly. Also, we discovered a finely tuned mechanism whereby stress-activated MAP kinase Sty1 negatively regulates Orb6 kinase and Orb6 C-terminal phosphorylation during heat stress. Orb6 inhibition by Sty1 increases the sensitivity of the cell to heat stress in a temperature-specific manner, fostering increased stress resilience and metabolic adaptation. These observations highlight the role of NDR kinase in the process of heat adaptation and thermotolerance during environmental cell exposure to elevated temperatures.

B276/P0854

The 53BP1-USP28-p53 Signalling Axis in Cellular Stress Responses in Normal and Malignant Cells

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Tumour suppressor binding protein 53BP1 is known for its role in DNA repair, but its function in p53 regulation is less defined. We previously demonstrated that 53BP1, in complex with the deubiquitinase USP28, promotes the binding of tumour suppressor p53 to target gene promoters, triggering p21-mediated cell cycle arrest - independently of its canonical DNA repair functions. This alternative 53BP1–USP28–p53 pathway is triggered by spindle assembly checkpoint activation in response to centrosome defects and/or prolonged mitosis, forming the so-called “mitotic stopwatch”. Interestingly, we and others previously demonstrated that this pathway is additionally vital for optimal p53 transactivation following treatments with the MDM2 inhibitor Nutlin-3a or ionising radiation in MCF-7 breast adenocarcinoma cells (Brummelkamp et al, 2006), but not in non-transformed hTERT RPE-1 cells, suggesting a greater p53-53BP1 co-dependence in cancer cells. Yet, what drives p53-53BP1 co-dependency at the molecular level remains unclear. Here, we monitored DNA damage-induced p53 responses triggered by camptothecin (CPT) and ionising radiation treatments, focusing on their dependence on 53BP1 and USP28 in cancer and normal cells. Our data shows that both RPE-1 and MCF-7 cells possess functional G2/M checkpoints, but in MCF-7 cells, it is less efficient, with unrepaired DNA damage persisting into mitosis. RPE-1 cells’ response to CPT, including activation of the G2/M DNA damage checkpoint, is dependent on p53, yet independent of 53BP1. By contrast, both 53BP1 and p53 losses confer CPT resistance in MCF-7 cells, highlighting their interdependence. Thus, p53 influences the G2/M checkpoint in RPE-1; while in MCF-7, it also functions in a 53BP1-dependent manner, akin to the mitotic stopwatch in RPE-1. Given the high proliferative index of cancer cells, we also investigated whether p53-53BP1 co-dependency might also characterise physiological contexts with high proliferative demands. Concentrating on murine embryonic stem cells, which are highly proliferative with transient gap-phases, our results confirm that p53-53BP1 interplay is

active at early developmental stages, coordinating cellular response to both centrosome defects and DNA damage. Collectively, our results reveal unexpected mechanistic variations in 53BP1/USP28-dependent p53 regulation among cell types, with potential implications for targeted therapies.

B271/P0849

The importance of DNA and chromatin integrity and stability differences detected in blood samples from outpatient and hospitalized patients

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Introduction: Netosis is an immune process where granulocytes release DNA to capture microbes. Gamma H2AX (gH2AX), a molecule activated during DNA double-strand breaks, helps recruit repair proteins to the damaged DNA sites. p16 is a marker of cellular senescence. Both DNA damage and senescence can be indicators of age and disease. Elevated ssDNA levels in cells can indicate diseases such as carcinoma. Aims: We aimed to compare outpatients with hospitalized ones. To evaluate netosis, we focused on citrullinated H3 (H3Cit) and myeloperoxidase (MPO). We also wanted to assess DNA damage and senescence state in patients using gH2AX and p16 and analysed dsDNA and ssDNA content to evaluate the DNA state in patients. Materials & Methods: Peripheral blood was collected from healthy (n=6), outpatient (n=7) and hospitalized (n=8) patients. Macrophages (M) and granulocytes (G) were isolated. In G cells H3Cit and MPO were labelled with fluorescent antibodies, while gH2AX was labelled in M cells. Additionally, p16 were labelled in both M and G cells of out and hospitalized patients. Both cell groups were treated with RNase and incubated with acridine orange (AO). Sony SH800 flow cytometer was utilized to measure fluorescence for the observed markers and nucleic acid intensity. Results: Compared to healthy controls and outpatients, hospitalised patients had higher numbers of H3Cit (1454/100000 counted cells), MPO (78/100000 counted cells), and gH2AX positive cells (351/100000 counted cells), suggesting the presence of netotic structures in granulocytes and DNA double-strand breaks in macrophages. These markers were also higher in outpatients compared to healthy control. Moreover, we observed higher number of p16 positive cells (22/100000 counted cells) in the M cells of outpatients. Additionally, differences in nucleic acid content were observed in both cell types between patient groups. The ssDNA content of G cells were much higher in hospitalized patients compared to outpatients. The change was significant ($p=0.0014$). Conclusion: We observed significant differences between outpatients and hospitalized patients regarding DNA double-strand breaks, netosis and senescence markers, and DNA contents. These findings provide a broader understanding of how certain diseases can impact the expression of various markers and affect the state of blood cells.

B269/P0847

Transcription Factor Control Under Oxidative Stress

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Cells exposed to stressful levels of hydrogen peroxide (H_2O_2) must activate certain transcription factors (TFs) to counteract oxidative damage, yet the mechanisms coordinating this response over varying H_2O_2 doses remain poorly defined. Here, we show that translation inhibition is tightly linked to regulation of the TF p53. Low doses of H_2O_2 activate p53, but at high doses of H_2O_2, activation of the integrated stress response (ISR),

inhibition of mTORC1 signaling, and stimulation of the kinase eEF2K coincide with reduced p53 accumulation and attenuated translation. No single pathway was necessary to block translation or suppress p53 accumulation, indicating a robust translational block under severe oxidative stress. However, ISR activation alone was sufficient to prevent p53 accumulation at lower H_2O_2 doses, supporting the conclusion that p53 levels are controlled at the level of translation. Importantly, inhibition of transcription, autophagy, or proteasome activity did not rescue p53 accumulation, reinforcing the idea that translational control maintains low p53 under high H_2O_2 levels. These findings reveal a coordinated mechanism linking translational suppression to TF regulation, providing new insight into how cells integrate oxidative stress signals.

B266/P0844
Unravelling Stress Granule Regulation via mTORC-S6K2 Pathway
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Living organisms frequently encounter stresses such as temperature shifts, oxygen fluctuations, irradiation, infections, or injuries. Cells respond via protective or destructive mechanisms depending on stress type and duration. A common protective response is transient inhibition of protein synthesis, leading to stress granule (SG) formation. SGs are membraneless cytoplasmic assemblies of untranslated mRNAs, signalling factors, and proteins that regulate mRNA localization, degradation, and signalling. Dysregulation of SGs is linked to viral infections, neurodegenerative diseases, and cancer. SGs interact with signalling pathways, notably the mammalian target of rapamycin (mTOR), which controls survival, proliferation, and metabolism via mTORC1 and mTORC2. S6 kinases (S6K1 and S6K2), effectors of mTORC1, display distinct roles under oxidative stress: S6K1 promotes SG formation via eIF2α phosphorylation, whereas S6K2 sustains SG persistence. S6K2—but not S6K1—is also activated by an alternative mTORC complex (mTOR–mLST8–MEAK7). We investigated how SG regulation affects cellular stress responses via the mTORC1 pathway. S6K2 domains critical for SG localization were mapped using deletion mutants, and the functional interchangeability of S6K1 and S6K2 was tested via domain-swapping constructs. The C-terminal intrinsically disordered region (IDR) of S6K2 enhances its SG localization and independently drives accumulation. A chimeric S6K1–S6K2 construct recapitulated this behaviour, showing increased SG accumulation and persistence. S6K2 levels within SGs progressively rose under mild oxidative stress in HeLa cells. MEAK7 was identified as a regulator of SG formation via S6K2. These findings reveal the distinct regulatory role of S6K2 and its C-terminal IDR in SG dynamics, identify MEAK7 as a key upstream modulator, and provide mechanistic insights into SG maintenance that may inform therapeutic strategies for stress-related disorders.

Cell Stress and Quality Control - Organelle-specific quality control and stress response

B297/P0875
Cardio-protection via Non-canonical Actin-Mediated Mitophagy
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GJA1-20k, an internally translated isoform of the gap junction protein connexin 43, is stress-responsive and reduces ischemia-reperfusion injury. GJA1-20k localizes to the outer membrane of mitochondria and stabilizes

actin to trigger a protective mitochondria fission, which and reduces mitochondrial oxidative damage. However, the mechanism by which GJA1-20k and actin work towards mitochondrial protection remains unknown. Here, we report that GJA1-20k recruits actin to mitochondria to form rich actin sheaths, which we call mitochondrial actin cages. Importantly, we are able to reconstitute the formation of mitochondrial actin cages in a reductionist cell-free suspension with only purified GJA1-20k, actin, and isolated mitochondria, underscoring the specificity of GJA1-20k's mediation of mitochondrial actin formation. The enrichment of actin at mitochondria, driven by GJA1-20k, results in increased formation of mitochondrial autophagosomes, whereas GJA1-20k with impaired actin-binding ability leads to a reduction of autophagosome formation and autophagic flux. These data suggest that GJA1-20k protects mitochondria by forming mitochondrial actin cages necessary for protective mitophagy.

B304/P0882

Cell-type-resolved effects of Yellow 5 on cortical Ca^{+} signaling during laser-induced shockwaves

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We tested the ability of the food dye Yellow 5 (tartrazine) to affect the calcium responses in neurons and glia upon injury by laser-induced shockwave (LIS). Prior work in mixed cortical cultures did not separate these two cell types. Primary mouse cortical cultures were prepared and loaded with Fluo-4 AM and bathed in Ethidium Homodimer III to exclude non-viable cells. Imaging was acquired about every 3 seconds for ~20 minutes. Cells were treated with 100 µM Yellow 5 dissolved in culture medium prior to shockwaves. LIS was generated by a pulsed near-IR laser focused above the substrate, and calcium traces were collected before and after LIS. Cells in mixed cultures were assigned as neurons or astrocytes by morphology, and single-cell $\Delta F/F_o$ traces were quantified. LIS alone evoked robust calcium spikes in both neurons and astrocytes. Yellow 5 produced greater calcium changes in both neurons and astrocytes when compared to medium controls. In control conditions Astrocytes showed greater calcium elevations compared to neurons. However, these differences disappeared in Yellow 5 treated cells. The duration of the transient was affected by Yellow 5 in astrocytes compared to control astrocytes and neurons treated with Yellow 5. Separating neurons and astrocytes reveals that Yellow 5 differentially reshapes mechano-evoked Ca^{2+} dynamics by potentially buffering some of the Ca^{2+}. Future investigations will probe the effects of cell survival on both cell types in the presence of Yellow 5.

B298/P0876

Characterization of mutations in the nuclear-encoded RNASEH1 reveal significant loss of mitochondrial genome integrity and innate immune activation

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Mitochondria, beyond their energy-producing function, play critical roles in cellular homeostasis and stress response. Mitochondria possess their own unique genome, mito-genome – mitochondrial DNA (mtDNA) and RNA (mtRNA) – which are distinct from nuclear DNA (nDNA). Proper mitochondrial functions depend on the integrity of mtDNA as well as adequate regulation and supply of mitochondrial proteins from the nDNA. This interplay, or the lack thereof, is characteristic of various mitochondrial (dys)functions and disorders. Mitochondrial diseases are profoundly marked by mutations in nDNA that result in mitochondrial dysfunctions, due to loss of structural and/or genome integrity of the mitochondria. Recent studies have demonstrated the

contributions of mito-genome instability in activating innate immunity signaling pathways, highlighting the immunogenic capacity of mitochondrial genetic material and their contributions to inflammation and disease. However, the genetic features that modulate mito-genome integrity and their contributions to the pathology of mitochondrial diseases are not fully understood. In this study, we examined pathogenic mutations in the nuclear-encoded gene Ribonuclease H1 (RNASEH1) and downstream contributions to a mitochondrial-driven innate immunity. RNASEH1 is critical for genome maintenance as it functions in clearing DNA:RNA hybrids in both the nDNA and mtDNA. Additionally, mutations in RNASEH1 give rise to various mitochondrial disorders marked by mtDNA instability. Using in vitro genetic models in conjunction with patient–derived fibroblasts, our characterizations of RNASEH1 mutations confirmed a significant loss of mito-genome stability – decreased mtDNA and mtRNA copy number. Additionally, we observed a severe accumulation of mitochondrial 7S RNA, indicative of RNASEH1 dysfunction. Coupled with this mito-genome instability, we observed abnormalities in mitochondrial respiration and ATP generation, and atypical mitochondrial morphology. Our investigation into the inflammatory status downstream of RNASEH1 mutations revealed an innate immune activation with significant upregulation of interferon stimulated genes like ISG-15, IL-6, IFI6 and other inflammatory signaling markers. Interestingly, our examination of the immunogenic stimuli involved in this innate immune activation revealed a significant accumulation of dsRNA in RNASEH1 mutant cells, with proportional localization of dsRNA foci in the cytosol versus foci colocalized with the mitochondrial network. Cumulatively, our findings highlight pathogenic mutations in RNASEH1 as a modulator of a mitochondrial-driven immunity via an RNA-mediated activation of innate immunity downstream of mito-genome instability.

B303/P0881

Characterizing Parkin-dependent mitophagy and NF-?B signaling in iPSC-derived dopaminergic neurons

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Neurons are long-lived cells with high metabolic demands, relying on autophagy to degrade damaged organelles and aggregated proteins. Mitochondrial autophagy (mitophagy) is a highly regulated process essential for homeostasis. Disruptions in mitophagy, such as mutations in the proteins PINK1, Parkin, or Optineurin, are linked to multiple neurodegenerative diseases, including Parkinson's Disease (PD) and Amyotrophic Lateral Sclerosis, emphasizing the critical role of mitophagy in homeostasis and health. Our previous work discovered that in addition to canonical PINK1/Parkin mitophagy, mitochondrial damage also leads to Parkin-dependent recruitment of the IκB kinase gamma (IKKγ, or NEMO) to damaged mitochondria, which activates NF-κB signaling in HeLa cells and murine astrocytes. NF-κB activation is critical for cell survival and immune responses but is also associated with autoinflammatory disease, highlighting the careful regulation of NF-κB. PINK1/Parkin mitophagy has been relatively uncharacterized in dopaminergic (DA) neurons, which are selectively lost in PD, and the role of NEMO recruitment to mitochondria and subsequent induction of NF-κB signaling has not been explored in this cell type. Using spinning disc confocal microscopy and biochemical assays, we seek to characterize Parkin-dependent mitophagy and subsequent NEMO recruitment and NF-κB activation in iPSC-derived DA neurons, as well as to assess how induction of inflammatory signaling alters neuronal responses to mitochondrial stress, given the inflammatory pathology consistently observed in PD and other neurodegenerative diseases. Our preliminary findings demonstrate that NEMO is recruited to damaged mitochondria in iPSC-derived neurons. Furthermore, our initial results indicate that DA neurons gene-edited to express a PD-linked mutation in Parkin exhibit lowered Parkin expression. We find that proinflammatory stimuli following mitochondrial damage impedes cellular recovery without inducing cytotoxicity, which may align with neuroinflammatory phenotypes in PD. These initial data support the importance of further characterization of Parkin-dependent neuronal responses to mitochondrial stress and

inflammation that are critical for homeostasis and are perturbed in neurodegeneration. Through our studies, we aim to shed new light on disease progression to help inform future therapeutic development.

B306/P0884
Direct visualization of ER-associated degradation by correlative, real-time single protein tracking.
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Targeted degradation of proteins is an essential aspect of life, as old, damaged, or misfolded proteins must be degraded to avoid the buildup of nonfunctional or aggregating forms. In eukaryotes, approximately 25-35% of proteins are translated into or through the membrane of the endoplasmic reticulum (ER), and these proteins must be extracted back into the cytoplasm for effective degradation by the proteosome if they are damaged. This process is collectively known as ER-associated degradation (ERAD), and it serves as a major quality control system in eukaryotic cells. Despite decades of research, the mechanism of ERAD remains a fundamental mystery, since the process is sufficiently precise to distinguish functional and nonfunctional forms of the same protein, yet it works for a very large range of proteins with no apparent relationship in sequence or structure. The core of ERAD is the process of retrotranslocation, where substrates are trafficked back across the ER membrane by a set of large protein complexes centered on a conserved set of membrane-associated E3 ubiquitin ligases. In this work, we utilize our new multicolor, high-speed single protein tracking system to directly visualize the dynamic assembly and disassembly of the Hrd1 retrotranslocon on the ER memebrane in living cells. Using quantitative single molecule bleaching analysis, we show that Hrd1 complexes are frequently in higher oligomeric states than the existing monomer/dimer models predict, and these higher order structures are entirely dependent on the HAF-H domain. Additionally, we show for the first time the real-time translocation of single misfolded proteins, and association with other ERAD factors including the p97/VCP ATPase, several Derlin pseudoproteases, and the Hrd3 substrate adaptor.

B307/P0885
Effects of E-Cigarette-Derived Phthalates on Testicular Gene Expression
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Phthalates are common endocrine-disrupting chemicals associated with declining male reproductive health, yet their molecular effects on testicular tissue remain poorly understood. Electronic cigarette (e-cig) devices and e-liquids contain multiple phthalates, including dibutyl phthalate (DBP), which may enter systemic circulation when aerosolized and inhaled. The goal of this study is to characterize how e-cigarette–derived phthalates alter gene expression patterns related to apoptosis, oxidative stress, and spermatogenesis in male reproductive tissues. Male Sprague–Dawley rats were exposed to vaporized DBP using a controlled e-cigarette exposure system for 72 hours. Following exposure, testicular tissue was collected and total RNA was isolated. The next phase of analysis will quantify expression of ten target genes across three functional pathways using RT-qPCR: apoptosis (Casp3, Casp8, Casp9), antioxidant defense (Hmox1, Sod2, Gpx1), and spermatogenic support/fertility (Tex14, Gopc, Shcbp1l, Hspa2). Relative expression will be analyzed using the ΔΔCt method normalized to Gapdh, enabling comparison between DBP-exposed and control groups. We hypothesize that DBP exposure will upregulate pro-apoptotic and oxidative stress–response genes while downregulating genes

required for spermatogenic maintenance, reflecting early molecular disruptions to testicular homeostasis. This study aims to provide mechanistic insight into how vaping-associated phthalate exposure may contribute to impaired male reproductive function.

B299/P0877

Evidence of directional UPR communication between mitochondria and endoplasmic reticulum in astrocytes

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Protein quality control predominantly takes place in the endoplasmic reticulum (ER), where detection of misfolded proteins activates the unfolded protein response (ERUPR). The ERUPR restores homeostasis by increasing the expression of molecular chaperones and proteases. Whereas protein accumulation in the ER engages the ERUPR, protein accumulation in the mitochondria engages the mitochondrial UPR (mtUPR), a pathway that similarly results in translational attenuation and degradation of mislocalized proteins, albeit in the mitochondrial matrix. Both ERUPR and mtUPR remain homeostatic in a narrow window, and prolonged activation of either program can cause cell death, contributing to the neuronal dysfunction and dysregulated calcium activity reported in neurodegenerative diseases. Recent work has shown important roles for ERUPR and mtUPR signaling in astrocytes, the most abundant glial cell type in the brain. UPR signaling in astrocytes has been shown to be transmissible between cells and lead to inflammation. Our work investigates UPR signaling and stress response coordination in astrocytes. We report that pharmacological induction of mitochondrial stress using gamitrinb triphenylphosphonium triggers mtUPR activation, followed by ERUPR activation, shown by increased levels of ER chaperone BiP. In contrast, induction of ER stress using thapsigargin and tunicamycin elicits ERUPR activation, but does not lead to subsequent mtUPR activation, as measured by levels of mtHsp60, mtHsp70 and Cpn10. This crosstalk suggests asymmetrical signaling between the organelles. We propose that the translational repression associated with ER stress may not extend to mtUPR targets. This may be due to differences in translational machinery or regulatory features of mtUPR transcripts. Our findings highlight a directional hierarchy in organellar stress responses, with implications for understanding cellular response to proteostatic stress across compartments.

B302/P0880

Role of VIMP in C. elegans Organellar Homeostasis and Cellular Stress Response

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Valosin-containing Protein-interacting Membrane Protein (VIMP, SelenoS) is a member of the ER-Associated Degradation (ERAD) machinery that can interact directly with the adaptor protein, p97, which shuttles misfolded proteins to the proteasome for degradation. Despite VIMP's central position in ERAD protein complexes, its precise roles in ERAD and cellular stress responses are still unclear. VIMP levels increase during ER stress, but some studies suggest that cells that lack VIMP have reduced response to ER stress as measured by the induction of Unfolded Protein Response (Capelle et al., 2021; Men, et al., 2018). In C. elegans, a putative ortholog to VIMP is the gene F26F4.9 (ceVIMP), which has high sequence homology with mammalian VIMP except for at its C-terminus. Two deletion alleles of ceVIMP (tm2430 and tm2433) remove a large portion of the N-terminus and the start codon of the gene, respectively. Under basal conditions, neither allele activates the ER UPR via xbp-1, based on the UPR reporter Phsp-4::GFP. Although the deletion alleles do not cause Phsp-

4::GFP activation, we find that they do lead to accumulation of the ERAD substrate, CPL-1W32A;Y35A::YFP. This suggests that the VIMP ortholog is required for normal ERAD function and clearance of misfolded proteins. Previous research showed that the overexpression of VIMP causes changes in ER morphology in a tissue culture model (Noda, et al., 2014). While we do not see evidence of changes in the C. elegans ER morphology in the absence of F26F4.9, we are currently using cemOrange-tagged organelle markers to determine if other organelles are affected by the tm2433 deletion. To investigate if and where ceVIMP is upregulated in C. elegans, we are developing a promoter-reporter that will allow us to define in which cells the promoter is active and how ceVIMP's expression changes during stress response. Because these experiments can be carried out in whole, live animals, we expect to be able to dissect tissue and cell-specific changes in ceVIMP that will provide a more wholistic perspective on VIMP's role in ER homeostasis.

B300/P0878
SLC33A1 exports oxidized glutathione to maintain endoplasmic reticulum redox homeostasis
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The endoplasmic reticulum (ER) requires an oxidative environment to support the efficient maturation of secretory and membrane proteins. This is in part established by glutathione, a redox-active metabolite present in reduced (GSH) and oxidized (GSSG) forms. The ER maintains a higher GSSG:GSH ratio than the cytosol; however, the mechanisms controlling ER redox balance remain poorly understood. To address this, we developed a method for the rapid immunopurification of the ER, enabling comprehensive profiling of its proteome and metabolome. Combining this approach with CRISPR screening, we identified SLC33A1 as the major ER GSSG exporter in mammalian cells. Loss of SLC33A1 leads to GSSG accumulation in the ER and a liposome-based assay demonstrates that SLC33A1 directly transports GSSG. Cryo-EM structures and molecular dynamics simulations reveal how SLC33A1 binds GSSG and identify residues critical for its transport. Finally, an imbalance in GSSG:GSH ratio induces ER stress and dependency on the ER-associated degradation (ERAD) pathway, driven by a shift in protein disulfide isomerases (PDIs) toward their oxidized forms. Altogether, our work establishes SLC33A1-mediated GSSG export as a key mechanism for ER redox homeostasis and protein maturation.

B308/P0886
The E3 ligase PRPF19 mediates the ubiquitination of defective 60S ribosomal biogenesis intermediates
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Ribosomes are the complex molecular machines responsible for the highly regulated process of protein production. Their assembly requires the coordination of hundreds of factors that take part in dozens of error-prone steps. Mistakes during ribosome biogenesis may result in the accumulation of defective ribosomes, which can impair protein synthesis and, if unresolved, cause diseases such as cancer and neurodegenerative disorders. To counteract these deleterious effects, cells have evolved quality control mechanisms that detect and clear defective ribosomes. However, we still do not know all the factors involved in the process and the detailed molecular mechanism of faulty ribosome recognition and degradation. We have developed a cell line-based model of large subunit biogenesis failure. We discovered that defective 60S ribosomal precursors are degraded via the Ubiquitin-Proteasome System (UPS). To further study how defective large ribosomes are tagged for removal, we utilized Di-Gly-based mass spectrometry to map ubiquitinated lysine residues on large

ribosomal proteins. We identified PRPF19 as a candidate E3 ligase in a whole-genome CRISPR screen for quality control factors. We observed that knockdown of PRPF19 prevents the degradation of defective 60S subunits. Currently, we are purifying PRPF19 and performing in vitro ubiquitination assays to confirm the ability of this E3 ligase to ubiquitinate defective ribosomes. Together, these mechanistic studies will provide insight into how defective pre-ribosomes are targeted by the UPS to ensure quality control during ribosome biogenesis.

B301/P0879

T-tubule Microdomains Facilitate Mitophagy in Stressed Cardiomyocytes

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Background: Heart failure with preserved ejection fraction (HFpEF) represents about 50% of all HF incidence, yet with limited therapeutic options. Downregulation of protective mitophagy in cardiomyocytes is a poorly understood HFpEF pathophysiology. Abnormal remodeling of cardiomyocyte t-tubule microdomains organized by cardiac bridging integrator 1 (cBIN1) contributes to HFpEF progression in mice and can be rescued by cBIN1 gene therapy, which also reverses the accumulation of heart mitochondrial proteins. Objective: We tested the hypothesis that t-tubule cBIN1-microdomains maintain mitochondrial function in HFpEF cardiomyocytes by facilitating mitophagy. Methods: 9-week-old db/db mice were injected with AAV8-cBIN1 (2x10e12 vg/kg) and terminated 8 weeks later for heart acquisition for imaging, biochemical, and functional analysis. Transmission electron microscopy (TEM) was used to image mitochondria and t-tubule membrane structure. Seahorse and Oroboros were used to determine mitochondrial function. Cardiomyocytes from Bin1-deleted mice expressing mt-Keima were used to determine mitophagic flux by confocal imaging of mt-Keima lysosomal/mitochondrial signal ratio. To establish a mechanistic link, we evaluated FCCP-induced mitophagy in H9c2 cells with BIN1 siRNA knockdown (KD) +/- cBIN1 rescue by detecting activated autophagosomes (LC3II) in mitochondrial fractions and by imaging mt-Rosella flux. Results: In db/db mice treated with AAV9-cBIN1, abnormal t-tubule ultrastructure and mitochondrial accumulation are corrected. Reduced mitochondrial calcium retention, elevated oxidative stress, and impaired oxygen consumption in db/db cardiomyocytes are normalized by cBIN1 therapy. With improved mitochondrial function, AAV9-cBIN1 increases activated LC3II but not PGC1α expression, indicating an effect on mitophagy rather than mitogenesis. Imaging of mt-Keima reveals impaired mitophagic flux in Bin1-deleted cardiomyocytes when compared to wild-type, indicating BIN1 is required for mitophagy. In H9c2 cells, FCCP-induced mitophagy is diminished by BIN1 KD and normalized with siRNA-resistant cBIN1, supporting the role of cBIN1 in promoting mitophagy. Conclusion: In stressed cardiomyocytes, normalization of cBIN1-microdomains promotes mitophagy to remove damaged mitochondria and preserve healthy mitochondrial function, contributing to functional rescue in mice with HFpEF.

B305/P0883

Tumor-promoting UBR4 coordinates impaired mitophagy-associated senescence and lung adenocarcinoma pathogenesis

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Cellular senescence, an irreversible cell cycle arrest, plays a pivotal role in development, aging, and tumor suppression. However, the fundamental pathway coordinating senescence and neoplastic transformation remains unclear. Here, we describe the tumorigenic involvement of ubiquitin protein ligase E3 component n-recognin 4 (UBR4), an E3 ubiquitin ligase of the N-degron pathway, in lung adenocarcinoma (LUAD). Public

genome databases revealed high UBR4 expression in LUAD patients, associated with a dysregulated cell cycle and impaired mitochondrial homeostasis. UBR4 knockout (ΔUBR4) in A549 lung cancer cells induced cellular senescence with defective mitochondria. Restoration of UBR4 or antioxidant treatment reversed the ΔUBR4 phenotypes caused by impaired mitophagy. Mitochondrial stress exacerbated mitochondrial dysfunction in ΔUBR4 cells, contributing to diverse cellular phenotypes. Additionally, ΔUBR4 cells exhibited substantially slow tumor growth in mouse xenograft models. In LUAD patients, UBR4 levels correlated with tumor stage, mitophagy markers, and poor survival. These findings suggest a tumor-promoting function of UBR4 in LUAD by regulating mitochondrial quality control. Further research into the pharmacological inhibition of UBR4 could open promising avenues for developing effective antitumor therapies targeting LUAD.

Cell Stress and Quality Control - Protein quality control and proteostasis

B282/P0860
Analysis in vivo using a new method, ARGO (Analysis of Red-Green-Offset), reveals complexity, cell-type specificity, and requirement for TFEB/HLH-30 in presynaptic turnover of synaptic vesicle protein Synaptogyrin/SNG-1
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The maintenance of proper synaptic vesicle (SV) cycling throughout a neuron's extended lifespan requires SV protein turnover – the production and delivery of newly synthesized proteins balanced with the removal and degradation of damaged or excess proteins. How SV protein degradation is regulated in vivo remains incompletely understood, largely due to the prior lack of robust methods to quantify protein turnover with subcellular spatial resolution. We developed the ARGO method, a genetically encoded, ratiometric fluorescence imaging approach that visualizes and quantifies protein turnover in vivo with subcellular resolution. With ARGO, the protein of interest is cell-specifically, co-translationally labeled with both RFP and GFP. Steady-state imaging reveals whether and where the protein is degraded within lysosomal compartments. Next, a Cre/Lox 'pulse' removes the gfp gene from the argo cassette, and periodic ratiometric imaging quantifies the amount of old (RFP+GFP) versus total (RFP) protein during the 'chase'. The approach is modular, inexpensive, and scalable for use in genetically tractable experimental organisms. Using ARGO, we examine the turnover of a SV protein, Synaptogyrin/SNG-1, in adult C. elegans neurons. Our data support the model that SV proteins are sorted for degradation at the synapse and then trafficked to the neuron cell body to complete degradation within lysosomal compartments. We find that Transcription factor EB (TFEB)/HLH-30 is required to generate proper neuronal lysosomal capacity and promote presynaptic protein turnover. Specifically, loss of HLH-30 leads to slowed SNG-1::ARGO turnover in the presynapses without increased steady-state SNG-1 abundance or accumulations in somatic lysosomal compartments. These findings suggest that the neuron may possess feedback mechanisms to accommodate, at least initially, defects in protein turnover. In addition, we show that SNG-1 turnover rate is consistent across synapses within an individual neuron but varies between neuron classes. Notably, in at least one neuron type SNG-1 exhibits biphasic turnover within each presynaptic compartment. This result suggests that the presynapse contains two chronically distinct, non-intermixing populations of SNG-1. This separation could be an intrinsic property of the SNG-1 protein or indicative of two distinct, non-intermixing pools of SVs. In summary, ARGO enables routine, spatially resolved protein turnover quantification and reveals previously hidden complexity in SV protein maintenance.

B296/P0874

Characterizing the Molecular Composition and Dynamic Behavior of Non-Canonical Stress Granules

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Biomolecular condensate formation is a fundamental feature of cellular physiology, orchestrating diverse regulatory processes. Stress granules (SGs), one such condensate, are non-membrane-bound, protein-rich assemblies that form in response to stress and are implicated in various disease states, including neurodegeneration, cancer, and viral infection. While canonical SGs contain polyadenylated messenger RNA (poly-A mRNA), emerging evidence points to non-canonical (NC) SG subtypes with distinct compositions. Notably, ultraviolet (UVC) radiation and the eIF4A inhibitor Rocaglamide A (RocA) generate SGs that are distinguished by the absence of poly-A mRNA. The deficit of this primary SG component marks a significant deviation from canonical SG architecture, challenging conventional models of SG biology. To investigate the composition and dynamics of these NC SGs, we characterized their formation, regulation, and component recruitment in U2OS cells. We confirmed that RocA and UVC SGs lack an oligo-dT FISH signal, suggesting a deficit of poly-A mRNA. However, these NC SGs still require G3BP1/2 for nucleation, demonstrating their assembly via conserved protein scaffolds despite divergent composition. Localization analyses reveal unexpected diversity in protein recruitment patterns: RACK1 segregates to RocA and arsenite (AsIII) SGs but not UVC SGs, while DHX9 is uniquely recruited to RocA and UVC SGs. UVC, but not RocA or AsIII SG formation, is cell-cycle dependent, linking select NC SGs to cell-cycle regulation. Together, these findings uncover novel distinctions in SG composition and regulation, with the shared absence of mRNA setting RocA and UVC SGs apart from canonical AsIII SGs. We next examined how these NC SGs interact with other cytoplasmic condensates. Under UVC and RocA stress, we observed changes in Processing body (P-body) association and mean P-body size, indicating that crosstalk between SGs and P-bodies may vary depending on stress conditions. We also found that RocA SGs are subject to clearance through autophagy, as inhibition of these pathways delayed their disassembly. This supports a potential role for RocA SGs in protein quality control, consistent with their lack of mRNA and protein enrichment. Collectively, these studies establish a conserved requirement for G3BP scaffolding and, importantly, demonstrate that SGs cannot be universally defined by the presence of mRNA. Rather, RocA and UVC SGs represent a distinct class of protein-rich condensates that likely function in protein sequestration and signaling modulation instead of mRNA triage. This work, together with future studies, will broaden our understanding of SG biology, yield new mechanistic insights into SG dynamics, and advance our knowledge of cellular stress responses.

B284/P0862

Derlins in control: shaping sphingolipid metabolism and mitochondrial dynamics

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The ER and mitochondria coordinate key processes including protein quality control, calcium signaling, and energy metabolism. While Derlin proteins are classically linked to ER-associated degradation (ERAD), their individual roles in organelle homeostasis remain unclear. Using CRISPR-engineered HEK293T cell lines, we find that Derlin-2 knockout (KO) causes pronounced cellular defects, including increased ER stress, reduced proliferation, and increased apoptosis, unlike Derlin-1 or Derlin-3 KO. Derlin-2 loss also disrupts mitochondrial

integrity, resulting in fragmented morphology, decreased calcium uptake, impaired respiration, and alters mitochondria-ER contact sites (MERCs), highlighting a unique role in inter-organelle communication. Furthermore, lipidomic profiling revealed distinct membrane lipid alterations specific to Derlin-2 KO cells. When we conducted a proteomic screen, we identified a negative regulator of sphingolipid metabolism, ORMDL3, as a likely Derlin-2 substrate, which was then confirmed through degradative assays. These findings uncover a non-redundant function for Derlin-2 in maintaining ER-mitochondria homeostasis and challenge the prevailing view of Derlin paralog redundancy.

B290/P0868

Embryonic Protein Turnover is More Rapid and Widespread in Flies than Frogs

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Dynamic proteome regulation drives lineage specification and organogenesis, and mass spectrometry is typically employed to quantify the thousands of protein levels underlying these processes. However, protein abundances derived from mass spectrometry often correlate poorly with mRNA levels, highlighting the importance of post-transcriptional regulation through translational control and protein turnover. Ribosome profiling has established the significance of translational regulation during embryogenesis, but the contribution of protein turnover in shaping embryonic proteomes has remained debated and unmeasured at a systems scale. Here, we combine heavy isotopic labeling with 18O-water and multiplexed proteomics to generate a resource of protein half-lives in frog and fly embryos. Following fertilization, proteolytic hydrolysis incorporates 18O into the free amino acid pool, which is subsequently used by ribosomes for protein synthesis. This metabolic labeling distinguishes newly synthesized proteins from preexisting, degrading species. In frog embryos, we find that the proteome is largely stable, with only 0.1% turnover per hour, although 18% of proteins undergo measurable degradation, including E3 ligases, transcription factors, and microtubule-associated proteins. Tracking the first mitotic division provided an orthogonal validation for cell-cycle–associated proteins, yet nearly all degrading proteins did so independently of cell cycle, underscoring the value of our 18O-based approach. Extending this analysis to fly embryos revealed far more pervasive turnover: 55% of all identified proteins were degraded. Proteins that were degraded in both species turned over 3-fold faster in flies than in frogs, consistent with global protein stability scaling with developmental tempo. Notably, however, the greater fraction of the fly proteome subject to turnover indicates that degradation plays a more pronounced regulatory role in flies than developmental speed alone would predict. Together, these results establish a broadly applicable strategy for quantifying protein half-lives in embryos and uncover fundamental species-specific differences in proteome regulation.

B289/P0867

ER Stress-induced Oxidoreductase ERO1 drives myopathy in a loss-of-function RyR1 Mutant

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Objective: Loss-of-function mutations in the RYR1 gene, such as the I4898T variant in humans (I4895T in mice), are among the most common causes of congenital myopathies. Despite advances in understanding RyR1-related diseases, no FDA-approved therapies exist. Studying the underlying mechanisms of disease may help identify molecular targets for the development of effective pharmacological treatments. Previous work linked this mutation to ER stress and the consequent unfolded protein response (UPR), but the molecular determinants and precise mechanisms driving pathogenesis remain unclear. Methods: To investigate this, we developed a compound mutant mouse model carrying the I4895T RyR1 mutation (RyR1IT/WT) and a knockout of CHOP, a pro-apoptotic UPR effector. We performed in vivo respiratory function assessments, ex vivo analysis of ER stress/UPR markers, transmission electron microscopy to examine the structure of the sarcoplasmic reticulum (SR), mitochondria and their interface, mitochondrial oxidative phosphorylation assays, and compartment-specific Ca^{2+} imaging. Pharmacological modulation of ER stress was tested using novel pyrazolone-based ER oxidoreductase, ERO1 inhibitors and the chemical chaperone TUDCA. Experiments in HEK293 cells expressing RyR1IT or RyR1wt after ERO1α inhibition were exploited to assess whether ER stress–induced ERO1 promotes increased SR Ca^{2+} channel activity and mitochondrial Ca^{2+} overload, impairing mitochondrial function and triggering apoptosis through mitochondrial permeability transition pore formation. Results: Aged RyR1IT/WT mice exhibited elevated expression of ERO1α in diaphragm tissue, along with impaired respiratory function. These phenotypes were ameliorated in the CHOP-deficient compound mutants. Isolated RyR1IT/WT myotubes displayed increased basal and ER stress–induced ERO1α levels compared to wild-type. ERO1 inhibition or TUDCA treatment improved myotube differentiation. Conclusions: Chronic ER stress-induced upregulation of ERO1α in RyR1IT/WT mutants drives myopathy progression. Ongoing studies test if increased ERO1α acts by promoting pathological SR Ca^{2+} leak, mitochondrial calcium overload and ensuing apoptosis. Inhibition of ERO1 improves muscle cell function, highlighting ERO1α as a promising therapeutic target for RyR1-related myopathies.

B281/P0859

Extracellular Vesicle-Mediated Delivery of 20S Proteasomes Enhances Tau Degradation in Recipient Cells

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The 26S proteasome holoenzyme comprises 20S catalytic and 19S regulatory complexes. Accumulating evidence suggests that the majority of proteasomes in the extracellular space exist as free 20S proteasomes; however, their origin and pathophysiological function remain to be determined. Here, we report that cellular proteasomes are effectively packaged into the lumen of extracellular vesicles (EVs) and secreted in a structurally intact and enzymatically active 20S form. We further demonstrate that EV-encapsulated 20S proteasomes are delivered to recipient cells and facilitate the degradation of overexpressed tau proteins without disrupting global proteolytic pathways. These findings highlight a novel cell-to-cell communication system that transports the proteasomes to target cells for the clearance of proteotoxic substrates. Further characterisation of this homeostatic mechanism will improve our understanding of organismal stress response mechanisms and may provide a therapeutic approach to treat various proteinopathies, including Alzheimer's disease.

B291/P0869

Heat acclimation modulates skeletal muscle water transport via AQP4

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Repeated heat exposure induces heat acclimation (HA), which enhances heat tolerance and adjusts whole-body water homeostasis to promote efficient heat dissipation. Although skeletal muscle is a major water reservoir, it remains unclear whether HA modifies its water transport function. In this study, we investigated the effects of HA on skeletal muscle water transport and potential biomarkers of HA in mice. To establish a mouse HA model, C57BL/6 mice were exposed to repeated heat stimulation for 14 days (HA group). Compared to controls, HA mice showed a significant reduction in body weight, and the acute heat-induced upregulation of Hspa1a was attenuated. Because water transport is crucial for heat-dependent adaptation, we analyzed the expression of aquaporin 4 (AQP4) in HA mice skeletal muscles. Aqp4 mRNA was significantly increased in several skeletal muscles after HA. In addition, immunofluorescence revealed AQP4 localization at the sarcolemma. In parallel, when C2C12 myotubes underwent 1–5 days of repeated heat exposure, AQP4 expression progressively increased. To determine whether HA alters myokine expression, we performed extensive profiling in myokine expression analysis in animal and cellular models to assess whether HA affects the expression profile of myokines. We identified C-C motif chemokine ligand 5 (CCL5) as a myokine respond to HA. Serum CCL5 concentration was significantly decreased in HA mice, and CCL5 expression in skeletal muscles of HA mice and C2C12 cells declined progressively with each heat exposure. Together, these findings indicate that myokine CCL5 is downregulated by HA both in vivo and in vitro. In conclusion, our results suggest that HA in mice potentially modulates water transport via AQP4 and that CCL5 may serve as a useful biomarker of acclimation status.

B292/P0870

Human Limb Girdle Muscular Dystrophy mutations disrupt Hsp104-mediated prion elimination, but not propagation, of specific prions in yeast

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The overexpression of Hsp104 efficiently cures yeast cell populations of the prion [PSI+] by a currently-debated mechanism. Previously, we showed that this process requires the essential J-domain protein (JDP) Sis1 for strong, but not weak, variants of [PSI+], indicating that the mechanisms for curing may differ for distinct prion variants. Strong variant curing is impaired when cells lack either Sis1's GF region or the JDP Apj1. Surprisingly, only the first 90 residues of Apj1, which include the J-domain but not the GF region, are required for curing. Mutations in these same regions (J-domain and GF) of the human Sis1 homolog DNAJB6 cause Limb Girdle Muscular Dystrophy Type D1 (LGMDD1). To date no point mutations in Sis1 have been reported that disrupt Hsp104-mediated prion elimination. To address this, we individually examined seven LGMDD1 point mutations in Sis1 that still support cell viability in [PSI+] strains. While none impaired the propagation of strong [PSI+] variants, three dramatically impaired Hsp104 curing of these variants. These results provide the first direct evidence that Sis1's J-domain is important for Hsp104 curing and imply that Sis1 is acting with an Hsp70 partner in this process. They also underscore that Apj1 and Sis1 act through different parts of the respective proteins as just a single residue change in the GF region of Sis1 can disrupt curing while this same region of Apj1 is dispensable. All mutations also propagated weak [PSI+] variants but no mutation impacted the curing of these variants. This supports our assertions that JDPs are not required for Hsp104 curing of weak [PSI+] variants and that weak and strong [PSI+] variants are cured by biochemically distinct mechanisms. Finally, we asked whether these mutations might affect the propagation of the prion [RNQ+], which is more sensitive to alterations in Sis1's GF region than [PSI+]. No loss of [RNQ+] nor any change in [RNQ+] aggregate size was detected. Overall, these results indicate that some mutations in human DNAJB6 that result in LGMDD1 alter JDP function in a way that specifically affects Hsp104 prion curing in yeast. Understanding this function of Sis1

may help to elucidate the mechanism by which DNAJB6 functional alterations result in disease. Additional mutational scanning coupled with structural analysis to search for interaction surfaces is ongoing.

B293/P0871
KLHL41 regulates the calcium homeostasis of the sarcoplasmic reticulum in skeletal muscle
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Cullin3(CUL3)-RING E3 ubiquitin ligases (CRL3s) play crucial roles in various cellular processes through Bric a brac, Tramtrack, and Broad-Complex (BTB)-domain proteins, which recognize target substrates for ubiquitylation. Evidence supporting the involvement of CRL3s in the endoplasmic reticulum (ER) emerged from a proteomic study of skeletal muscle-specific Cul3 knockout (KO) mice, which revealed elevated levels of SERCA1 and RYR1, two sarcoplasmic reticulum (SR) resident proteins in skeletal muscle cells. We hypothesized that KLHL41, a BTB domain protein that is linked to nemaline myopathy, might be a good candidate, as a pool of KLHL41 is present at the SR in myofibers. Indeed, knockdown of Klhl41 expression in mouse C2C12 myotubes resulted in a significant increase in the protein levels of SERCA1. Similarly, zebrafish klhl41 KO mutants exhibited elevated Serca1 protein levels, and the SR was notably enlarged in double KO mutants (klhl41a; klhl41b). Further investigation revealed that the SR was overloaded with Ca2+ in the absence of KLHL41 both in C2C12 myotubes and klhl41 dKO mutants. Taken together, the results of the present study support a critical role for KLHL41 in maintaining SR Ca2+ homeostasis by regulating SERCA1 levels in skeletal muscle.

B286/P0864
Off-target effects of azole antifungals drive autophagy in fission yeast
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Miconazole, an azole antifungal on the WHO Model List of Essential Medicines, primarily targets ergosterol biosynthesis. However, concerns about toxicity and predominant topical use have limited its broader uptake compared with newer agents, while it remains widely utilized in many low- and middle-income countries due to cost considerations. To inform clinical safety and repurpose opportunities, we systematically probed potential off-target effects of miconazole in fission yeast (Schizosaccharomyces pombe). We performed a genome-wide screening using a heterozygous deletion library, analyzed sensitive candidates by next-generation sequencing (NGS), and conducted susceptibility/interaction assays along with functional and structural mechanism assessments. Our data suggest an off-target interaction with protein quality-control machinery, compatible with disturbance of ATP-driven folding and proteostasis-linked autophagy. These findings suggest a mechanistic basis for safety evaluation and mechanism-guided repurposing of miconazole

B287/P0865
Protein Interaction Analysis Reveals the Roles of Selenoprotein K in Protein Quality control, Trafficking and Autophagy

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Human selenoprotein K (selenok) contributes to protein quality control, signaling, autophagy, and the immune system. Evidence suggests that it plays a role in both cancer progression and the development of neurodegenerative diseases. To understand the role of selenok, we investigated its membrane orientation as well as its protein interaction network. Selenok is a small, intrinsically disordered, endoplasmic reticulum (ER)-associated membrane protein that was previously described as a single-pass transmembrane protein with its N-terminus in the ER lumen and C-terminus in the cytoplasm. However, we showed that it is associated with the ER membrane as a peripheral membrane protein, exposing both N- and C-terminus to cytoplasm. A major implication of a peripheral topology for selenok is that its N- and C-terminus and its amphipathic helix will have cytoplasmic protein partners. We mapped the interactome of selenok using affinity purification coupled with mass spectrometry in combination with crosslinking and we identified protein partners that suggest broader cellular roles for selenok than previously recognized. We found that selenok participates in intracellular trafficking and contributes to the organization of organelle membranes and their associated protein complexes, expanding its known functional repertoire. Notably, the intrinsically disordered segment of selenok, which contains the reactive selenocysteine is essential for interactions with specific partners.

B283/P0861

The p97 cofactors UBXD2 and UBXD8 play a conserved role during the ER-associated degradation (ERAD) of a model misfolded membrane protein

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Protein quality control is essential for maintaining cellular homeostasis by ensuring the proper folding and degradation of non-native proteins. This process is especially important during membrane protein biogenesis, as nearly all membrane proteins are synthesized at the surface of the endoplasmic reticulum (ER) and must fold across distinct environments—the ER lumen, the ER membrane, and the cytoplasm. While ER-associated degradation (ERAD) facilitates the disposal of misfolded membrane proteins via the ubiquitin-proteasome system, the mechanism by which proteins selected for this pathway are retained in the ER, and thus prevented from accessing later compartments in the secretory pathway, remains poorly understood. UBX domain-containing proteins are ideally constructed to interact with ERAD machinery, since they possess a ubiquitin-like (i.e., UBX) domain, which allows them to interact with the AAA+ ATPase p97 (mammals)/Cdc48 (yeast), and some may also interact with ERAD substrates via ubiquitin-binding domains (e.g., UBA). Indeed, we previously showed that the yeast Ubx2 protein promotes the ER retention of a model misfolded membrane protein, SZ*. To examine whether mammalian UBX domain-containing proteins function similarly, we investigated the roles of mammalian UBXD2 and UBXD8 in the degradation and retention of SZ* after induced expression in HEK293 cells. We find that CRISPR-Cas9-mediated knockout of UBXD8 and, to a lesser extent, UBXD2 results in SZ* stabilization. Overexpression of UBXD2 or UBXD8 in mammalian cells similarly leads to SZ* stabilization and the accumulation of ubiquitinated protein, indicating potential interference with degradation machinery. Interestingly, this effect relies on functionally distinct domains. In addition, expression of UBXD8 and UBXD2 in ubx2Δ yeast rescues the SZ* degradation defect, consistent with functional conservation amongst these proteins. Our findings suggest a conserved role for ER-localized UBX domain-containing proteins in membrane protein quality control and highlight their influence on ERAD across species.

B288/P0866

The proteostasis network is a critical hub for viral functions and a target for broad-spectrum antiviral discovery

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Cells rely on the proteostasis network (PN), the integrated system of protein synthesis, folding, trafficking, and degradation, to maintain protein homeostasis and adapt in response to stress. Viral infection is a powerful perturbation to this system, as all viruses depend on and co-opt the PN to replicate. This dependency provides insight into how cells adapt when homeostasis is challenged. By disrupting components of the PN during infection by medically relevant dsDNA viruses (herpesviruses and poxviruses), we identified conserved dependencies that highlight critical nodes for infection within this network. A targeted compound screen uncovered translation initiation (EFT226/eIF4A), protein folding in the ER (HA15/BiP), and protein translocation (Apratoxin S4/SEC61) as broad-spectrum vulnerabilities for these viruses. A parallel CRISPRi screen of more than 3,000 PN-related genes revealed virus-specific dependencies such as the Exocyst complex for HSV-1, mitochondrial ATP synthase for HCMV, and the amino acid–sensing GATOR2 complex for Vaccinia. Remarkably, the three viruses converged on the Conserved Oligomeric Golgi (COG) complex, underscoring vesicular retrograde trafficking as a shared viral dependency. We validated this observation through chemical targeting with retrograde transport inhibitors. Together, these complementary approaches open an opportunity to demonstrate the potential of targeting the PN as a strategy for host-directed, broad-spectrum antiviral intervention, and highlight viruses as valuable tools to continue exploring the rewiring of the PN in response to extrinsic perturbations.

B295/P0873

Using ER-degrons to understand the mechanics of ERAD substrate selection and transport

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The majority of secreted proteins and integral membrane proteins are folded within the lumen of the endoplasmic reticulum (ER). However, many of these proteins never properly fold or become unfolded within the ER. An accumulation of these misfolded or unfolded proteins can cause ER stress, which can be toxic to the cell. One way eukaryotic cells have evolved to mitigate this proteotoxic stress is through ER-associated degradation (ERAD), a pathway that facilitates proteasomal degradation of misfolded ER lumenal and membrane proteins. A critical step in ERAD is the recognition of substrates. However, substrate recognition for this pathway remains poorly understood. Degrons, or sequence features sufficient to trigger degradation, may serve as a key piece in understanding this process. Recent work in our lab identified the first degrons (ER-degrons) sufficient to target proteins for degradation from the ER lumen in S. cerevisiae. These degrons work through ERAD but do not require all known ERAD components previously implicated in substrate recognition. This suggests that additional factors may be required for substrate targeting and recognition. Here, we will discuss our efforts to understand the mechanics of substrate recognition using ER-degrons as tools.

Condensates - Biophysical Mechanisms

B392/P0382
Biomolecular Condensates Dictate the Folding Landscape of Proteins
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Biomolecular condensates, which are typically thought to form via phase separation driven by disordered protein domains, concentrate a variety of disordered and structured biomolecular components. Here, we ask whether the unique physicochemical environment of condensates can manipulate the folding landscape of simple protein structures–namely, the protein alpha-helix. To this end, we have developed and validated a reside-resolution coarse-grained model to describe protein alpha-helices. Using this model, we probe how model condensates formed of short scaffold peptides influence the folding thermodynamics and kinetics of individual alpha-helices. We extend the model to probe the behavior of disordered proteins, such as the TDP43 and the Androgen Receptor protein low complexity domains, within condensates formed of long disordered peptides. Our findings indicate that condensates which concentrate a high number of residues that participate in strong multivalent interactions, for example aromatic and charged residues, counteract to varying degrees the crowding effect of a dense environment and favor a coil state within these helical motifs. Further, these domains experience a high degree of kinetic frustration with folding transition times that differ among condensates of distinct compositions. These results serve as a foundation for understanding the biophysical implications of condensates on folded protein domains and the role of these helical motifs in condensate associated protein aggregation diseases.

B398/P0388
Cytoplasmic Crowding and Diffusion Dynamics Regulate Biomolecular Condensation
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The physical microenvironment of the cytoplasm—including crowding, fluctuations, and spatial organization—critically influences biomolecular dynamics such as liquid-liquid phase separation (LLPS), yet the underlying mechanisms remain unclear. By combining single-particle tracking with novel approaches to manipulate cytoplasmic properties, we systematically investigate how intracellular dynamics and crowding govern LLPS. We demonstrate that reduced molecular diffusion promotes initial stress granule (SG) nucleation, while microtubule-driven active transport facilitates subsequent droplet coalescence. Intriguingly, the formation of SG droplets contributes to increased spatial heterogeneity within the cell as well. To dissect crowding effects without perturbing cell volume (e.g., via osmotic compression), we develop a method for in situ synthesis of abiotic supramolecular polymers directly in living cells, and reveal that elevated crowding decelerates droplet coarsening kinetics. Our findings highlight how distinct physical parameters of the cytoplasm regulate condensate dynamics, offering insights into the interplay between metabolic activity and membraneless organelle formation.

B389/P0379
Dissecting the Role of Phosphorylation and Block-Copolymer Architecture in Reflectin Biomolecular

Condensates

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Cephalopods possess a unique family of proteins known as reflectins, which are central to the structure and optical function of iridescent nanostructures in specialized skin cells called iridocytes. Reflectin proteins are intrinsically disordered cationic block copolymers, consisting of highly conserved domains interspersed with less conserved, positively charged linkers. The tunable iridescence observed in certain squid species is thought to arise from the regulated and reversible assembly of reflectin biomolecular condensates formed through liquid–liquid phase separation (LLPS). In tunable iridocytes, condensate formation correlates with the phosphorylation state of different reflectin subtypes: Reflectins A1 and A2 are phosphorylated, while Reflectin B—more abundant in tunable cells—is dephosphorylated during induction of iridescence in cephalopods. To investigate the molecular mechanisms underlying reflectin condensate behavior in eukaryotic cells, we generated deletion and phosphomimetic mutants of Doryteuthis opalescens reflectins A1, A2, and B, each fused to fluorescent proteins and expressed in HeLa cells. Expression, localization, and condensate morphology were examined via confocal microscopy, and fluorescence recovery after photobleaching (FRAP) analyses were performed to assess their dynamic properties. Distinct reflectin mutants exhibit characteristic differences in cellular localization, condensate formation, and dynamics as assessed by confocal imaging and FRAP analyses. Together, these findings support a model in which the phosphorylation and structural block-copolymer architecture of different reflectin subtypes contribute to reflectin condensate assembly and dynamics, offering insights into understanding the tunable and reversible optical properties of iridocytes

B391/P0381

Fluorescent protein dimer-based biosensor for quantitative hydrophobicity mapping of biomolecular condensates in live cells

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Biomolecular condensates (BMCs), formed by liquid–liquid phase separation (LLPS), are dynamic organizers of cellular biochemistry, creating specialized membrane-less compartments that regulate gene expression, RNA metabolism, signalling, and stress responses. Their assembly and disassembly provide cells with the flexibility to adapt to environmental and metabolic change, while their misregulation is increasingly linked to neurodegeneration, cancer, and viral pathogenesis. Although electrostatic and hydrophobic interactions are key determinants of condensate stability, tools to non-invasively quantify hydrophobicity in living cells are lacking. We present the first genetically encoded Förster Resonance Energy Transfer (FRET) biosensor that enables high-resolution spatial mapping of hydrophobicity in real time. Exploiting the moderate affinity between two fluorescent proteins which form dimers but can easily break apart, the sensor is designed to partition into condensates and dynamically reports on their internal microenvironments. Lower FRET efficiency signifies higher hydrophobicity. First, we directly probed sensor responsiveness to hydrophobicity in glycerol solutions, subjecting cells to a hydrophobic fluidic environment. As expected, we observed decrease (37%) in FRET efficiency in 75% glycerol solution compared to PBS. Then we tested whether the sensor could partition into BMCs consisting of poly-l-lysine and ATP. Interestingly, higher concentration of the sensors was at the surface of the condensate compared to its interior. Moreover, a 16% decrease in FRET efficiency was detected in the condensate, suggesting an increase in hydrophobicity. When expressed in breast cancer cells (MDA-MB-231), negligible phototoxicity was detected, indicating suitability for live-cell measurement. Furthermore, the sensor uniformly distributed across the cytoplasm, facilitating continuous mapping of hydrophobicity of the whole cell. Our first-of-its-kind sensor can be used to monitor hydrophobicity not only in condensates but also

other subcellular compartments. It can also be used in combination with other biosensors for multiplexing to survey the relationship of multiple biophysical and biochemical parameters in cells.

B393/P0383
Kinase KEY1 controls pyrenoid condensate size throughout the cell cycle by disrupting phase separation interactions
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Biomolecular condensates spatially organize cellular functions, but the regulation of their size, number, dissolution, and re-condensation is poorly understood. The pyrenoid, an algal biomolecular condensate that mediates one-third of global CO2 fixation, typically exists as one large condensate per chloroplast, but during cell division it transiently dissolves and reconfigures into multiple smaller condensates. Here, we identify a kinase, KEY1, in the model alga Chlamydomonas reinhardtii that regulates pyrenoid condensate size and number dynamics throughout the cell cycle and is necessary for normal pyrenoid function and growth. Unlike wild type, key1 mutant cells have multiple smaller condensates throughout the cell cycle that fail to dissolve during cell division. We show that KEY1 localizes to the condensates and promotes their dissolution by disrupting interactions between their core constituents, the CO2-fixing enzyme Rubisco and its linker protein EPYC1, through EPYC1 phosphorylation. We develop a biophysical model that recapitulates KEY1-mediated condensate size and number regulation and suggests a mechanism for controlling condensate position. These data provide a foundation for the mechanistic understanding of the regulation of size, number, position, and dissolution in pyrenoids and other biomolecular condensates.

B397/P0387
Moving in the right direction: a statistical analysis of single-molecule dynamics in the synaptonemal complex during crossover designation
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Meiosis is a crucial process in sexually reproducing organisms, physically exchanging genetic material between homologous pairs during crossover (CO) formation. COs are required for proper segregation of homologous chromosomes: improper CO formation can lead to infertility and aneuploidy. However, only a subset of the many double stranded breaks (DSBs) present during early meiosis go on to become fully fledged COs. How are CO designation and formation regulated? Enter the synaptonemal complex. The synaptonemal complex (SC) is a ~100 nm thick liquid-like protein assembly that forms between paired meiotic chromosomes. It has been proposed that the SC functions as a conduit for diffusing molecules during CO designation, but it remains challenging to study its biophysical dynamics in vivo. To investigate the dynamics of molecules involved in CO designation, we performed super resolution single-particle tracking (SPT) in live meiotic nuclei within the C. elegans gonad. To analyze the dynamics of SC components, we used the SC mutant syp-1K42E to examine impacts in SYP-3 motion. syp-1K42E mutants do not fully abolish the SC but rather partially form SC on paired homologs (among other phenotypes). These disturbances likely disrupt the efficiency of molecular diffusion along the SC conduit. To test this “conduit” idea, we used uTrack (a SPT tracking algorithm) and NOBIAS (a Hidden Markov Model) to explore the subpopulations of motion between/within SYP-3 molecules. We found

that SYP-3 had two modes of motion: 1) fast, nucleoplasmic, Brownian diffusion and 2) slower, SC-confined exploration and binding. Though SYP-3 diffuses quicker in syp-1K42E mutants overall, there were significantly lower proportions of SC particles and higher unbinding rates; the SC became a “leaky” conduit. We then asked how CO formation is impacted by perturbations in the SC. Given that protein dosage is hypothesized to affect designation, we used a syp-5(-) mutant to measure the variation in GFP-COSA-1 foci (a cytological marker for CO formation). Interestingly, the variation in intensity of COSA-1 foci in syp-5(-) mutants was higher compared to wildtype, indicating weaker CO formation. Thus, when the SC is a “leaky” conduit, CO formation is not as efficient. Taken together, these results suggest that the SC acts as a conduit for molecules to engage in CO formation, and perturbations in the SC impair the ability of molecules like SYP-3 to accumulate at CO sites. To further establish the SC’s role as a conduit for CO signaling, we are currently investigating how SC-recruited signaling proteins are impacted by defects in SC organization. Understanding the dynamics of the SC sheds light on how crossovers are regulated, and these clues can help reveal the underlying causes of meiotic problems like infertility.

B390/P0380

No Causal Relationship Between Intrinsically Disordered Region Binding With Nucleic Acid and Phase Separation

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Protein phase separation facilitates the formation of biomolecular condensates (BMCs) which concentrate and organize pathway specific factors in a dynamic fashion within the cell. A key determinant of phase separating proteins is multivalency which is commonly contributed by intrinsically disordered regions (IDRs). In complex coacervation, two oppositely charged multivalent polymers form a network of interactions to drive phase separation. Consistently, nucleic acids (polyanions) have been identified as key players in many phase separating systems and the prevailing view is that IDR nucleic acid binding and phase separation are intimately linked. Extending our work on the DNA binding and phase separating IDRs of DNA replication factors, we have completed an unbiased assessment of the causal relationship between nucleic acid binding and phase separation in biological IDRs. Using a bioinformatic search scheme we identified 66 IDRs from across the D. melanogaster proteome that share biochemical properties with the DNA replication factor IDRs. We directly tested a subset of these IDRs on their ability to bind nucleic acid (gel shift analysis) and undergo nucleic acid dependent phase separation (confocal microscopy). All tested IDRs bound to double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), and RNA, however binding affinity varied widely (nM to µM) and could not be fully explained by IDR net charge. Despite high-affinity nucleic acid binding, none of the purified IDRs formed condensates with dsDNA, only a third phase separated with ssDNA and RNA, and one IDR phase separated exclusively in the presence of RNA. Notably, we found no direct relationship between nucleic acid binding affinity and phase separation activity. Finally, we used quantitative fluorescence microscopy to relate in vitro IDR results with cellular behavior. We observed striking agreement between in vitro and in cellula data, demonstrating chromatin-binding ability of all IDRs (FRAP) and a non-uniform distribution of each IDR shown to phase separate in vitro. Our work here demonstrates that nucleic acid binding and IDR phase separation are not inseparably linked suggesting that the biochemical properties encoding an IDR’s phase separation propensity are not decipherable by any current method as they are more complex and occur less often than anticipated.

B395/P0385
Ordered and disordered interactions coordinately govern material properties and functions of biological condensates
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The proteomes of living species are enriched with oligomerization domains which are often preceded with or followed by intrinsically disordered regions/sequences (IDRs). Recent studies suggest that IDRs are drivers of protein condensate formation via phase separation. Oligomerization of folded proteins is known to be able to form filamentous fibers for diverse cellular function, but uncontrolled growth of ordered filaments is deleterious to cells. We discover in this study that IDRs, in their length- and phase separation propensity-dependent manner, can soften material properties of condensates formed by ordered protein oligomerizations. IDRs tune material properties of protein condensates by diluting densities of specific protein-protein interactions and breaking linear growth of filaments with their non-directional weak interactions and weaking percolation levels of the condensates. Thus, IDRs play critical roles in preventing uncontrolled growth of filamentous fibers formed by open protein polymers. We also demonstrate that adding IDRs to a-synuclein can slow down or even prevent its amyloid aggregate formation, a finding that may inspire new strategies in battling human diseases caused by non-reversible amyloids.

B396/P0386
Quantitative image analysis pipelines for studying the flow of mitochondrial gene expression
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Biomolecules undergo phase separation to form liquid-like structures called biomolecular condensates within the cell. Recently, phase separation has also been described in the mitochondria, particularly in condensing the mitochondrial genome into mt-nucleoids and its RNA products into mtRNA granules. However, relatively little is known about the biophysical principles governing the phase behavior within mitochondria. To create a quantitative framework for studying mitochondrial phase behavior, we developed an image analysis pipeline in python that identifies key morphological properties of both endogenous and synthetic condensates from super-resolution images. First, we applied our image analysis on optogenetically induced condensates and found that they were relatively small (diameter ~200 nm) and non-spherical (aspect ratio ~1.5), oriented along the direction of tubular mitochondria. These results point to the notion that mitochondrial architecture influences condensate morphology. Next, we applied this analysis across multiple channels, allowing us to compare both endogenous condensates relative to nascent and steady-state RNA transcripts. We then measured the nearest-neighbor distances of the identified puncta in three dimensions and found these components range from 0.1 um to 1 um apart. Such small distances challenge the idea that mt-nucleoids and mtRNA granules exist entirely as disparate structures, but rather they sample a variety of organizations. The measurements from these image analysis pipelines enable the quantitative study of the flow of mitochondrial gene expression.

B400/P1804

Tuning the Emergent, Time and Length Scales Dependent Viscoelasticity of Biomolecular Condensates

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Biomolecular condensates are “membraneless organelles” that can specifically organize together functionally related biomolecules and confer spatial and temporal control over cellular processes. The unique physicochemical and material properties of biomolecular condensates, distinct from their surroundings, are increasingly recognized as key determinants of biological functions in both native and disease contexts. Yet, how these material properties and their regulation are related to the underlying molecular and mesoscale interactions and structures remains unclear. Thus, in this study, we aim to establish mechanistic principles that link the molecular determinants of condensate formation to their emergent viscoelastic properties. Guided by the “stickers-and-spacers” model, we engineered polySH3 and polyPRM mutants to systematically and independently tune sticker and spacer interactions. Our optimized microrheology pipeline enabled precise measurement of condensate viscoelasticity across a wide range of interaction strengths. We show that stronger sticker and spacer interactions increase both the magnitude of condensate viscoelasticity (quantified by zero-shear viscosity) and its characteristic timescale (quantified by terminal relaxation time). Importantly, we observe coupling between sticker and spacer interactions, such that changes in one interaction type show diminished effects when the other is already strong. Extending this framework to biologically relevant systems, we examined condensates formed by the SARS-CoV-2 nucleocapsid (N) protein and the splicing factor PTBP1, both of which recapitulated our previous findings. Our results further reveal that condensates exhibit complex rheological behaviors, with nonlinear responses across timescales that indicate departures from simple Maxwellian mechanics. These behaviors also display length scale dependence: when spacer interactions are sufficiently strong, condensate viscoelasticity is dependent on length scales > 100 nm, indicating heterogenous network organization. To gain structural insight, we performed cryogenic electron tomography (cryoET), which revealed that enhanced spacer interactions promote higher-order structures and heterogeneity at the mesoscale. Altogether, this work provides a generalizable framework in which molecular level interactions define not only the presence of phase separated condensates, but also their complex, time and length scale-dependent rheological behaviors through mesoscale organization. These mechanistic principles advance understanding of how the emergent material properties of biomolecular condensates are tuned and regulated for specific functions, and how their dysregulation may contribute to disease.

B394/P0384

Unraveling the connection between meiotic function and condensate protein dynamics

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Meiosis is the cellular process responsible for generating haploid cells used in sexual reproduction. One of the key steps in this process is recombination, which begins with the formation of the synaptonemal complex (SC) condensate that synapses homologous chromosomes together. Once synapsed, double stranded breaks are generated in order to allow strand invasion for genetic exchange between the chromosomes. These events are known as crossovers, and the mechanism behind the selection of which subset of double stranded breaks are

chosen, as well as how the liquid-like properties of the SC contribute are still unknown. Previous research from our lab has shown that both the SC scaffold protein SYP-3 and the crossover signaling protein ZHP-3 diffuse along the SC condensate. Our work aims to address the mechanisms behind these impacts, determining what biophysical properties are required for both the stability of the condensate and the mobile nature of signaling molecules. The von Diezmann lab combines classical C. elegans genetics techniques such as fertility measurements and immunofluorescence with single-molecule tracking microscopy of meiotic proteins for a comprehensive methods tool-box. Combining these techniques allows us to pair the overall biological impact directly to changes in protein dynamics and localizations. Preliminary data suggests targeted disruptions to amino acid composition negatively impact both meiosis and condensate dynamics. This appears to be true in both structured regions in scaffold proteins as well as flexible disordered regions in signaling proteins. We hypothesized that perturbations to these regions would disrupt specific meiotic processes in distinct ways. Both minor truncations of the disordered region of ZHP-3, as well as small epitope tag additions to the C-terminus cause fertility defects, demonstrating the importance of disordered regions for meiotic health. In comparison, a lysine to glutamic acid substitution in the beginning of a coiled-coil region in the SC scaffold protein SYP-1 appears to destabilize the condensate, impacting diffusion of SYP-3 along the SC. While the global negative consequences of changes to these regions is clear, the biophysical mechanism behind these changes is still unknown. These results indicate the complex nature of biophysics that dictate meiotic outcomes, and linking the change in protein dynamics to meiotic impacts can directly relate biophysical properties to fertility health.

B399/P0389
Wetting Interaction between Actin and Vimentin regulates Vimentin Assembly
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Vimentin, a type III intermediate filament, is a key component of the cell cytoskeleton and is involved in a range of biological processes ranging from wound healing to cancer metastasis. Vimentin undergoes phase separation to form liquid droplets which wet actin stress fibers. In this work, we report that the phase behavior of vimentin influences its assembly in a variety of ways. During assembly, when the concentration of vimentin is low, it remains dissolved in the cytoplasm. With increasing concentration, they first form droplets and eventually filaments. However, the phase separation of vimentin is governed by the wetting interaction between vimentin and actin. As vimentin concentration increases, droplets are first observed on actin stress fibers before they appear in the rest of the cytoplasm. The wetting interaction allows vimentin to condense on actin stress fibers and form droplets at a lower concentration than is required for their bulk phase separation. As vimentin intermediate filaments (VIFs) grow out of these droplets, they remain colocalized with actin for short length-scales before lifting off. This behavior indicates that the droplet to filament phase transition of vimentin also affects its interaction with actin. Combined, these results suggest that weak interaction-driven phase behavior of vimentin exerts a much greater influence on its assembly and behavior than simply concentrating subunits.

Condensates - Cellular functions of condensates

B419/P0407
A novel RNP compartment boosts translation in growing mammalian oocytes to avoid cytoplasm dilution
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Mammalian oocytes undergo an extended growth phase in the ovary, during which transcriptional activity gradually declines. To support successful meiotic divisions and early embryonic development, growing oocytes must accumulate maternal stores and tightly regulate translation. Through a combination of immunofluorescence, mass spectrometry, and electron microscopy, we identified a novel and transient compartment in late-growing mouse oocytes, which we named the Zollo Body. This structure, composed of ribonucleoprotein particles (RNPs) and organelles, bears morphological similarities to the Balbiani body observed in many vertebrate species. However, unlike the Balbiani body, the Zollo Body exhibits active nascent translation and enrichment of phosphorylated mTOR (mammalian target of rapamycin). RNA sequencing and dry mass measurements of oocytes with or without this compartment further support its critical role in enhancing translation. This boost in translational capacity allows growing mammalian oocytes to counteract cytoplasmic dilution during their rapid volumetric expansion, thereby preserving their developmental competence.

B425/P0366

Architectural proteins organize replication into multiphasic condensates

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Genomes are organized across several hierarchical levels. Recently, phase transitions have been shown to play a role in condensing genomes, localizing fundamental genomic processes, including transcription and DNA repair. However, less is known regarding how phase transitions influence replication, in which long strands of both double- and single-stranded DNA (dsDNA, ssDNA, respectively) need to be coordinated. Here, we investigated the molecular interactions driving the phase behavior of the core biopolymers associated with mitochondrial replication. We designed several DNA templates varying in sequence features, length, and strandedness, and combined them with the dsDNA-binding protein TFAM and the ssDNA-binding protein mtSSB to induce in vitro phase separation. With super-resolution imaging, we observed multiphasic droplets, in which each architectural protein co-localized preferentially with its cognate nucleic acid. Indeed, we observed similar degrees of miscibility within mt-nucleoids as a function of their replication activity. To better understand the molecular driving forces, we performed single-molecule experiments using dual optical traps and generated force-extension curves, showing how the two architectural proteins cooperate to package tethered DNA into a condensate. Coarse-grained molecular dynamics simulations captured the formation of multiphasic condensates in protein-nucleic acid mixtures, providing molecular-level insight into our experimental findings. Together, these studies shed light on how phase separation of the architectural proteins facilitates segregation of the exposed single-stranded DNA from double-stranded DNA. Overall, the multiphasic organization of mt-nucleoids has implications for how replication can be spontaneously organized in cells.

B414/P0402

Assembly state of human phosphofructokinase-1 regulates localization and activity in cells

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Glycolysis is an evolutionarily conserved metabolic pathway that breaks down glucose to generate energy and biosynthetic precursors to sustain cellular functions. Pathway flux is controlled by rate-limiting enzymes, one of the most important being phosphofructokinase-1 (PFK1), which catalyzes the step committing glucose to breakdown. While PFK1 has been extensively studied in vitro, its regulation in cells remains incompletely understood, in part because most cultured cells express all three human isoforms which complicates structure-function analysis. To overcome this limitation, we generated a CRISPR PFK1 liver isoform (PFKL) knockout HepG2 cell line with <5% of normal PFK1 activity. Proteomic and metabolomic analyses revealed that loss of PFKL induced severe energetic stress, which was rescued by re-expression of FLAG-PFKL. Using this cell line, we asked how alternative assembly states of PFKL regulate activity and function. PFK1 activity is regulated by allosteric ligands and assembly state. While tetramers have long been viewed as the canonical active form, recent studies suggest that higher-order filaments regulate spatial localization and that dimers may retain catalytic activity in cells. Both wild type (WT)- and filament-incompetent PFKL (FI-PFKL) rescued glycolytic flux, indicating filaments are not required for basal activity. However, WT-PFKL assembled into biomolecular condensates in a filament-dependent manner. Further, WT-PFKL localized to lamellipodia, while FI-PFKL remained cytoplasmic. To investigate the role of this filament-dependent localization, we expressed WT- and FI-PFKL in migratory MDA-MB-231 human breast cancer cells and observed that FI-PFKL impaired chemotaxis, consistent with a model in which higher-order assemblies supply ATP to regions of high energetic demand. We next asked whether PFKL dimers are catalytically active in the cellular environment. Re-expression of tetramer-incompetent PFKL (TI-PFKL) partially restored glycolytic flux in knockout cells. Native PAGE showed TI-PFKL exists as monomers and dimers and was unable to form higher-order assemblies like the WT enzyme. In vitro activity assays showed TI-PFKL had reduced activity, but moderate activity under activating levels of AMP and fructose-1,6-bisphosphate. In conclusion, we identified a novel function of PFKL filaments in spatial regulation, and provide evidence for a catalytically active PFK1 dimer in cells. Additionally, our results establish a new cell platform to perform structure-function analysis of PFK1 and investigate isoform-specific roles of PFK1 in cells.

B422/P0410

Characterizing the effects of liquid-liquid phase separation on Abl2 kinase-mediated cytoskeletal regulation

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Abl2 is a cytoplasmic non-receptor tyrosine kinase which has been shown to participate in transducing a variety of extracellular stimuli into intracellular responses, including remodeling of the actin cytoskeleton and the regulation of microtubule dynamics (MT). Recent work from our group has shown that Abl2 can undergo liquid-liquid phase separation (LLPS), a process through which macromolecules can partition from a dilute phase (cytosol) into a dynamic dense phase (condensate), effectively increasing the concentration of the macromolecule in a spatially defined location. A growing body of work has linked LLPS to a variety of biological functions, including regulation of the cytoskeleton. Abl2 can regulate MT dynamics and co-condense with tubulin dimers on pre-assembled microtubules in vitro, but whether this happens in live cells is still an open question. To address this question, a combination of live cell imaging and biochemistry experiments was performed to assess if Abl2 is capable of undergoing LLPS in cells, and the functional outcomes of said LLPS

competency in terms of changes in MT dynamics. Using the OptoDroplets system, we were able to show that the N-terminus and C-terminus regions of Abl2 contribute to its LLPS competency in cells, with the N-terminus driving specific subcellular localization and the rounded morphology of the condensates, and the C-terminus providing the bulk of LLPS potential to the full-length protein, an observation that is in line with results obtained from disorder prediction algorithms. Furthermore, treating full-length Abl2-GFP expressing cells with DPH, an Abl kinase activator, results in the formation of cytoplasmic clusters that partially resemble OptoDroplet condensates, thus providing a demonstration of clustering that more closely resembles how Abl kinase LLPS might occur under endogenous conditions. Interestingly, these clusters localize on, and appear to be stabilized by microtubules. Our results thus far have given us a better understanding of the Abl2 sequence determinants that underlie its capacity to undergo LLPS in a cellular context. We are currently working on studying the Abl2-condensate proteome by combining the OptoDroplets system and TurboID-mediated proximity biotinylation.

B420/P0408
COMPARATIVE ASSESSMENT OF THE NOTCH MEMBRANE LESS ORGANELLES (BIOMOLECULAR CONDENSATES).
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Although biomolecular condensates have been known to exist in cells for almost one hundred years, only recently did the broad importance of biomolecular condensates come to be understood. It is believed that some condensates form on specific genetic loci and accumulate a population of transcription factors and co-factors to drive transcription. Notch proteins are cell-cell communication proteins that play critical roles in the development of all animals through their transcriptional activities. Mammals express four unique Notch proteins that are activated in an identical manner and bind to identical sites in the promoters of the genes they regulate. Despite these similarities, the four mammalian Notch proteins are well-known to exhibit both overlapping and non-overlapping functions but the mechanistic basis of the non-overlapping functions of Notch proteins remains a mystery. Recently, we discovered that the four mammalian Notch proteins form biomolecular condensates with unique characteristics suggesting that the non-overlapping Notch functions may be due to differences in the biomolecular condensates formed by the various Notch proteins. Based on our preliminary results, we hypothesize that condensates formed by the four mammalian Notch proteins contain both unique and overlapping proteins and that the composition of the different Notch condensates is responsible for the non-overlapping functions of Notch proteins. The research goal of this study is to use proximity ligation to capture proteins from Notch condensates, then compare the captured proteins to discover protein signatures that define each different Notch condensate. We expect that of some of the captured proteins will be distinct to each Notch condensates and that immunostaining results will reveal that they prevent the overlapping of the condensates.

B417/P0405
Connecting biomolecular condensate material properties to function at synapses
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Biomolecular condensates organize a variety of structures throughout the cell. In neurons, synapses are hot spots of condensate formation, with the presynaptic active zone, synaptic vesicle pool, and postsynaptic density each forming condensates. We have discovered that the presynaptic active zone, the core structure

responsible for clustering and priming synaptic vesicles and ion channels, forms a liquid condensate during development which solidifies into a stable structure. Active zone condensates are critical to build a functional synapse, and blocking condensate formation causes synapse assembly defects and synaptic transmission failures. Despite their demonstrable function in building synapses, mechanistically how and why these condensates are important remains unclear. In fact, it has been challenging to connect condensate properties to specific functions in many different cellular contexts. Here, we have investigated the function of the material properties of synaptic active zone condensates. We have decoded the molecular basis of SYD-2/Liprin-α condensate phase separation propensity, liquidity, and solidification. We identified the underlying cation-pi interactions supporting phase separation and design mutations that alter phase separation critical concentration over four orders of magnitude. The sequence basis of SYD-2 condensate liquid dynamics and solidification properties is also identified, and we engineer mutants that maintain condensate liquidity or solidify rapidly. Together, we have assembled a mutant toolkit to alter specific condensate material properties. Integrating these mutations into SYD-2/Liprin-α in vivo reveals functional connections between condensate properties and synaptic active zone assembly.

B411/P0399

Contact sites at the interface of membraneless organelles and membranes

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Phase separation is a major mechanism for organizing macromolecules, particularly proteins with intrinsically disordered regions, in compartments not limited by a membrane or a scaffold. The cell can thus be viewed as a dynamic emulsion, composed of numerous biomolecular condensates alongside classical membrane-bound organelles. The condensate-membrane interfaces emerge as chemical microenvironments that couple diffusion and the material properties of condensates to biochemical processes occurring in the membranes. For example, synapsin/synaptic vesicle condensates – a membraneless organelle that is in fact packed with membranes – can harbor ion potential differences at their interfaces and may act as a charge center in synapses. Another prominent example of large membrane areas that are tightly juxtaposed in cells are mitochondria and ER. Our recent data show that PDZD8, the ER-resident protein identified as a tether between the ER and mitochondria, can undergo liquid-liquid phase separation via its intrinsically disordered region. Endogenously-labeled PDZD8 forms condensates on membranes both in vitro and in vivo. These observations highlight how phase separation at membrane interfaces can create adhesion zones that tether vesicle cohorts or stitch neighboring organelles, revealing new principles for mesoscale cellular organization.

B413/P0401

CYCLON, a novel intrinsically disordered nucleolar protein, regulates pan-cancer cell fitness through nucleolar and splicing condensates.

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Cancer cell fitness—the ability of malignant cells to sustain growth, adapt to stress, and resist therapy—is central to tumor progression and adverse clinical outcomes. Fitness is mainly driven by dynamic regulation of

nuclear processes, including ribosome biogenesis and RNA processing. Intrinsically disordered regions (IDRs), known for mediating biomolecular condensates and regulatory plasticity, are key facilitators of these functions. In this context, the nuclear protein CYCLON, characterized by a large IDR and dual nucleolar/nucleoplasmic localization, has emerged as a candidate regulator of cancer cell fitness. It is overexpressed in multiple cancers, linked to aggressive disease and poor prognosis, especially in lymphoma where exclusive nucleoplasmic localization associates with unfavorable outcomes. Despite its clinical relevance and classification as essential for tumor survival in DepMap, its nuclear functions are poorly understood. Using inducible silencing, we found that CYCLON maintains nucleolar integrity. Its loss reduces nucleoli number but increases their size, delocalizes nucleolin to the nucleoplasm, and triggers hallmark features of nucleolar stress: p53 stabilization and nucleolar vacuoles, indicating disrupted phase-separated compartments. Molecularly, we demonstrated that CYCLON directly binds the 28S rRNA, ribosomal proteins and processing factors, aiding 32S pre-rRNA processing. Silencing impairs ribosomal subunits assembly, alters functional polysomes, reduces global protein synthesis, and leads to reduced proliferation and clonogenicity, highlighting CYCLON's critical role as a regulator of cancer cell fitness. These phenotypes are not associated with cell cycle arrest or apoptosis. Instead, they are characterized by prolonged interphase and mitotic durations, leading to a slower overall cell cycle progression. To further explore CYCLON's dynamics between the nucleolus and nucleoplasm, we identified minimal localization signals using truncation mutants. A nucleoplasm-restricted variant preferentially interacted with nuclear speckle–associated splicing factors. CYCLON depletion altered nuclear speckles morphology was rescued by expressing this variant, suggesting a moonlighting role in pre-mRNA processing that may contribute to oncogenic splicing program besides its function in sustaining ribosome biogenesis. Taken together, our findings establish this multifunctional IDR protein as a key link between nucleolar and splicing condensates, crucial for sustaining cancer cell fitness and promoting malignant growth.

B416/P0404

Development of a Molecular Tool to Explore the Physiological Roles of Biomolecular Condensates

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Biomolecular condensates (BCs) such as nucleolus and stress granules are unique organization of proteins and nucleotides. While many BCs are known to assure cellular homeostasis, their aberrant regulation often associates with disease conditions including neurodegenerative disorders and cancers. Despite the physiological significance, the exact role of individual BCs in cellular processes still remains elusive, primarily due to a lack of experimental tools for achieving their loss of function. For example, depletion of BCs from cells can be achieved by traditional approaches including knock down and knock out of genes encoding a key component of the target BCs. However, this type of approaches cannot easily distinguish the role of condensates themselves from that of the depleted molecules in observed phenotypes, as many of such key components are multitasking in a given BC, or have a role in other BCs. A chemical such as hexanediol could efficiently deplete BCs at the cost of poor target specificity and unacceptable cytotoxicity. Therefore, development of a new technique is required to enhance our understanding of BCs. Ideally, such a tool should disperse target BCs inducibly, efficiently and selectively, and should have minimal effect on other BCs. We have previously demonstrated that recruitment of ActA, an actin nucleation promoting factor, to stress granules by targeting its major component G3BP1 can induce their dispersion. Unfortunately, the use of ActA inevitably associated with actin polymerization which could unfavorably trigger adverse effects on cell homeostasis. By engineering the ActA-based prototype, we here report a new molecular tool termed "induced Dispersion of Granules (iDoG)". iDoG consists of the disordered regions of ActA that were built upon a structural platform made of alpha helices. Upon chemical addition or light illumination, iDoG was rapidly recruited to stress

granules and led to their complete dispersion, resulting in co-resolution of other stress granule components such as mRNA and PABP1 into cytosol. Of note, the dispersion occurred without causing actin polymerization or dispersion of non-targeted BCs such as P-bodies. The modular design principle of iDoG allowed us to target other BCs such as FUS and P-Bodies. With the demonstrated utility of iDoG, we can now begin studying physiological roles of individual BCs.

B418/P0406

Exploring the role of adaptor proteins in EGFR phase separation

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Epidermal Growth Factor Receptor (EGFR) is a type of receptor tyrosine kinase bound in the membrane of a cell. When activated, it triggers a signaling cascade that promotes cell proliferation, growth, and survival. EGFR is activated via binding with the ligand Epidermal Growth Factor (EGF), causing EGFR to dimerize and autophosphorylate the tyrosines on its cytoplasmic C-tail. This recruits several adaptor proteins, causing the proteins to cluster and phase separate to form small droplet-like condensates on the cell membrane. To better understand if these condensates affect EGFR signaling and what role of these adaptor proteins play in EGFR phase separation, we first examined EGFR C-tail phase separation in vitro using supported lipid bilayers. We co-incubated phospho-EGFR C-tails with Growth Factor Receptor-Bound Protein 2 (Grb2) at increasing concentrations on a supported lipid bilayer to determine at what concentration of Grb2 phase separation occurs. We also added Grb2-Associated-Binding Protein 1 (Gab1) to pre-formed pEGFR and Grb2 condensates on another bilayer to study how Gab1 affected the clusters. Through this, we found that EGFR and Grb2 phase separate to form condensates on bilayers starting at 2μM of Grb2, and that EGFR-Grb2 condensates are disrupted and dissolved by the presence of Gab1. From there, we chose to explore how different adaptor proteins affect EGFR condensates in cells. To determine if the concentrations of Gab1 and Grb2 change during EGFR activation, we used serum-starved MDA-MB-468 cells—a triple-negative breast cancer cell line with EGFR overexpression—and treated them with increasing concentrations of Epidermal Growth Factor (EGF) for 24 hours. We then collected the cells and performed fluorescent western blots on the lysates. We found Gab1 levels increased by 50% when stimulated with just 10nM of EGF compared to the untreated control, while Grb2 levels decreased by 35%. Grb2 also required a larger concentration of 1μM EGF than Gab1 for its levels to change. Through these experiments, we confirmed that EGFR is capable of phase separation with Grb2 on lipid bilayers, and that Gab1 disrupts these clusters. We also found evidence of a trend between Gab1 and Grb2 during EGFR activation where, as EGFR activation increases, Gab1 levels increase and Grb2 levels decrease. Future directions for this project include probing cell lysates treated with increasing amounts of EGF on a western blot for Protein Kinase B (AKT) bulk and phosphorylation levels to determine if increased EGFR activation affects downstream signaling in the pathway. We will also immunostain MDA-MB-468 cells for Gab1 and EGFR under different stimulation conditions to see if the proteins colocalize and determine how their respective enrichment correlates in clusters in cells.

B415/P0403

Investigate the role of condensate formation in actin organization during myoblast fusion

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Cell surface receptors often form nanometer- to micrometer-scale clusters to initiate signal transduction in response to environmental cues. At the sites of Drosophila myoblast fusion known as the fusogenic synapse, Ig domain-containing cell adhesion molecules (CAMs), Dumbfounded and Sticks and stones (Sns), form clusters, and the latter recruits over a dozen additional proteins into a “focus” to promote actin polymerization and bundling. The actin cytoskeletal regulators, in turn, regulate the formation of dynamic and mechanically stiff invasive protrusions to promote cell-cell fusion. How Sns recruits various actin regulators to the fusogenic synapse remains a longstanding question. The SH2 and SH3 domain-containing adaptor proteins, Crk, Drk, and Dock, are known to link CAMs and the actin cytoskeleton, and the mammalian homologs of Drk and Dock have been implicated in condensate formation with Ig domain-containing CAMs. Using an in vitro phase separation assay with purified proteins, we found that the cytodomain of Sns (SnsCT) formed condensates by itself, and that Crk, but not Drk or Dock, partitioned into the SnsCT condensates. Consistent with this, Crk was recruited to the SnsCT condensates in cultured S2R+ cells and was required for myoblast fusion in Drosophila embryo, suggesting that Sns-Crk condensate formation may play a role in cell fusion. By making truncated forms of SnsCT, we mapped an essential region for both SnsCT condensate formation and Crk recruitment. Deleting this region compromised cell fusion in cultured cells and in Drosophila embryo, supporting a role for condensate formation in cell fusion in vivo. Phenotypic analysis by electron microscopy revealed abnormally wider invasive protrusions in crk mutant embryos, indicating that the actin filaments within these protrusions are disorganized. Indeed, two actin-bundling proteins, Dynamin and WASP-interacting protein/Solitary (Sltr), which normally partitioned into the Sns-Crk condensates, were no longer recruited to the fusogenic synapse in crk mutant embryos. Taken together, our study demonstrates that Sns recruits Crk and actin-bundling proteins into condensates, which increases the local enrichment of these proteins at the fusogenic synapse to upregulate the mechanical strength of the actin cytoskeleton for invasive protrusion formation during cell fusion.

B421/P0409

MPS1 licenses robust spindle checkpoint via phase separation

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The spindle assembly checkpoint (SAC) safeguards faithful chromosome segregation by monitoring kinetochore-microtubule attachment. While the kinase MPS1 is known to initiate SAC signaling at kinetochores, the regulatory mechanisms ensuring its rapid and robust activation are not fully understood. Here, we identify liquid-liquid phase separation (LLPS) as a critical mechanism driving MPS1 activation. We demonstrate that the C-terminal intrinsically disordered region (IDR) of MPS1 is capable of mediating LLPS. The recombinant C-terminal 342-amino-acid fragment undergoes phase separation in vitro, a process enhanced by a kinase-dead mutation (D664A), suggesting that multivalent interactions facilitate condensate formation. Functionally, this IDR-driven LLPS is essential for the rapid activation of MPS1 kinase activity and is required for the full strength of the SAC response. Our findings reveal that MPS1 licenses a robust spindle checkpoint through a phase separation mechanism, providing a new paradigm for the assembly of mitotic signaling complexes.

B423/P0411

Proteomic analysis of arsenite-induced stress granules by high-precision microscopy-guided photo-biotinylation

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Deciphering the protein composition of stress granules (SGs) has long posed a challenge due to their transient, dynamic, and membrane-less nature. Here, we employed optoproteomics, an advanced technology integrates microscopy-guided automated photo-biotinylation, pulldown and mass spectrometry, to isolate and identify proteins localized at the arsenite-induced SGs. Two-photon illumination was directed to the locations of the G3BP1 signals, to trigger photo-induced biotinylation at a high precision. This process is automatically repeated across thousands of FOVs to photolabel proteins in SGs. Subsequently, ample biotinylated SG proteins are obtained for streptavidin pulldown and mass spectrometry analysis. Within the top 50 abundant proteins mapped with the known stress granules database, functional enrichment analysis highlights 13 non-SG associated proteins as high-confidence core interactors within the stress granule network. Using immunofluorescence validation, we found that 11 proteins (PDLIM7, EIF3CL, YWHAE, RPSA, MTA2, UGDH, DDX17, ANLN, PSMD3, PSMA6, and MCM2) were co-localized with G3BP1, thus elevating the specificity eventually to 96% SG association. Overall, our study illustrates that optoproteomics enables hypothesis-free, comprehensive mapping of subcellular proteomes at user-defined regions of interest, shedding light testable hypothesis for further functional understanding of SGs.

B412/P0400

Spatiotemporal organization of mitochondrial condensates in live cells

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Phase separation drives the assembly of many membrane-less cellular structures, including genomes. Mitochondria package their own genome (mtDNA) into nucleoprotein complexes called mt-nucleoids, which also behave as biomolecular condensates. Yet, little is known about how the higher-order structure of mt-nucleoids contributes to their activity. Here, we examined the phase behavior of mt-nucleoids in the context of two fundamental processes associated with genomes: replication and transcription. In doing so, we linked the activity of mt-nucleoids to the degree of miscibility of their components. For example, we found that that actively replicating mt-nucleoids had high partitioning of the main packing protein of single-stranded DNA. To identify the molecular interactions influencing component partitioning, we reconstituted in vitro the four core biopolymers associated with mt-replication: dsDNA, ssDNA, and their two respective packaging proteins. Strikingly, we found that these components assembled into multiphasic droplets, where each architectural protein preferentially partitioned with its cognate nucleic acid. Analogously, transcribing nucleoids appeared to undergo dynamic mixing events with neighboring mtRNA granules, likely coordinating the switch between transcription and subsequent RNA processing. Moreover, inhibiting transcription led to dissolution of the mtRNA granules, suggesting RNA acts a key scaffold molecule that sustains the steady-state organization observed for these two disparate condensates. To understand the biophysical driving forces that support mitochondrial phase behavior, we developed an optogenetic construct (mt-OptoIDR) that could form synthetic condensates upon light activation within live mitochondria. The optogenetically induced condensates exhibited similar morphological features as endogenous condensates, indicating the mitochondrial environment largely determines the properties of its condensates. The structure-function relationships that emerge from mitochondrial condensates appear to be generalizable to other multicomponent condensates in cells.

Condensates - Condensates in Disease and Aging

B008/P1140
Alpha-synuclein condensates impact mitochondrial homeostasis and lipid droplet turnover resulting in energy failure
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Background Alpha-synuclein (αSyn) is an intrinsically disordered protein that misfolds and aggregates within neurons in Parkinson's disease (PD), forming inclusions known as Lewy bodies. Recent evidence suggests that aberrant liquid-liquid phase separation might be involved in the early stages of αSyn dysregulation in PD. Objective: To research how aberrant phase separation is involved in the early stages of αSyn dysregulation. Methods: To model αSyn inclusions, we used human neuroblastoma M17D cell lines engineered to overexpress YFP-tagged αSyn under doxycycline induction. We had two separate cell lines for each of the following versions of αSyn: 1) wild type αSyn, 2) a PD autosomal dominant E46K mutant ("1K"). Results: Wild type and 1K αSyn did not spontaneously form inclusions, but did so after treatment with oleic acid, which induces the formation of lipid droplets. The resulting inclusions entrapped lipid droplets. Fluorescence recovery after photobleaching (FRAP) experiments showed that they were liquid, consistent with biomolecular condensates. They were readily dissolvable through treatment with 1,6-hexanediol, which is an alcohol that breaks apart hydrophobic interactions, further supporting their liquid status. A pulse chase assay showed that lipid droplets entrapped within αSyn condensates had a reduced turnover rate in comparison to lipid droplets in the cytoplasm, suggesting that αSyn condensates could impair energy homeostasis in the cell. Finally, mitochondria around αSyn condensates were depolarized, which indicates that αSyn condensates could be toxic species affecting mitochondrial function. Conclusion: αSyn forms biomolecular condensates in lipid droplet-rich environments, which impair lipid droplet turnover and can contribute to energy failure.

B005/P1137
Alpha-synuclein condensates precede aggregate formation and are dissolvable by chloroquine
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Alpha-synuclein (αSyn) is an intrinsically disordered protein that is involved in the pathogenesis of Parkinson's Disease (PD) through the formation of intraneuronal inclusions called Lewy bodies. Objective: The objective of this study is to investigate the mechanism underlying the formation of αSyn inclusions. Methods: This project uses a M17D neuroblastoma cell line stably over-expressing doxycycline-inducible αSyn that spontaneously forms inclusions with structural features that are similar to Lewy bodies. Results: Fluorescence Recovery after Photobleaching (FRAP) experiments were conducted on αSyn inclusions after 16h (when they first become visible), 48h, 72h, 96h and 120h of doxycycline induction. By measuring their immobile fraction (Fi), we determined that the inclusions at 16h have diverse material properties and range from completely liquid to solid, which indicates that they form through a combination of liquid-liquid phase separation (LLPS) and aggregation. After 48h, inclusion immobile fraction increases, indicating that they expand through aggregation.

Next, we investigated the effect of chloroquine (an autophagy inhibitor) on αSyn inclusions, as it has been previously shown that chloroquine treatment reduces the risk for development of PD1. We found that there was a significant decrease in the number of inclusions in cells treated with chloroquine. Flow cytometry experiments showed no significant decrease in αSyn levels. Furthermore, chloroquine treatment in autophagy deficient cells (through FIP200 knockdown) compared to control cells did not show a change in removal of αSyn inclusions, which indicates that clearance of inclusions by chloroquine is autophagy-independent. FRAP experiments on cells treated with chloroquine showed significant changes in the Fi of inclusions. Finally, nuclear magnetic resonance (NMR) experiments showed that there is a weak interaction between chloroquine and αSyn in vitro. Conclusion: In conclusion, LLPS is involved in the early stages of the formation of αSyn inclusions. Chloroquine treatment reduces the number of αSyn inclusions in an autophagy-independent manner, likely by modulating the LLPS of αSyn through direct interaction. Further studies are needed to determine the precise mechanism of action of chloroquine as well as the optimal timepoint of administration to derive maximal clinical benefit in animal model systems for PD. Part of the results have been published in2.
1. Paakinaho, A. (2022). Neurology. 2. Eubanks, E. (2025). Cell Rep.

B003/P1135

Altering the material properties of condensates to impair oncogenic functions of TFE3 fusion oncoproteins in translocation renal cell carcinoma

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Biomolecular condensates are dynamic, membrane-less compartments essential for diverse cellular functions. Their dysregulation contributes to diseases including cancer. While therapeutic strategies to disrupt condensate formation or composition are under investigation, whether altering condensate material properties can be therapeutically exploited remains unclear. Here we focus on TFE3 fusion oncoproteins (FOs), generated by chromosomal translocations, which we previously showed drive translocation renal cell carcinoma (tRCC) through formation of oncogenic condensates. We hypothesized that arresting condensate dynamics could impair their oncogenic function. To test this, we used a 17–amino acid micropeptide, “killswitch,” derived from a mutant HMGB1 protein, to selectively target and reduce the dynamics of NONO::TFE3 condensates. Killswitch expression in the tRCC patient derived cell line decreased endogenous TFE3 FO condensate number, solidified these condensates showing reduced fluorescence recovery after photobleaching (FRAP), and downregulated TFE3 FO-specific target genes. These findings suggest that modulating condensate material properties offers a novel therapeutic avenue for tRCC and other condensate-driven cancers.

B004/P1136

Coarsening of the Mitochondrial Genome Redistributes Mitochondrial Chaperones and Proteases

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Mitochondria are semi-autonomous organelles that contain their own genome. The mitochondrial genome is packaged into ~100 nm nucleoprotein complexes known as mt-nucleoids. Recently, we showed that mt-nucleoids behave as biomolecular condensates. Consistent with these findings, mt-nucleoids undergo liquid-like fusion events upon exposure to various forms of stress, coarsening into prominent condensates and reaching sizes over 1 μm in diameter. The functional consequences of mt-nucleoid coarsening are still largely

unclear. Here, we investigated how mt-nucleoid coarsening leads to local reorganization of mitochondrial components. First, we induced the coarsened phenotype by treating human fibroblasts with a small molecule inhibitor of mitochondrial fission. With super-resolution microscopy, we detected significantly enlarged mt-nucleoids, particularly within highly swollen, bulbous mitochondria (mt-bulbs). Strikingly, we noticed that coarsened mt-nucleoids were also associated with a local two-fold enrichment of core components of the mitochondrial unfolded protein response (UPRmt), including chaperones and proteases, within mt-bulbs. To test if defects in protein import were contributing to the recruitment of chaperones to coarsened mt-nucleoids, we measured changes to mitochondrial membrane potential. Indeed, we found an imbalance in the mitochondrial membrane potential in mt-bulbs compared to surrounding tubular mitochondria. These findings support a mechanism by which coarsening of mt-nucleoids disrupts the spatial distribution of mitochondrial-encoded OXPHOS products. As a result, mt-bulbs become the primary sites of OXPHOS assembly, facilitating a high membrane potential that supports protein import and leading to accumulation of chaperones and proteases near coarsened mt-nucleoids. Overall, mt-nucleoid coarsening may be a self-regulating mechanism that ensures mitochondrial quality control.

B002/P1134

Identification of cellular regulators of an oncogenic nuclear condensate using high-throughput imaging screens

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NUP98-HOXA9 is an oncogenic fusion protein in pediatric leukemias. This oncofusion protein forms via chromosomal translocation by joining the Intrinsically Disordered Regions (IDR) of the nuclear pore protein NUP98 and the DNA binding domain of the homeobox transcription factor HOXA9. In line with the recent realization that IDRs are major drivers of phase-separation, NUP98-HOX9A forms prominent condensates in the cell nucleus. These condensates drive aberrant chromatin looping, induce formation of super-enhancer clusters, and upregulate leukemogenic gene expression. The biomolecular regulators of NUP98-HOXA9 condensates are unknown. To identify them, we have performed a high-throughput imaging-based CRISPR screen targeting 1068 chromatin proteins and assayed for changes in condensate number and texture. We found that loss of several subunits of RNA Polymerase I (RNAP1) resulted in a sharp decrease in condensate number and altered condensate texture. Concomitantly with dissolution of nucleoplasmic condensates, NUP98-HOXA9 gradually redistributed to the nucleolus and accumulated in nucleolar granular centers. Interference with RNAP1 transcription using a small molecule inhibitor phenocopied these effects. RNAP1 inhibition also reduced the expression of several leukemogenic genes which are misregulated by NUP98-HOXA9 condensates. These effects were specific to NUP98-HOXA9 as other transcriptional condensates and splicing condensates remained unchanged upon RNAP1 inhibition. Taken together, we have identified RNAP1 as a major regulator of NUP98-HOXA9 oncofusion nuclear condensates. Our findings lay the foundation for the exploration of the mechanistic basis of NUP98-HOXA9 function and, given the clinical relevance of oncofusion condensates, they open up new avenues towards therapeutic intervention in leukemia by targeting nuclear condensates.

B006/P1138

Proteostress-induced Neuronal Vesicle Extrusion: Exophers Preferentially Recruit Protein Condensates with

High Viscoelasticity

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Propagation of pathological protein species is a hallmark of neurodegenerative disease progression. The mechanism of cell-to-cell transfer and the nature of the pathological protein species that spread in disease are not well understood and are challenging to study in the context of a human brain. We model spreading biology in our study of C. elegans touch neuron exophers, which we hypothesize act as a potential mechanism for pathological proteins to exit the cell. Exophers are large extracellular vesicles discovered in C. elegans neurons and later observed in mammalian systems. Exophers can contain protein aggregates and damaged organelles. Notably, exophers preferentially recruit overexpressed mCherry and Htt-polyQ128 aggregates while excluding soluble GFP. Recruitment of exopher cargo may be sequence dependent or aggregation state dependent. Furthermore, many proteins implicated in neurodegenerative disease also form liquid or hydrogel condensates, and it is unknown if exophers recruit these condensates which have intermediate material properties between soluble and aggregated protein. We hypothesize that condensates with enhanced viscoelasticity such as hydrogels will be recruited to exophers more than dynamic liquid condensates given that solid aggregates are preferentially recruited to exophers. To address this question, we used PGL-3, a native C. elegans germline protein that forms dynamic liquid condensates, as a model substrate. We mutated the intrinsically disordered region (IDR) of PGL-3 by swapping the Arg or Lys residues or by replacing the IDR with synthetic sequences. Five variants were purified, and material properties were characterized using fluorescence recovery after photobleaching (FRAP) and microrheology. Mutation of the IDR generated of a panel of variants with a range of material properties. We expressed the PGL-3 variants in C. elegans touch neurons and measured condensate properties using FRAP and fluorescence correlation spectroscopy. Variants with enhanced viscoelasticity in vitro showed similar trends in vivo. We quantified the ratio of the PGL-3 variants in touch neuron exophers versus soma and found that variants with enhanced viscoelasticity were recruited to exophers more. These findings further our understanding of exopher cargo criteria and condensate biology in large vesicle extrusion in neurons of living animals.

B007/P1139

TEAD1 condensates are transcriptionally inactive storage sites on the pericentromeric heterochromatin

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TEA domain transcription factor 1 (TEAD1), a Hippo pathway transcription factor important in cellular homeostasis and development, is increasingly implicated in cancer biology. Here, we reveal a novel role for TEAD1 in organizing nuclear condensates, independent of active transcription. Using high-resolution imaging, ChIP-seq, RNA-seq and proximity-based proteomics, we demonstrate that in patient-derived renal cell carcinoma cells, TEAD1 forms micron-sized foci by binding to the heterochromatic pericentromeric regions using its DNA-binding domain. These TEAD1 foci do not mediate transcription but instead serve as depots for excess TEAD1. This contrasts with TEAD1 organization in other genomic regions of both RCC and normal kidney cells, where TEAD1 associates with markers of active transcription. Our findings provide a mechanistic framework for TEAD1's dual regulatory roles, offering new insights into its contribution to transcriptional dysregulation and tumor progression.

Condensates - Cytoplasmic Condensates

B410/P0398
DDX3 Activity Shapes RNA Pools in Stress-Related Ribonucleoprotein Condensates and Controls Stress Granule Abundance, Size, and Compartmentalization
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RNA–protein condensates are widespread macromolecular assemblies that serve as catalytic hubs, sites for RNA maturation, and adaptive regulators of signaling pathways. In mammalian cells, cytoplasmic RNA–protein condensates such as stress granules (SGs) and P-bodies play central roles in stress responses. SGs, in particular, have been shown to delay apoptosis, senescence, and modulate signaling pathways and metabolism during cellular stress. Dysregulation of SGs is linked to cancer, neurodegenerative disorders, and viral infections. The diverse functions and related pathologies of SGs stem from the complex and heterogeneous organization of RNA–protein assemblies. These assemblies include a broad range of proteins—such as RNA-binding proteins, prion-like proteins, translation factors, and roughly 50% uncharacterized proteins—as well as an RNA pool involving mRNAs associated with ATP binding, transcription, cell cycle regulation, and splicing, along with non-coding RNA species believed to serve as key structural elements of the condensates. Recent studies indicate that ATP is vital for SG assembly, fusion, and dynamic behavior. Helicases from the RNA-dependent DEAD-box ATPase family have been shown to influence the formation and disassembly of biomolecular condensates by binding to or unwinding RNA secondary structures. Here, we report that inhibiting the ATPase activity of DEAD-box helicase 3 (DDX3) with the small-molecule inhibitor RK-33 alters both the RNA composition and assembly of SGs during arsenite-induced oxidative stress in U2OS cells. First, RNA sequencing shows that DDX3 inhibition reduces stress-responsive RNAs and depletes long non-coding RNAs, including NORAD, which has been reported to contribute to SG formation, as well as circular RNAs such as circMPP6 and ARID1A, which are believed to interact with RNA-binding proteins associated with SGs and P-bodies. Second, STED microscopy reveals that treatment with the inhibitor decreases SG formation and produces smaller SGs that lack the DDX3-patterned compartmentalization observed in SGs formed under stress without RK-33. Notably, in RK-33–treated cells, SGs still recruit FXR1, which normally forms condensates even without stress and colocalizes with SGs under stress. Finally, analysis of cytoplasmic viscosity, through tracking 40-nm GEM nanoparticles, characterizes the physical properties of SGs and their surrounding cytoplasm and suggests potential effects of DDX3 ATPase inhibition. Overall, our findings suggest that DDX3 activity influences SG dynamics, RNA composition, structure, and physical properties by reshaping the RNA landscape, with a reduction in stress-related and protein-binding RNAs. NIGMS Grant #: 5P20GM103499 USC SPARC Grant

B405/P0393
Germ granules transform into multiphasic structures by molecular condensation
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Biomolecular condensates assemble a specific repertoire of macromolecular components to promote specialized biochemical reactions. Owing to their properties as phase delimited entities, condensates have the

ability to rapidly form, break down, and to change configuration or composition by exchanging protein content with the environment as intracellular conditions fluctuate. Here, we investigate the relationship between condensate function and composition using germ granules in Drosophila melanogaster which are RNA/protein condensates that experience diverse intracellular changes during development. Drosophila germ granules concentrate mRNA regionally in the oocyte and then deploy the protein products of these mRNAs for the formation and the specification of the primordial germ cells (PGCs) in the embryo. Moreover, through mechanisms that are not fully understood, germ granules and their components ensure that the initial proteins required for PGC development are produced from germ granule mRNAs at the right place, at the right time, and to the proper levels. We hypothesized that components of germ granules evolve as intracellular differences arise in development leading to changes in granule function. To test this hypothesis, we tracked changes to proteins in proximity of germ granules across oogenesis and embryogenesis using a proximity-dependent biotinylation approach. The core germ granule protein Oskar was conjugated to TurboID, an engineered biotin ligase that catalyzes promiscuous biotinylation of proteins within a radius of several nanometers. Proteins biotinylated by Oskar-TurboID were quantified with multiplexed TMT mass spectrometry in a comprehensive time course comparing two time periods during oogenesis and six sequential time intervals after fertilization spanning the first 270 minutes of embryogenesis. Our results demonstrate widespread remodeling of the germ granules and their environment upon two developmental transitions, first when the egg is fertilized and second during the formation of the PGCs. By analyzing proteins that change significantly over time near germ granules, we discovered a novel remodeling event involving the recruitment of the CCR4-NOT complex to the surface of germ granules that is elicited by molecular condensation of the universal germline determinant Nanos. Assembly of a Nanos rich protein domain around germ granules promotes granule fusion and transitions germ granules into a hybrid state distinguished by a shell containing P-body components. We find that that this switch-like transformation of the germ granules is required for the robust survival of PGCs by timing a key cell state transition.

B409/P0397

Inference of Molecular Interactions Mediating Cortical Condensate Dynamics in C. elegans Oocytes

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A central challenge of cell biology is to connect the dynamics of molecular interactions to emergent cellular structures and functions. A paradigmatic emergent structure is the actomyosin cortex, which must be constructed from cytoskeletal proteins and their interactors to generate cell-scale contractile forces starting early in development for successful embryogenesis and all future cell divisions. However, the manner in which these microscopic components dynamically interact to construct and remodel the cortex and mediate its cellular functions is not well understood. To address these questions, we imaged the cortex during its construction in C. elegans oocytes using live-cell fluorescence microscopy. We focused on recently identified transient structures that appear in the cortex, called cortical condensates, which are short-lived assemblies of branched F-actin and the branch nucleators Arp2/3 and WSP-1 (N-WASP) that are potentially associated with sites of endocytosis. Previous work successfully modeled the average dynamics of F-actin and WSP-1 in condensates with coarse-grained interactions. However, these interactions cannot account for observations of a large variance in F-actin dynamics during condensate disassembly. We find that, specifically, the molecular mechanism by which cortical condensates dissolve remains unexplained. We show that the coronin-family F-actin de-branching protein, POD-1, is an additional component of cortical condensates. We characterize its dynamics and demonstrate that active F-actin disassembly is sufficient to explain the variance of F-actin dynamics during condensate dissolution. Finally, we use conditional dependence tests on co-observations of F-actin, WSP-1, Arp2/3, and POD-1 dynamics to determine the direct molecular interactions which enable POD-1

to dissolve cortical condensates. We further test these plausible direct interactions using RNAi experiments to perturb protein levels and observe their impacts on condensate dynamics. Our final model describes the dynamics of the direct molecular interactions mediating non-equilibrium condensates in live cells.

B402/P0390

Intracellular pH regulates lactate dehydrogenase expression and subcellular localization

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Transient intracellular pH (pHi) dynamics have been shown to regulate normal cell behaviors such as cell cycle progression, differentiation, apoptosis and metabolism. The White Lab has previously shown that increasing pHi in normal epithelial cells increases the expression of Lactate Dehydrogenase A (LDHA) at both the mRNA and protein level. Lactate Dehydrogenase (LDH) is a tetrameric enzyme that reversibly converts the final product of glycolysis, pyruvate into lactate. Importantly, increased LDHA expression also drives increased lactate production at high pHi compared to control cells, suggesting that high pHi may be a sufficient driver of metabolic reprogramming. Here, we show that experimentally raising pHi in normal cells (MCF10A) endogenous LDH forms a higher order structures within the cytoplasm that may drive metabolic reprogramming. We find that LDHB, an enzyme that catalyzes the reversible conversion of lactate into pyruvate, with a preference for the forward direction, forms distinct regions of high expression (puncta) within the epithelial cell cytoplasm across all pHi conditions. However, LDHA only forms puncta under high pHi conditions. Under high pHi, LDHA puncta colocalize with existing LDHB puncta, but the LDHA:LDHB structures do not colocalize with stress granule marker (G3BP1). This indicates that LDHA:LDHB subcellular structure formation is not a generic cellular stress response. The LDHA:LDHB structures partially colocalize with Golgi (Golgin97) and lysosomes (LAMP1) but have the strongest colocalization with mitochondria (TOMM20). Our work suggests that pH-sensitive formation of higher-order LDHA:LDHB structures within the cytoplasm may be important for driving subcellular membraneless compartmentalization of glycolytic enzymes and may be a key driver of pH-dependent metabolic reprogramming. Ongoing work will characterize the dynamics of LDHA:LDHB puncta formation and investigate the metabolic changes associated with this subcellular organization.

B404/P0392

Label-Free Characterization of Biomolecular Condensate via Light Interference Energetics (LIFE) Microscopy

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Biomolecular condensates (BMCs), formed through liquid–liquid phase separation (LLPS), are non-membrane-bound compartments enriching proteins involved in the same signaling pathways, thereby regulating important cellular processes including gene expression and DNA damage response. Abnormal BMC formation is associated with cancer and neurodegenerative diseases, such as Parkinson's, Huntington's, and Alzheimer's disease. Characterization of BMCs in live cells might elucidate the mechanisms by which BMCs achieve homeostasis or cause pathology. Currently, few in cellulo characterization methods exist besides labeling BMCs with fluorophores. However, the fluorophores might alter the electrostatic state of BMCs and introduce artifacts. In this project, we explored a label-free method to study BMC dynamically, by imaging BMCs consisting of poly-l-lysine (PLL) and ATP using the Light Interference Energetics (LIFE) microscope. We

configured the microscope so that the BMC sample was illuminated by a 1040-nm IR laser from above. Part of incident light was scattered by BMCs, while another part continued to travel until it was reflected by the cover glass underneath. The scattered and the reflected light formed interference detectable by a camera. If the imaged objects are motile, the interference pattern will change over time. Different motilities of the moving objects can be distinguished because the rates of the change in interference pattern also differ. Indeed, we observed different frequency peaks of interference pattern change at the surface of the BMCs, in their interior, and in the buffer excluded from the BMCs. Specifically, the PLL-ATP condensates in buffer exhibited 7% higher power between 26.7 Hz to 28.1 Hz and up to 17% higher power between 88 Hz to 89.5 Hz compared to the buffer-only condition. Such differences could also be clearly observed in the spatial images of the interference pattern. We note that we employed quantum-limited low-noise laser, so that the interference pattern changes are unlikely to be caused by random noises. Our preliminary results are an encouraging sign that LIFE microscopy can be a useful tool to characterize molecular motilities related to BMCs in a minimally invasive manner.

B406/P0394

Mechanical perturbation induces tensin 3 phase separation

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Phase separation has emerged as a key mechanism by which cells organize and regulate membraneless subcellular organelles known as biomolecular condensates. Previously, we demonstrated that tensin 1, a focal adhesion protein, forms condensates during focal adhesion disassembly in a cell cycle-dependent manner. While tensins have long been recognized for linking actin filaments to integrins and transmitting mechanical tension, our findings reveal that they can also undergo phase separation - highlighting a previously underexplored mechanism for regulating adhesion dynamics. Here, we report that GFP-tagged tensin 3, the third member of the tensin family, also phase-separates to form biomolecular condensates in epithelial cells. Notably, this process is induced by microneedle-applied force, often rapidly propagating to neighboring cells. Furthermore, live-cell stretch assays and fluorescence recovery after photobleaching analysis showed that the tensin 3 condensates dissolve after tension release and readily recovers their fluorescence, thus demonstrating the condensates' dynamic, liquid-like nature. Importantly, the deletion of alpha-helical sequences within tensin 3 significantly enhances this force-induced condensate assembly, suggesting that these motifs normally restrain its phase-separation. Together, our findings provide the first demonstration that mechanical perturbation induces phase separation in living cells, suggesting that tensin 3 condensation may serve as a mechanism to regulate focal adhesion dynamics during cell migration.

B408/P0396

Membranes Arrest the Coarsening of Mitochondrial Condensates

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Mitochondria are double membrane bound organelles that contain their own genome within their interior. The mitochondrial genome is a small 16.5 kb, circular form of DNA that is packaged into membraneless nucleoprotein complexes called mt-nucleoids. Recently, we showed that mt-nucleoids behave as biomolecular condensates. Normally, these condensates are small (~100 nm) and uniformly distributed across the

mitochondrial network, yet are prone to coalescing upon various forms of stress. However, how mitochondria maintain the emergent properties of mt-nucleoids is not well understood. Here, we engineered an optogenetic tool (mt-optoIDR) that undergoes a concentration dependent phase transition upon light activation within live mitochondria upon. Using super-resolution microscopy, we visualized the formation of synthetic mitochondrial condensates (mt-opto-condensates) and characterized their phase behavior. Strikingly, the synthetic condensates exhibited comparable morphologies and dynamics as endogenous mitochondrial condensates. Specifically, the mt-opto-condensates appeared to be a few hundred nanometers in size and elongated in the direction of the mitochondrial network, suggesting the mitochondrial membranes may be restricting their growth. To identify the contribution of mitochondrial membranes, we overexpressed a dominant negative form of the fission protein Drp1, leading to swollen, bulbous mitochondria. Indeed, such perturbation to membrane structure resulted in the rapid coarsening of mt-opto-condensates into prominent droplets compared to small, evenly distributed condensates observed in wildtype mitochondria. Further quantitative analysis of mt-condensate diffusion and nucleation dynamics confirmed that mt-membranes act as a physical barrier limiting the dynamics and growth of mitochondrial condensates. Our findings reveal that mitochondrial membranes define the morphology of the condensates they enclose.

B407/P0395

Structural basis for assembly of a metabolic condensate in purine biosynthesis

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Higher-order assembly of enzymes into condensates and filaments has emerged as an important mode of regulation in intermediary metabolism, particularly at rate-limiting steps and branch points of biochemical pathways. In Saccharomyces cerevisiae, the PRPP amidotransferase (PPAT) Ade4 catalyzes the rate-limiting step in de novo purine biosynthesis and phase separates into dynamic condensates under purine-depleted conditions. Previous work showed that cells unable to form Ade4 condensates had growth defects, but the mechanism and structural basis for Ade4 condensation remained unclear. Here, we purified Ade4 and reconstituted its assembly in vitro, finding that Ade4 polymerizes into filamentous structures. We solved a high-resolution cryo-EM structure that revealed the filament is composed of tetramers interacting through one of two symmetric interfaces, which allows the filament to branch in either direction upon the addition of a new tetramer. Despite the rigid interaction between two tetramers, this assembly mechanism results in filaments lacking long-range order that could act as a scaffold to locally concentrate Ade4 into phase-separated condensates. Based on our structural data, we rationally designed mutations at the filament assembly interface to generate filament-incompetent mutants. Compared to wildtype (WT) Ade4, these non-assembly mutants showed reduced catalytic activity and reduced affinity for the substrate glutamine in vitro. We then generated budding yeast strains expressing WT or mutant Ade4 fused with mNeonGreen (mNG). While WT-Ade4-mNG cells rapidly assembled Ade4 condensates within minutes of washing into purine-depleted media, we did not detect condensate assembly in mutant-Ade4-mNG cells, demonstrating that Ade4 filaments we observe in vitro are the basis for condensate assembly in vivo. We are currently investigating phenotypic differences in the yeast strains and employing fluorescence microscopy-based methods for biophysical characterization of Ade4 condensates.

Condensates - Nuclear organization and gene regulation

B431/P0417
A long noncoding RNA encodes a protein binding logic to assemble multicomponent condensates
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Biomolecular condensates are subcellular assemblies that organize essential functions. Many contain layered internal architectures which are critical for their activity, yet how this layering is controlled is largely unclear. We investigated the rules for assembling multilayered nuclear paraspeckles, which regulate gene expression in many cell types. Paraspeckles segregate the proteins FUS/NONO into a core and TDP-43 into a surrounding shell. This layering is mediated by a long noncoding RNA scaffold called NEAT1. A previously established model proposed that FUS/NONO directly bind specific core-localized NEAT1 segments to assemble layered paraspeckles, but we lack direct biochemical evidence for this model. Using cell-free reconstitution, we unexpectedly found that FUS/NONO instead prefer shell-localized NEAT1 segments. This preference arises from high local densities of cognate RNA binding motifs for FUS/NONO within these shell segments of NEAT1. However, the shell protein TDP-43 also binds abundant motifs in these same segments, creating a binding competition for NEAT1. Critically, TDP-43 is insoluble with FUS/NONO, forcing the proteins to separate like oil and water. Physics-based modeling revealed this combination of competitive binding and mutual insolubility redirects FUS/NONO to suboptimal core NEAT1 segments, mimicking their native layered organization. Our study reveals that NEAT1 encodes a complex binding logic that spatially arranges insoluble proteins. We hypothesize that other RNA scaffolds may similarly encode condensate architecture in a variety of cellular contexts.

B428/P0414
Beyond Cytoplasmic Regulation: Drosophila Keap1 Oxidative Response Factor Accumulates in the Nucleus and Interacts with Lamin
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The Keap1-Nrf2 pathway plays a crucial role in cell protection by mediating transcriptional responses to oxidative and xenobiotic stresses. Misregulations of KEAP1 and NRF2 have been linked to a variety of diseases including cancer. Under basal conditions, Keap1 binds to Nrf2 in the cytoplasm and targets it for proteasomal degradation. Oxidative and xenobiotic stimuli disrupt this interaction, allowing Nrf2 to enter the nucleus and activate detoxifying genes. Our previous studies demonstrated that Drosophila Keap1 (dKeap1) can also localize to the nucleus, bind chromatin and activate transcription, indicating novel biological functions of Keap1 proteins. However, the detailed molecular mechanisms of nuclear dKeap1 remain unclear. Our recent study found that YFP-dKeap1 accumulates in the nucleus and forms condensate-like foci in response to oxidative stress. Both in vitro and in vivo analyses indicate that foci formation is mediated by its C-terminal domain of dKeap1, which contains two intrinsically disordered regions (IDRs). In contrast, the Kelch domain, which mediates dKeap1's interaction with actin filaments, prevents foci formation. FRAP assays revealed that YFP-dKeap1 exhibits reduced mobility within these nuclear foci. We also discovered that nuclear accumulation of dKeap1 leads to mislocalization of B-type lamin (Lamin Dm0) into intranuclear regions, which correlates with the spreading of the heterochromatin marker H3K9me2 into euchromatin. Directly interactions between

dKeap1 and Lamin were confirmed by both in vitro assays and BiFC imaging. Knocking down of dKeap1 partially rescued the lethality induced by Lamin overexpression, supporting a genetic interaction between dKeap1 and Lamin in developmental regulation. These results reveal a novel mechanism whereby dKeap1 regulates nuclear architecture and gene expression. Taken together, we revealed novel mechanisms of dKeap1 in the nucleus, which will provide new insights into the physiological and pathological functions of the Keap1-Nrf2 pathway.

B430/P0416

Mechanisms of FUS Nucleolar Localization under Transcriptional Stress

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The nucleolus is a dynamic nuclear compartment whose organization changes dramatically in response to cellular stress. Fused in sarcoma (FUS) is a nuclear RNA-binding protein involved in DNA damage response and RNA metabolism, both of which are implicated in various neurodegenerative diseases. Previous studies reported that, under DNA damage stress, FUS translocates into the nucleolus and/or assembles at dark nucleolar caps (DNCs), crescent-shaped structures at the periphery of disassembling nucleoli, suggesting a stress-induced response. However, the mechanisms governing this phenomenon and the specific nucleolar functions of FUS remain elusive. Here, we show that among several DNA-damaging agents tested, only camptothecin (CPT), a topoisomerase I inhibitor, induces FUS nucleolar translocation and DNC assembly. This phenotype is recapitulated by inhibition of RNA polymerase II (RNAP2), which is sufficient for nucleolar localization, followed by the subsequent inhibition of RNA polymerase I (RNAP1) which is required for DNC formation. Using RNA fluorescence in situ hybridization (RNA-FISH), immunofluorescence, and RNA immunoprecipitation followed by sequencing (RIP-seq), we demonstrate that these compartmental changes coincide with altered RNA-binding, with FUS showing increased association with nascent RNA transcripts involved in ribosomal biogenesis. Importantly, we reveal that nucleolar translocation is primarily driven by the RNA binding domain (RBD) while the DNC formation depends on the low complexity domain (LCD) of FUS and is conserved across the FUS/EWSR1/TAF15 (FET) protein family. These findings provide new insights into RNA-binding protein dynamics in nucleolar stress response and may have implications for their roles in neurodegeneration.

B427/P0413

Mitochondrial Transcription Sustains Phase Co-Existence of Mitochondrial Nucleoids and RNA Granules

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Gene expression is organized into biomolecular condensates in many contexts from transcription of rRNA by Pol I in the multiphasic nucleolus to dynamic bursts of mRNA transcription by Pol II within liquid-like transcriptional clusters. How phase separation affects the flow of mitochondrial gene expression remains largely unknown. Indeed, mitochondria package their own genome (mt-genome, mtDNA) into nucleoprotein complexes called mt-nucleoids, which serve as the transcription sites of long polycistronic mitochondrial RNA (mtRNA). mtRNA transcripts quickly leave mt-nucleoids and undergo further processing and maturation within neighboring ribonucleoprotein complexes known as mtRNA granules (MRGs). Both structures have been shown to behave as biomolecular condensates. Here, we investigated the phase behavior of mt-nucleoids and

their neighboring MRGs in relation to mitochondrial gene expression. With super-resolution imaging, we examined the endogenous spatial organization of these two mt-condensates relative to steady-state mtRNA in fixed human cells. Using nearest neighbor analysis, we identified wide distributions of localization, ranging from the mt-nucleoids and MRGs being a few microns apart to being fully mixed. To test if the mixing events are associated with transcription, we performed a series of experiments. Labelled nascent transcripts with EU and overexpression of fluorescently tagged mitochondrial RNA polymerase (POLRMT), showed enrichment of these markers within well-mixed mt-nucleoids and MRGs, confirming that mixing of mt-condensates is associated with active transcription. Moreover, with super-resolution live imaging, we observed frequent mixing and de-mixing events between mt-nucleoids and MRGs, resembling kissing behavior characteristic of other transcriptional systems. In support, small molecule inhibition of POLRMT led to reduced mtRNA levels, and correspondingly, to the dissolution of MRGs, indicating that RNA is a driver of MRG stability. Together, our results suggest that RNA produced upon active transcription sustains the phase coexistence between mt-nucleoids and mtRNA granules, and in this way, organizes the flow of mitochondrial gene expression.

B432/P1805

Rainbow Nucleus Charts Dynamic Interactome of Membrane-less Organelles

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Membrane-less organelles (MLOs) are dynamic cellular structures that carry out essential biological functions without lipid membranes. While individual MLOs have been extensively studied, how MLOs interact and coordinate with each other to carry out cellular functions is unknown. In this work, we developed the “Rainbow Nucleus”, a multi-spectral imaging tool to simultaneously visualize the interactions among five nuclear MLOs. First, through multiplexed imaging, we discovered that different MLOs occupy characteristic nuclear spaces and harbor unique cellular biophysical properties. Additionally, we identified two distinctive MLO interaction dynamics, stable and transient interactions, existing among different MLO pairs using time-lapsed spectral imaging. Stable interactions persisted for longer durations with few breakoffs, and occur frequently between functionally coordinated MLOs, such as between Cajal body and nuclear speckle. By contrast, transient interactions formed between functionally unrelated MLOs and exhibited “kiss and run” contact patterns. Furthermore, by plotting the inter-MLO interactome network, we found that the MLO interactions are not random: nuclear speckle serve as the central hub that organizing pairwise interaction networks with Cajal body, histone locus body, and PML nuclear body, while the nucleolus is excluded from the main MLO interaction network. Finally, we demonstrated that active transcription is fundamental for maintaining inter-MLO interactions, as inhibiting transcription completely rewired the MLO interactome network and caused markedly reduced MLO interaction frequency. Collectively, enabled by multi-spectral imaging using “Rainbow Nucleus”, our study uncovers different modality of MLO interaction dynamics, and maps the first comprehensive MLO interactome network of five MLOs under different conditions. These findings establish a foundation for future efforts to reprogram the MLO organization as a therapeutic strategy.

B429/P0415

Targeting PHF19 condensates reduces multiple myeloma tumorigenicity

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Overexpression of PHD finger protein 19 (PHF19) drives multiple myeloma tumorigenesis by dysregulating polycomb repressive complex 2 (PRC2) activity. However, the underlying regulatory mechanism remains unclear. Here, we demonstrate that PHF19 scaffolds PRC2 components into biomolecular condensates via liquid-liquid phase separation (LLPS), a process driven cooperatively by its H3K36me3-binding Tudor domain and intrinsically disordered region (IDR). PHF19 Y56A mutation completely disrupted its binding to H3K36me3, leading decondensation of the PHF19-PRC2 complex and aberrant transcriptional activation of PRC2-repressed genes. Fragment-based screening revealed Rotigotine as a selective PHF19 Tudor inhibitor (with 3 μM affinity; >100-fold selectivity vs non-PCL Tudors), exploiting its distinctive pocket plasticity. Structure-guided optimization yielded DYZ0099, achieving a 10-fold potency enhancement (with 0. 3 μM affinity). DYZ0099 disrupted the interaction between H3K36me3 and PHF19 Tudor using a H3K36me3 FRET sensor and promoted PHF19 decondensation in living cells. Treatment with DYZ0099 significantly suppressed multiple myeloma cell proliferation and tumor growth. Our work suggests that PHF19 recruits PRC2 to gene-activated loci to form polycomb body condensates, compartmentalizing catalytic activation of PcG target genes with high efficiency.

Cytoskeleton and Motility - Actin Cytoskeleton

B045/P0041

A new family of pointed end actin regulators (Aip5/SH3BGRL) conserved between animals and fungi

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The two ends of the actin filament (“barbed end” and “pointed end”) have distinct properties and are differentially regulated in cells. While our mechanistic understanding of actin regulation at barbed ends is reaching maturity, our understanding of pointed end regulation in cells is comparatively limited. For instance, pointed end cappers have been found in the animal kingdom but not the fungal kingdom, calling into question how universal a role pointed end capping plays in controlling cellular actin dynamics. Here, we identify yeast Aip5 as a member of a new family of thioredoxin-related domain pointed end-associated proteins (Aip5/SH3BGRL), conserved between the animal and fungal kingdoms. This discovery was made by studying actin nucleation mechanisms in yeast. We found that two fungal proteins, Aip5 and Bud6, directly interact to form a novel 'composite nucleator', in which the pointed end of the nascent actin seed is bound by the thioredoxin-related domain of Aip5, and the sides by two WH2-related domains in Bud6. The Aip5-Bud6 complex assembles actin seeds with free barbed ends and recruits formins to processively elongate and protect the barbed ends from capping protein. Live imaging reveals that the actin interactions of Aip5 and Bud6 are critical for maintaining proper thickness of actin cable bundles in vivo, which in turn prevents premature cable detachment from the bud neck and defects in secretory traffic. Single molecule imaging shows that after actin nucleation Aip5 remains associated with pointed end of the filament, stabilizing it against depolymerization. Further, live imaging reveals Aip5 puncta streaming inward from cortical polarity sites toward the back of the mother cell via actin cable retrograde flow, which depends on Aip5-actin interactions. From a structural homology search, we found that the thioredoxin-related domain of Aip5 is very similar to the widely-expressed vertebrate protein SH3BGRL. Further, other groups have recently shown that SH3BGRL interacts with the pointed ends of actin filaments. Together, these findings expand the known diversity of actin nucleation mechanisms and reveal a new family of pointed end-associated proteins (Aip5/SH3BGRL) conserved between yeast and metazoans.

B048/P0044

A novel sex-dependent actomyosin-mediated mechanical mechanism of cranial mouse neural tube closure

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During development, cells must create and coordinate forces to fold tissues into three-dimensional shapes. These forces are generated by the cytoskeletal protein, F-actin, coupled to the motor protein, Myosin IIB, linked across cells to create a supracellular network. Such a network on the apical mouse neural plate facilitates its folding from a flat tissue into the neural tube (NT), the precursor to the brain and spinal cord. This network is essential to cranial NT closure (NTC), as chemical or genetic perturbations of actomyosin or its regulators lead to defects in cranial NTC. Curiously, these defects have a higher penetrance in females in mammals. Yet, despite the critical nature of NTC to vertebrate development, we have a limited understanding of the mechanical forces driving NTC in mammals, much less how they fail and cause NT defects. In mice, the larger embryo size, more complex shape, and extended time of development have limited a quantitative analysis of the actomyosin networks that can inform on tissue mechanics. Therefore, we developed a computational workflow to project a 2D apical surface from 3D image stacks. By stitching together projections, we reconstructed the cranial NT apical actomyosin network and related cellular actomyosin distribution to broader tissue morphologies. Using this framework, we found that the cranial mouse NT uses a lateral tension mechanism to fold. Here, cells on the lateral NT, enriched with actomyosin, apically constrict and introduce high tissue tension. In contrast, cells at the bending NT midline, depleted in actomyosin, don't constrict. The result is a regionalized pattern of NT stiffness that resembles a hardcover book. The stiff lateral NT sides (the book covers) are elevated by an external pushing force, while the flexible NT midline (the book spine) bends. We confirmed this model through targeted laser ablation in live mouse embryo NTs, which showed that the initial recoil velocity after ablations are higher in the lateral NT than at the midline. Additionally, while embryos of both sexes exhibit regionalized actomyosin patterns and tension, the extent of apical constriction, actomyosin enrichment, and tension anisotropy vary significantly. Our work has uncovered a novel actomyosin based mechanism of tissue folding employed by the mouse NT. We have also shown the first cell-level and mechanical differences between sexes in the NT, providing a framework to understand the sex-dependent penetrance of NT defects.

B029/P0025

A novel short isoform of Tensin1, eTNS1, is required for enucleation of human erythroblasts via regulation of actin assembly and integrin-mediated adhesion.

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Erythropoiesis is a complex process in the bone marrow in which hematopoietic stem cells (HSCs) undergo terminal differentiation to generate enucleated reticulocytes that mature into functional red blood cells (RBCs). A critical step in this process is enucleation, where erythroblasts expel their nucleus. While actin filament (F-actin) assembly and integrin-mediated adhesion are both known to contribute to enucleation, the precise molecular mechanisms remain unclear. To address this, we analyzed publicly available RNA-seq and proteomics datasets and identified a dramatic upregulation of Tensin1 (TNS1), a focal adhesion protein that links the actin cytoskeleton to integrins, during terminal erythroid differentiation at both the transcriptional and translational levels. In human CD34+ HSCs differentiated into erythroid cells, we identified a novel short isoform of TNS1

(eTNS1), which lacks the N-terminal actin-binding domain due to an internal translation start site within the TNS1 mRNA. To assess its function, we used CRISPR/Cas9 to knock out eTNS1 in CD34+ cells prior to differentiation and examined the effects on terminal erythroid differentiation. Using the Zeiss CellDiscoverer 7 microscope, we observed reduced enucleation frequency in eTNS1-deficient erythroblasts, despite unchanged glycophorin-A levels. Loss of eTNS1 resulted in diminished F-actin intensity and loss of the characteristic perinuclear F-actin foci (enucleosome) required for nuclear extrusion. Interestingly, eTNS1-knockout cultures exhibited elevated levels of both α4 and β1 integrins, as shown by western blotting. These cultures also demonstrated increased adhesion to fibronectin and VCAM-1 compared to controls. Furthermore, Zeiss LSM 880 confocal microscopy revealed that β1 integrin was more abundant and more prominently localized near the plasma membrane in eTNS1-deficient erythroblasts within the heterogeneous knockout population. Together, these results suggest that eTNS1 is a novel regulator of terminal erythroid differentiation, promoting enucleation through promoting F-actin assembly into the enucleosome and modulating erythroblast adhesion through its interaction with integrins.

B049/P0045

A Synthetic Approach to Investigate Rho GTPase Self-Organization During Cell Repair

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Single cell wound repair is conserved among eukaryotes and impaired in certain types of muscular dystrophies. Therefore, better understanding the molecular mechanisms of cell repair will provide insight into a fundamental process and reveal avenues for improving healing. When a cell is wounded, calcium influx triggers activation of Rho GTPases at the plasma membrane (PM) around the damage site to remodel the cortical cytoskeleton and close the wound. We know that laser wounding Xenopus laevis oocytes results in activation of two Rho GTPases, Cdc42 and Rho, by visualizing the process via confocal time-lapse microscopy. Active Cdc42 (Cdc42-T) and active Rho (Rho-T) self-organize into mutually exclusive concentric rings in which Cdc42-T circumscribes Rho-T, which is confined to the wound edge. It is not well understood how Rho-T is confined, however, there is evidence that diminishing Cdc42-T causes the Rho-T zone to spread away from the wound edge. This suggests that Cdc42-T signaling is important for turning off Rho-T in the Cdc42-T zone, thereby confining Rho activity to the wound edge. I hypothesize that the following signaling pathway mediates Rho-T confinement: Cdc42-T binds and activates N-WASP, which activates the Arp2/3 complex, leading to F-actin branching that is stabilized by cortactin, which recruits P190RhoGAP, a protein that inactivates Rho-T. If this pathway is involved in repair, it suggests that Cdc42-T indirectly recruits P190RhoGAP to the Cdc42-T zone to turn off Rho-T there, leading to Rho-T zone confinement.I tested this hypothesis using a synthetic approach that relies on the PKCβ C2 domain, which binds to PM lipids when calcium is elevated. Since wounding triggers a local calcium elevation, C2 gets recruited to the PM around wounds immediately after wounding. We can exploit this by creating C2 fusion constructs that manipulate Cdc42 signaling in a spatiotemporally controlled manner. Using this approach, I found that Cdc42-T is necessary and sufficient to recruit N-WASP, Arp2/3, cortactin, and P190RhoGAP to wounds. Additionally, I found that bypassing Cdc42-T signaling and directly recruiting Arp2/3 is sufficient to recruit cortactin and P190RhoGAP, which deepens our understanding of the mechanism of P190RhoGAP recruitment to the wound. Lastly, I found that P190RhoGAP is important for turning off Rho-T in the Cdc42-T zone, as expressing a catalytically inactive P190RhoGAP mutant increases Rho activity around the wound and leads to Rho activity in the Cdc42-T zone. These data elucidate the Cdc42-T signaling pathway involved in repair and help us better understand how Rho GTPases self-organize to remodel the cortical cytoskeleton after wounding.

B056/P0052
Actin monomers influence the interaction between Xenopus cyclase-associated protein 1 and actin filaments
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Cyclase-associated protein (CAP) is a conserved protein that binds to both actin monomers and filaments and regulates multiple aspects of the actin dynamics including polymerization and depolymerization. CAP has been isolated from multiple species as a stable equimolar complex with actin monomer. However, functional significance of the CAP-actin complex is unknown. We previously purified a native complex of Xenopus cyclase-associated protein 1 (XCAP1) and actin from frog oocytes and demonstrated that they formed a 4:4 complex. Here, we successfully purified actin-free XCAP1 and characterized how actin-free XCAP1 and the XCAP1-actin complex interact with actin filaments using high-speed atomic force microscopy. We found that XCAP1-bound actin monomers influence dwell time and positional preference of XCAP1 on actin filaments. Actin-free XCAP1 bound to actin filaments transiently with a dwell time of ~0.2 seconds. The XCAP1-actin complex also bound to actin filaments transiently but with a 3- to 5-fold longer dwell time than actin-free XCAP1. Actin-free XCAP1 bound to both side and ends of actin filaments with moderate preference to ends. However, the XCAP1-actin complex bound preferentially to the side of actin filaments. These results indicate that binding of actin monomers to XCAP1 affects its binding modes to actin filaments, suggesting that this might be a novel mechanism to regulate the effects of CAP on actin filaments.

B011/P0007
An FGFR-ERK-FAK signaling axis drives actin-dependent cell motility in a densely packed epithelium
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Active cell motility is a defining feature of tissue morphogenesis, driving the sculpting of developing organs into their functional forms. Although cell migration has been extensively characterized on 2D substrates and within 3D extracellular matrices (ECMs), the mechanisms governing motility within densely packed epithelial tissues remain poorly understood. The embryonic mouse salivary gland offers an exceptional model for addressing this gap, as it exhibits robust intra-epithelium cell motility that can be readily visualized and experimentally manipulated. Using live imaging of sparsely LifeAct-GFP–labeled cells, we observed F-actin enrichment at the leading edge of migrating cells. Acute inhibition of actin polymerization with cytochalasin D rapidly abolished cell movements, confirming the essential role of actin dynamics in this process. Unexpectedly, however, inhibition of myosin contractility did not impair cell motility—contrasting with prior findings in cells migrating through 3D collagen matrices. To identify upstream regulators of this motility, we performed a pharmacological screen targeting receptor tyrosine kinases, kinases, and small GTPases commonly implicated in cell migration. We found that inhibition of FGFR, ERK, or FAK signaling, but not EGFR, markedly reduced cell motility. Surprisingly, blocking the small GTPases, Rac or Rho had little effect, suggesting potential redundancy or alternative pathways controlling actin dynamics. Together, our findings identify an FGFR–ERK–FAK signaling axis as a key driver of active cell motility within a densely packed epithelial tissue. This work reveals a mechanistic distinction between motility in cell-dense environments versus ECM-based migration and provides new insight into the principles governing tissue morphogenesis.

B044/P0040

Arp2/3-mediated bidirectional actin assembly by SPIN90 dimers

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Branched actin networks play critical roles in various cellular processes, from cell migration to intracellular transport. At the core of these networks is the Arp2/3 complex. When activated by VCA-containing nucleation-promoting factors, the complex nucleates the formation of a daughter filament from the side of a pre-existing mother filament. In contrast, when activated by WHSH/DIP/SPIN90 family proteins, Arp2/3 nucleates the formation of an unbranched actin filament. However, it remains unclear how the relatively small interface between WHSH/DIP/SPIN90 family proteins and ArpC4 is sufficient for full activation, especially given that branch formation requires extensive contacts with the mother filament. To address this question, we examined the interaction of human SPIN90 with human Arp2/3 complex using biophysical and structural approaches. We found that human SPIN90 is a dimer that can nucleate bidirectional actin filaments. To understand the basis for this, we determined a 3 Å resolution structure of human SPIN90–Arp2/3 complex nucleating actin filaments. Our structure shows that SPIN90 dimerizes through a three-helix bundle and interacts with two Arp2/3 complexes. Each SPIN90 molecule binds both Arp2/3 complexes to promote their activation. Our analysis demonstrates that single-filament nucleation by Arp2/3 is mechanistically more like branch formation than previously appreciated. The dimerization domain in SPIN90 orthologs is conserved in metazoans, suggesting that this mode of bidirectional nucleation is a common strategy to generate antiparallel actin filaments. Our work uncovers a new paradigm for how Arp2/3 is activated to shape actin cytoskeleton architecture.

B015/P0011

Azheimer's gene CD2AP regulates permeability of endothelial cells

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CD2AP is a branched actin regulator that is expressed in microvascular endothelial cells, including brain endothelial cells. Several genome-wide association studies have identified CD2AP as a significant risk factor in developing Alzheimer’s disease, through its vital role in maintaining the blood-brain barrier. In endothelial cells, branched actin networks maintain cell-cell junction morphology, dynamics, and permeability and are required for transcellular trans-endothelial migration (TEM) of immune cells. Branched actin networks require activated Arp2/3 complex and capping protein (CP) to form. Arp2/3 complex is activated by membrane associated nucleation promoting factors, such as WASp and WAVE complex; whereas, CP is activated by membrane-associated proteins containing CP-interacting (CPI) domains, such as CD2AP. We have shown previously that N-WASP is required for junction integrity, whereas WAVE2 is involved in TEM in human microvascular endothelial cells; however, the specific CPI-containing proteins required for junction integrity and TEM in microvascular endothelial cells have not been established. CD2AP has been shown to localize to specific plasma membrane subdomains through interaction of its SH3 domains. We show in microvascular endothelial cells, that CD2AP localizes to cell junctions, where it regulates permeability and cell junction dynamics. We also show that CD2AP at the apical surface of microvascular endothelial cells regulates TEM of immune cell trafficking through trans-cellular migration.

B068/P0064
Big Bad Bundlers: Mechanism of IQGAP1 Bundling
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Actin filament bundles of different dynamics, dimensions, and polarity exist in cells to build and maintain cellular protrusions like filopodia, lamellipodia, invadopodium and stress fibers—each of which are essential for cell functions that power cell movements, intracellular transport of diverse cargoes and maintain structural integrity. IQ-motif containing GTPase-activating protein 1 (IQGAP1), influences the growing ends of actin filaments and organizes polymerized filaments into higher-order structures known as bundles. Classic bundling proteins, such as fascin, α-actinin and villin facilitate filament-filament interactions through actin-binding motifs that engage in side-binding, often through canonical domains including calponin homology (CH), spectrin and β-trefoil folds. IQGAP1 is thought to bundle actin filaments via side binding interactions mediated by the dimerization of its CH domain, however whether the specific residues or the specific mechanism for its bundling ability is not fully understood. Here we identify and characterize several key residues within the CH domain of IQGAP1 that are potential mediators of actin filament bundling. Using pure purified proteins, we performed Total Internal Reflection Fluorescence Microscopy (TIRF) microscopy to assess the influence of IQGAP1 and a mutant IQGAP1 Deficient in Bundling (DiB) on actin filament bundling by measuring intensity of fluorescently labeled actin. To quantitatively assess actin filament bundling, we purified the bundling protein fascin, and established a dose-response curve correlating fascin concentration to the fluorescent intensity of bundles. This allowed us to identify single filaments and distinguish bundles containing defined numbers of filaments (e.g., two, three, or four filaments). Using this benchmark, we compared the bundling capacity of IQGAP1 and IQGAP1(DiB). IQGAP1 formed bundles composed mainly of 2-3 filaments, while the IQGAP1(DiB) primarily formed up to two-filament bundles or failed to bundle filaments altogether. We also observed a shift in bundling orientation, from predominately antiparallel with IQGAP1 to parallel with the mutant. In two-color TIRF assays, fluorescent IQGAP1 localizes to filament sides at bundling sites, whereas the IQGAP1(DiB) had reduced binding and localization to filament sides. Notably, IQGAP1(DiB) appears to associate more frequently with the growing ends of actin filaments, which further suggests impaired side-binding activities. These results imply that specific residues in IQGAP1 are critical for higher-order actin bundling and may have significant implications for cells by disrupting network architecture, mechanics, and functions of actin-rich contractile and protrusive structures.

B016/P0012
Branched Actin Regulates Endosomal Morphology
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Cellular membrane trafficking is critical for cell function: regulating processes such as cargo sorting and molecular transport, degradation, secretion, and response to pathogens. Branched actin localizes to a wide variety of intracellular membrane surfaces, here referred to as endomembranes, where it operates as a cellular force generator and membrane insulator. We hypothesize that both polymerization and depolymerization of branched actin regulate endomembrane trafficking and maturation. To elucidate the role of actin branch assembly and disassembly at endomembranes, we first documented the colocalization of Arp2/3-branched actin with various endolysosomal compartments. For these experiments, we are using a mouse dermal fibroblast cell line that has an endogenously-labeled essential subunit of the Arp2/3 complex, Arpc2, which can

also be conditionally deleted. Using this system, we are also evaluating organelle morphology and distribution in the presence and absence of the Arp2/3 complex. Upon genetic depletion of Arpc2, we observe a reduction in F-actin puncta and enlargement and perinuclear accumulation of early endosomes. These results suggest that F-actin puncta are largely comprised of branched actin and support the involvement of branched actin in the sorting and scission of early endosomes. Additional roles of branched actin in membrane trafficking were evaluated by labeling additional compartments, such as lysosomes and mitochondria. Finally, the results of complete deletion of Arp2/3 will be compared to the phenotypes observed upon depleting Arp2/3 activator WASH or branched actin stabilizer cortactin. Understanding the roles and regulation of actin-dependent membrane trafficking within and between organelles is valuable for understanding homeostatic cell function and disease states with underlying alterations in membrane trafficking, such as tumor metastasis, viral infection, and protein processing disorders.

B050/P0046

CAP1 mediates cancer cell proliferation and migration by regulating Src membrane localization and activity.

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Introduction Cyclase-associated protein 1 (CAP1) is a regulator of actin filament dynamics and a functional receptor of human Resistin. The actin filament dynamics and the membrane localization of Src is essential for cancer proliferation and metastasis. In this study, we identified that CAP1 interacts with Src and regulates its membrane localization and activation. Methods and Results CAP1-/- MDA-MB231 cells were defective both in cell growth and migration even treated with recombinant human epidermal growth factor (rhEGF). In vivo, the tumor growth measured by volume in NOD/SCID mice was significantly smaller with engrafting CAP1-/- MDA-MB231 cells than with CAP1+/+ MDA-MB231 cells ($p<0.0001$). CAP1-/- MDA-MB231 cells were arrested at G1 phase with induction of p53, p21, and p27 expression, and showed low phosphorylation of Src and high phosphorylation of LIMK and cofilin, leading to significantly shorter phalloidin and less lamellipodia than CAP1+/+ MDA-MB231 cells. Binding of CAP1 and Src was proved in the Super-Resolution Structured Illumination Microscopy (SIM) and immunoprecipitation assay. Complex of CAP1 and Src formed in cytoplasm is localized to membrane through geranylation or myristolylation of Src. We generated peptide based on the sequence at SH3BD of CAP1 to inhibit the binding of CAP1 and Src. This peptide effectively inhibited Src phosphorylation upon the rhEGF stimulation. Consistently in vivo, intravenous injection of this peptide of CAP1-SH3BD significantly reduced the tumor volume in NOD/SCID mice engrafted with WT CAP1+/+ MDA-MB231 cells. Conclusions CAP1 plays a key role for cancer cell growth and migration by binding to Src leading to membrane localization and phosphorylation of Src. Peptide based on CAP1-SH3 is an effective blocker of CAP1-Src binding and could be a good therapeutic candidate to treat Src-mediated breast cancer. Keywords ; cyclase-associated protein 1 (CAP1), Src, metastasis, geranylgeranylation

B024/P0020

Cdc42-mediated regulation of spindle positioning in a vertebrate model

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Proper mitotic spindle positioning and orientation of the cell division plane is imperative for tissue morphogenesis, development, and determination of cell fate. When the spindle is mislocalized or the division plane is askew, defects impacting the genetic content of daughter cells, tissue architecture, and development of tumors may arise. In vertebrates, cytoplasmic dynein and its cortically localized regulators, such as Leu/Gly/Asn-repeat containing protein (LGN) anchor the spindle and provide the mechanical force needed to move the spindle into its proper position and orientation. The small GTPase, Cdc42, has been repeatedly implicated as an upstream participant in spindle orientation in a variety of organisms and tissues, based largely on knockdown or knockout approaches. However, the means by which Cdc42 controls spindle positioning is poorly understood, in part because it can impact many processes known to be involved in spindle positioning including cell polarity, cell-cell junction formation, and cell-substrate adhesion formation. Further, almost nothing is known about Cdc42 dynamics during spindle positioning. Our data show that Cdc42 is present in propagating cortical waves during spindle positioning in Xenopus embryos, and that experimental suppression of these Cdc42 waves (via a titratable, optogenetic tool) at the cortex results in misoriented spindles and inappropriately positioned cleavage planes. Quenching of Cdc42 waves in Xenopus zygotes - a developmental stage which lacks cell-cell interactions and in which spindle orientation is insensitive to manipulation of polarity proteins – also results in abnormal spindle morphologies. Taken together, these data indicate that Cdc42 waves are required for normal spindle positioning, and the role of Cdc42 is apparently independent of cell-cell junctions and likely, polarity proteins.

B008/P0004

CdcD, the great protein extractor, as a regulator of cortical dynamics through proteasomal degradation

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The cytoskeleton is a highly dynamic network that responds actively to mechanical stress stimuli through disassembly and accumulation of contractility kits (CKs) components at the cortex of the cell. The cell cortex is a composite material of the cortical cytoskeleton and the plasma membrane. CKs serve as a means to package the CK components while they are in the cytoplasm, but they can then unpackage when they diffuse into the cortex. CKs are composed of non-muscle myosin II (NMII), cortexillin I (cortI), IQGAP1, IQGAP2, and many other unexpected proteins. The mechanoresponsive activity of these CK proteins is dictated by the IQGAP associated with the corresponding CK where IQGAP1 serves as a negative regulator while IQGAP2 serves as a positive regulator of the CK's mechanoresponsiveness. Through our labs' previous proteomic and genetic selection studies, we discovered that CKs include many other proteins that are associated with many cellular processes that are not traditionally associated with the cytoskeleton. One CK interactor is cdcD, which is a protein that is involved with the recognition system for polyubiquitylation and the presentation of ubiquitinated proteins to the 26S proteasome for degradation. We are in process of creating Dictyostelium strains with cdcD knocked down, knocked out, and over-expressed. We are also using small molecule inhibitors of cdcD to help decipher the role of cdcD. We are exploring the relationship of cdcD and CKs through live and fixed microscopy, protein degradation assays, differential expression western analysis, and more. We have already identified an interaction between the actin network and cdcD, which appears to help maintain a pool of cdcD in the nucleus.

B037/P0033

CIP4-marked asymmetric branched actin networks at apical clathrin-coated pits contribute to microvillar

biogenesis

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Microvilli are actin-based membrane protrusions that maximize the absorptive capacity of epithelial cells, yet the mechanisms initiating their assembly remain incompletely understood. Emerging evidence points towards apical clathrin-coated endocytic pits (CCPs) as key organizational hubs for microvillar growth, but only a subset of CCPs is permissive for this process. This raises the question of what distinguishes the pits that are competent to support protrusion growth. Here, we combined live-cell and super-resolution imaging to define the CCPs associated with microvillar initiation. We find that permissive CCPs are characterized by dense, Arp2/3-generated branched actin networks stabilized by cortactin. Importantly, these networks are asymmetrically offset from the CCPs, with nascent microvilli emerging from the actin-enriched side of the pit. The F-BAR protein CIP4 has previously been shown to mediate this asymmetry in non-epithelial systems by undergoing phase separation at CCPs and promoting localized Arp2/3 activation to generate unilaterally polarized actin networks. We find that CIP4 is enriched in intestinal crypts where microvilli are most actively growing and localizes to CCPs before protrusion growth. Strikingly, nearly all observed microvillar initiation events were marked by CIP4. Our findings suggest that epithelial cells could repurpose this CIP4-driven mechanism of asymmetric actin assembly to bias CCPs toward protrusion growth. Together, these results support a model in which CIP4-mediated actin asymmetry distinguishes the subset of CCPs competent to nucleate microvilli. This work builds on a mechanistic link between endocytosis and microvillar biogenesis to offer a broader evolutionary and systems-level perspective on how cells use limited molecular resources to build and maintain complex structures.

B006/P0002

CLaSSiNet: Integrative Analysis of Nanoscale Cytoskeletal Networks

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Quantitative analysis of nanoscale network architectures remains a central challenge in super-resolution fluorescence microscopy, where cytoskeletal and membrane-associated structures often appear as sparse localization points rather than continuous networks. Here, we introduce Classifier of Super-resolution Structural Networks (CLaSSiNet), a modular computational pipeline that integrates connectivity, one-dimensional (1D) periodicity, and two-dimensional (2D) regularity classifiers to segment and classify network-like structures in single-molecule localization microscopy (SMLM) images. This framework reliably distinguishes four organizational states, including 1D periodic networks, 2D polygonal ordered networks, disordered networks, and non-network regions, while remaining robust to experimental noise and compatible with different labeling strategies, including those that label filament junctions (“nodes”) or filament segments (“links”). We applied CLaSSiNet to the actin-spectrin-based membrane-associated periodic skeleton (MPS) as a model system, uncovering distinct organizational states distributed across subcellular zones: ordered 1D and 2D architectures were enriched at cell edges and junctions, whereas disordered or absent networks predominated in cell bodies. MPS organization was further influenced by actin stress fibers, with 1D periodic strips preferentially formed in their proximity and aligned perpendicular to fiber orientation, suggesting mechanical coordination between spectrin scaffolds and actin bundles. Across cell types, neurons favored extended 1D periodic structures, while epithelial and fibroblast cells displayed denser 2D ordered lattices, highlighting cell-type–specific adaptations of spectrin organization. Together, these findings establish CLaSSiNet as a versatile tool for mapping nanoscale network architectures and reveal how the spectrin-based MPS adapts to local mechanical environments. More broadly, this approach provides a foundation for dissecting general design principles of cytoskeletal, junctional, and organellar networks in health and disease.

B043/P0039

Cofilin controls the sorting of tropomyosin to distinct actin networks

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Proper cell physiology requires the co-assembly of multiple actin cytoskeletal networks that are tailored for specific functions. To distinguish between these networks, cells decorate them with distinct sets of actin-binding proteins (ABPs). This selective decoration is thought to arise from competitive and cooperative binding of ABPs to filament sides or growing barbed ends, but the role of actin disassembly factors is less well understood. Here, we used inducible CRISPR interference and quantitative live-cell imaging to test how disassembly factors control the size and ABP composition of different networks. We show that knockdown of cofilin (Cof1), a potent and highly conserved disassembly factor, leads to the formation of large, disordered actin networks decorated by inappropriate ABPs. Specifically, tropomyosin (Tpm1) is inappropriately recruited to branched networks due to defects in Cof1-mediated disassembly. Contrary to prevailing models of ABP sorting, these networks are co-decorated by Tpm1 and fimbrin (Sac6), and their assembly is independent of formin activity. Moreover, the formation of these disorganized, improperly decorated networks severely disrupts myosin-based intracellular transport. Finally, although dual knockdown of Tpm1 and Cof1 partially rescues polarized cable assembly, these cables fail to support transport. Together, our findings demonstrate a critical role for actin disassembly factors in maintaining cytoskeletal structure and function by regulating ABP sorting across distinct networks.

B065/P0061

Compartment-Specific Functions of Transgelins and Calponins in Organizing Actomyosin Bundles for Cell Adhesion, Migration and Morphogenesis

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Contractile actomyosin arrays are essential for normal physiology and development of multicellular organisms. The most prominent actomyosin structures in non-muscle cells are stress fibers, which contribute to various vital cellular processes, such as motility, adhesion, morphogenesis, and mechanosensing. Among the large spectrum of stress fiber components, the specific functions of Transgelins (TAGLNs), have remained elusive. Transgelins are small proteins (~22 kDa), which are abundant in smooth muscle and non-muscle cells and are widely associated to several malignant cancers and asthma. Structurally, Transgelins resemble Calponins (CNNs) by having an N-terminal Calponin homology (CH) domain. However, instead of three ‘Calponin-like’ (CLIK) repeats present in Calponins, Transgelins contain much smaller C-terminal region composed of only one CLIK repeat. By depleting Transgelins and Calponins individually, and in combination with each other, we reveal that Transgelins and Calponins display partially redundant roles in organizing actin filaments into contractile stress fibers. However, they operate at different cellular compartments, and their depletions led to opposite effects in cell migration and leading-edge dynamics. Whereas Calponins contribute to maintenance of stress fibers towards the cell center, Transgelins localize more prominently to cell periphery and are important for initial assembly of actomyosin bundles at cell edges. Consequently, simultaneous depletion of the Transgelin-1 and Transgelin-2 resulted in disorganized actin stress fibers at peripheral regions of the cell, increased dynamics of lamellipodial protrusions and elevated retrograde flow of filamentous actin. These cytoskeletal

defects are manifested as increased cell migration and defects in cell spreading, morphogenesis, and contractility. Together, these results identify Transgelins as important factors orchestrating the assembly of contractile actomyosin bundles at cell edges.

B063/P0059

Competition and synergy of Arp2/3 and formins in nucleating actin waves

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Dynamical patterns in the form of oscillations and waves in the cortex of immune cells are manifestations of excitable dynamics within the actin cytoskeletal network. One notable representation of this, is the emergence of pattern formation from the well-coordinated interplay and feedback mechanisms exhibited within non-linear dynamical systems. Such phenomenon of cortical excitability is dependent on the dynamic remodeling of a functional actin cytoskeleton in response to the activation of innate immune responses in immune mast cells. This is made possible by a repertoire of proteins capable of orchestrating actin assembly and turnover within the cortex in response to stimuli. Central to this are two different mechanisms of actin polymerization, namely the branched actin network nucleatedby the Arp2/3 complex, in contrast to the nucleation of linear actin filaments by the formins. However, the direct visualization of these actin nucleators in living cells of the same physiological conditions is often challenging, leading to our limited understanding of the crosstalk and feedback mechanisms of intertwined molecular networks within the dynamic actin cytoskeleton. Here, we employ the cortical actin waves exhibited by immune mast cells as a model, to understand how different actin nucleators collaborate within the same system to drive robust waves of actin assembly and disassembly. Our findings first reveal that the recruitment of formins, mDia3 and FMNL1, precedes the assembly of the Arp2/3 complex in Cdc42-mediated actin waves. We exploited experimental conditions that could abolish cortical actin oscillations without eliminating waves of membrane proteins, to understand the relative contribution of actin and membrane in their recruitment. Quantitative analysis of our results show that the recruitment of Arp2/3 complex as clusters of puncta in actin wave is dependent on the actin nucleating functions of formin FMNL1. Interestingly, the recruitment of FMNL1 and mDia3 was found to be enhanced in response to acute Arp2/3 inhibition by CK666. Our findings on the competition between the Arp2/3 complex and FMNL1 challenges the prevailing model of actin nucleators competing for a limited pool of G-actin monomers. Collectively, our works highlights the multifaceted interactions and the cooperative or competitive interplay between the nucleation mechanisms of formins and Arp2/3 complex, in the intricate and dynamic control of actin cytoskeletal network.

B074/P0070

Cyclase-associated protein 1 (CAP1) acts as a repressor of neuronal gene expression during mammalian brain development

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In vitro studies of the past few years unraveled multiple molecular activities of cyclase-associated protein (CAP) including acceleration of both dissociation of actin subunits from filamentous actin (F-actin) and nucleotide exchange on actin monomers, thereby establishing CAP as a key regulator of F-actin assembly and disassembly. However, the cellular and physiological functions of the two mammalian family members, CAP1 and CAP2, are

only poorly understood. The brain is unique in that it is the only tissue in which both CAPs are expressed at substantial levels, and recent studies linked their dysregulation to human brain disorders, emphasizing the need to elucidate their neuronal functions. By exploiting primary neurons from gene targeted mice, we previously reported mutual functional dependence of CAP1 and the key actin regulator cofilin1 in F-actin dynamics of axonal growth cones and excitatory synapses relevant for neuron connectivity and synaptic plasticity, respectively, and we demonstrated redundant functions for CAP1 and CAP2 in actin cytoskeleton differentiation during synapse maturation. Recently, we found that CAP1, but not CAP2 or cofilin1, controls neuronal gene expression mediated by serum response factor (SRF) and its co-activator myocardin-related transcription factor (MRTF), a transcriptional program essential for mammalian brain development. Mechanistically, CAP1 represses the MRTF-SRF pathway by promoting cytosolic MTRF relocation in an actin-dependent manner. The relevance of CAP1 for repressing this transcriptional program is evident from mass spectrometry and deep RNA sequencing, which proved specific upregulation of MRTF-SRF-mediated transcription in brains from embryonic CAP1-KO mice and led to the identification of previously unknown MRTF-SRF target genes. Our unpublished data revealed signaling cascades upstream of CAP1 in regulating the MRTF-SRF pathway in neurons, and they implicated dysregulation of CAP1-MRTF-SRF signaling in a human brain developmental disorder. Together, we identified CAP1 as crucial transcriptional repressor relevant for mammalian brain development.

B047/P0043

DAAM1 formin and VASP polymerize functionally distinct actin filament populations at focal adhesions

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Actin filaments form a large array of biochemically and functionally distinct structures to drive fundamental cellular processes such as endocytosis, migration and morphogenesis in eukaryotic cells. Our recent findings revealed that even specific cellular structures, such as focal adhesions, harbor at least three distinct actin filament layers, which are specified by α-actinin crosslinked filaments, as well as by actin filaments decorated with tropomyosin (Tpm) isoforms Tpm1.6 and Tpm3.2. How such functionally distinct actin filaments are assembled in cells remains an important outstanding question. Formins and Ena/VASP family proteins are key proteins that promote the elongation of linear actin filaments and have been proposed to contribute to actin filament assembly also in focal adhesions. However, the functional distinction between these two types of proteins in polymerizing linear actin filament arrays have remained elusive. Here, we reveal that depletion of DAAM1/DAAM2 formins from U2-OS cells resulted in focal adhesion defects similar to those reported earlier for Tpm3.1/Tpm3.2 knockout cells, and that Tpm3.2 protein levels were significantly reduced in DAAM1/DAAM2 double-knockout cells. On the other hand, siRNA-mediated silencing of the Ena/VASP family proteins resulted in reduced levels of α-actinin in focal adhesions and in the loss of focal adhesion -associated dorsal stress fibers. Importantly, artificial re-localization of DAAM1 to the outer mitochondrial membrane resulted in the preferential assembly of Tpm3.2-actin filaments on mitochondria, whereas mitochondrial-targeted Ena/VASP proteins (through their focal adhesion resident activator, zyxin) induced the specific assembly of α-actinin cross-linked actin filaments, but not tropomyosin-actin filaments, on mitochondria. Together, these results provide evidence that DAAM1/DAAM2 formins are responsible for the preferential assembly of Tpm3.2-decorated actin filaments, whereas the Ena/VASP-family proteins polymerize α-actinin crosslinked filaments at focal adhesions. More broadly, our study highlights specific roles for formins and Ena/VASP family proteins in assembling biochemically distinct linear actin filament arrays in cells.

B052/P0048

Defining the RoLE Pathway in Linking Rho Signaling to Ezrin-Mediated Cytoskeletal Remodeling

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Epithelial apical morphology depends on precise spatial control of RhoA signaling and its effectors. We define an integrated “RoLE” (Rho -» LOK/SLK -» Ezrin) pathway in which RhoA activates the kinases LOK and SLK to activate ezrin at the apical aspect of cells. Active ezrin then recruits the RhoA-GAP ARHGAP18 to locally tune RhoA. This creates a negative feedback loop that preserves apical architecture and polarity. Using CRISPR/Cas9 knockouts in human epithelial cell lines, RhoA-GTP pulldowns, atomic force microscopy, SORA spinning disk-live confocal imaging, and STORM/PALM super-resolution microscopy, we combine three advances into a single framework. First, we show that LOK and SLK are the main ERM (Ezrin, Radixin, Moesin)-activating kinases in human cells. Their loss abolishes the activating ezrin-T567 phosphorylation, removes microvilli, and converts junctional actin into apical actomyosin bundles with increased cell stiffness. Rescue with constitutively active ezrin restores apical organization, which demonstrates that ERM activation is the dominant output of LOK and SLK and that active ezrin restrains RhoA through feedback. Second, we identify how this feedback is built. Activation opens the ezrin FERM domain, creating a binding site for ARHGAP18. ARHGAP18 is recruited to microvilli and requires both ezrin binding and GAP activity to normalize microvilli density and lifetimes. ARHGAP18 prevents excess apical myosin-2 assembly while maintaining enough RhoA to sustain LOK and SLK activity and ezrin phosphorylation. This sharpens the boundary between microvilli and adjacent cytoskeletal structures of the cortex and junctions. Third, we connect the RoLE module to tissue-level polarity through the Hippo pathway. ARHGAP18 forms a complex with YAP (Yes-Associated Protein) and interacts with Merlin/NF2 to coordinate RhoA dynamics with YAP nuclear localization. Loss of ARHGAP18 causes abnormal RhoA signaling and mis-localized YAP. The result is translational changes in cytoskeletal and proliferation driving genes and major reorganization of cortical actin which we define using a combination of RNAseq and super-resolution microscopy. Together these findings define a novel Rho-Dependent signaling cascade that balances cellular force generation with membrane-actin linkage to build and maintain the apical domain. Similar effector-recruited GAP motifs may operate across Rho family pathways to partition actin structures in space and time. This mechanism provides new entry points to modulate epithelial barrier integrity and polarity.

B017/P0013

Differential regulation of actin paralogs as a conserved mechanism of stress response

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Actin is essential to eukaryotic life, and understanding how it has evolved and diversified is crucial to understanding the evolution and diversification of eukaryotes themselves. While some organisms encode a single actin gene, others possess multiple, sometimes highly divergent actin paralogs. Since the actin cytoskeleton is dynamic, functional differences in the actin paralogs can influence cellular processes, including cytokinesis and cell motility, and/or response to environmental changes. While the roles and regulation of a few intraspecies actin paralogs in mammals and plants have been studied, divergent actin paralogs in most eukaryotic species remain unexplored. Additionally, though many eukaryotes can sense and respond to actin cytoskeletal perturbations, it is unclear if and how actin paralogs are involved in response to perturbations. The amoeba Naegleria gruberi has 30 actin paralogs with pairwise sequence identities ranging from 30-100%,

making it an excellent system to study the functional consequences of actin paralog diversity. Despite this sequence variation, the N. gruberi actin paralogs phylogenetically group with actins rather than actin-related proteins (Arps). Under normal conditions, N. gruberi amoebae cells primarily express conventional actin, which is ~85% identical to Rabbit muscle actin, but the function and regulation of the divergent paralogs are unknown. To test if N. gruberi actin paralogs are differentially regulated in response to actin disruption, we performed RNA sequencing upon actin depolymerization by the drug Latrunculin B (LatB). We identified eight divergent actin paralogs that were upregulated in response to LatB, while other conventional actins or stress response genes (e.g., HSPs) were not. The gene with the highest fold change was a divergent actin that was 92% identical to the conventional actin. A similar mechanism exists in Chlamydomonas reinhardtii, which upregulates a divergent actin gene (~65% identical to its conventional actin) in response to LatB treatment. These findings suggest a common mechanism of actin stress response through the differential regulation of actin paralogs. Testing whether this occurs across eukaryotes will reveal if actin paralog diversification enhances resilience to chemical and genetic perturbations of the actin cytoskeleton. To evaluate this, I will extend these experiments to other actin-targeting drugs and species, including Dictyostelium discoideum. Identifying conserved strategies of actin paralog regulation will uncover new principles of actin cytoskeleton adaptability and could reveal novel cellular strategies used for responding to actin disruption.

B055/P0051

Direct actin monomer delivery is a WASP-specific requirement for human Arp2/3 complex activation

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WASP family proteins activate Arp2/3 complex to nucleate branched actin filaments in diverse cellular processes. To activate, WASP proteins recruit actin monomers to Arp2/3 complex through their conserved WASP homology 2 domains, but how these recruited monomers contribute to activation of Arp2/3 complex has been unclear. Previous studies suggested potential species-specific differences: work on human Arp2/3 complex implied that direct actin monomer delivery by WASP is required to assemble the filament nucleus, while studies on budding yeast Arp2/3 complex showed that direct monomer recruitment is required specifically for WASP-mediated activation of the complex and is not a fundamental requirement for activation. Here we show that mutations that shift the actin-related subunits (Arp2 and Arp3) in human Arp2/3 complex toward a filament-like conformation enable nucleation without WASP. This demonstrates that direct monomer tethering by WASP is not a fundamental requirement for formation of the nucleus, but instead a specific requirement for WASP-mediated activation—consistent with findings in yeast Arp2/3 complex. Using kinetic models of actin polymerization, we provide evidence that while the Arp2-Arp3 filament-like dimer is insufficient for nucleation by human Arp2/3 complex, actin monomers diffuse and bind to the complex to create the filament nucleus in the absence of tethering. We show that this diffusion-based mechanism can stimulate nucleation by mammalian Arp2/3 complex at rates similar to tethered actin monomer delivery. These results provide important mechanistic insights into Arp2/3 complex activation with or without monomer delivery and demonstrate that fundamental aspects of activation are conserved across fungal and mammalian Arp2/3 complexes.

B070/P0066

Dynamic reorganization of the actin cytoskeleton during FcγR-mediated phagocytosis and the roles of Myo1e/f in regulating phagocytic cup architecture

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Phagocytosis is a dynamic process that relies on the precise spatiotemporal remodeling of the actin cytoskeleton to assemble phagocytic cups, engulf phagocytic targets, and drive cup closure. During formation of phagocytic cups, class-1 myosins, Myo1e and Myo1f, localize to actin-rich adhesions that produce local forces on phagocytic targets. To investigate myosin 1 functions in phagocytosis, we generated a stable Myo1e/f double knock-out (dKO) RAW264.7 macrophage cell line using CRISPR-Cas9 gene editing. Myosin dKO cells exhibited a significant reduction in the uptake of IgG-coated beads compared to the wild-type (WT) cells. Using high spatiotemporal resolution Lattice Light-Sheet Microscopy (LLSM), confocal imaging, and deformable phagocytic targets, we investigated the dynamics of phagocytosis to dissect changes in actin organization during this process and the effects of Myo1e/f dKO on actin organization and force production. Live-cell and fixed-cell imaging revealed that in the WT cells, the early stages of target engulfment are characterized by the formation of actin-rich puncta at the base and rim of the cup and the extension of lamellipodia ahead of the advancing cup rim. As the cup matures to approximately 40-60% closure, we observe a coalescence of individual actin puncta into continuous actin rings. Both actin puncta and rings produce forces that deform and constrict phagocytic targets. Lamellipodia proceed along the upper hemisphere of the particle, eventually bending inward as the phagosome completes its closure. As the phagosome envelopes the particle, the actin ring is characterized by both an increase in actin intensity and constrictive forces. In contrast, dKO cells form fewer actin puncta and are prone to assembling a continuous actin ring prematurely. Engulfment attempts in which contractile rings form before ~40% cup closure appear to reduce the efficiency of phagocytosis. These findings suggest that Myo1e/f are critical for the early stages of phagocytosis, specifically for the establishment of actin-rich adhesions and the coordination of protrusive forces required for efficient particle engulfment.

B046/P0042

EPS8 dampens the growth dynamics and prolongs the lifetime of actin-based protrusions

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Actin-based protrusions such as filopodia, microvilli, and stereocilia support a variety of cellular functions, ranging from nutrient absorption to mechanosensation. Although their structure and composition are well characterized, mechanisms that promote their growth and stability remain unclear. The actin filaments that support these structures are bundled in a parallel and unipolar ‘barbed-end out’ manner, creating a core that is mechanically rigid enough to support membrane protrusion. At their distal ends, individual filopodia, microvilli, and stereocilia exhibit an electron dense tip complex, which could contain factors that gate actin assembly at filament barbed ends. One such factor is Epidermal Growth Factor Receptor Kinase Substrate 8 (EPS8), which localizes robustly to the distal tips of all three protrusions. Loss of EPS8 in cell culture and mouse models results in shorter microvilli and stereocilia, suggesting roles in elongation. Previous work from our group also showed that in epithelial cells, EPS8 puncta appear at the apical membrane prior to the growth of a new microvillus, remain at the distal tip for the lifetime of the structure, and then disappear right before the microvillus collapses, suggesting a role in protrusion survival on the cell surface. To more directly examine the role of EPS8 in protrusion elongation and survival, we used CRISPR/CAS9 to tag the endogenous copy of EPS8 in HeLa cells with mStayGold. As expected, endogenous EPS8 puncta are found at the distal tips of growing filopodia, which were highly dynamic. Using a transfection approach, we increased levels of mStayGold-EPS8 and examined the corresponding impact on filopodial growth and dynamics. Increasing the EPS8 concentration enhanced the amount of EPS8 per tip punctum, significantly reduced rates of filopodia elongation, and increased the lifetime of individual protrusions. Cytochalasin D (CytoD), which caps barbed ends, displaced

endogenous EPS8 from filopodial tips, and cells expressing higher levels of EPS8 were protected from this effect. Together, these data suggest that EPS8 binds directly to filament barbed ends where it likely plays a role as a 'leaky capper', allowing slow incorporation of new monomers, and offering long-term protection against actin bundle disassembly. In epithelial systems, such activity could promote the accumulation of large numbers of protrusions during differentiation.

B031/P0027

FHOD-1 and profilin collaborate to promote muscle growth and protect sarcomeres from contractile damage

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Formins are a class of proteins that nucleate, elongate, and often bundle actin filaments. Nucleation is done through a conserved formin-homology 2 (FH2) domain which binds to actin. Profilin is an actin monomer-binding protein which can interact with the formin-homology 1 domain (FH1) of formins to enhance actin filament elongation. Previously, our lab has found that the formin FHOD-1 in Caenorhabditis elegans, is involved in promoting body wall muscle growth, with fhod-1(-) null mutants having smaller muscle cells. In addition, fhod-1(-) mutants have irregular dense bodies, the sarcomere Z-line homologs of body wall muscle. We hypothesize that these phenotypes are a result of a loss of FHOD-1-mediated actin filament assembly. In this study, we demonstrate that introducing a point mutation predicted to eliminate actin nucleation activity in FHOD-1 results in identical reduced body wall muscle size and irregular dense bodies to that of a fhod-1(-) mutant. Additionally, we found a fhod-1 mutation that eliminates the FH1 domain results in similar irregular dense bodies and smaller body wall muscles seen in profilin pfn-2 pfn-3 mutations. Genetic analysis suggests profilin and FHOD-1 operate in separate and overlapping pathways. Further analysis of fhod-1 and profilin mutants revealed dense bodies of both were damaged by prolonged contraction and were less stable when compared to wild type animals. Overall, our data suggest that FHOD-1-mediated actin filament assembly is necessary for stable dense bodies, and that profilin contributes likely through aiding FHOD-1 actin assembly as well as through an additional separate pathway.

B036/P0032

FHOD3-related formins promote myofibril stability in vertebrate and invertebrate non-cardiac muscle.

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Formin HOmology Domain-2-containing (FHOD) proteins are a subfamily of actin-organizing formins that are particularly important for striated muscle function in many animals. Mammalian FHOD3 is critical for normal myofirbil formation in cardiac muscle, as is Drosophila FHOD in intermediate flight muscles, but FHOD proteins were thought to be unimportant for myofibril organization in vertebrate skeletal muscle. We have isolated novel loss-of-function mutants for two FHOD3 paralog genes in zebrafish. The skeletal muscle of 5 days post-fertilization (dpf) fish embryos lacking functional FHOD3a but not FHOD3b exhibit widespread fragmentation of myofibrils and reduced F-actin content. Interestingly, this defect appears to be in myofibril maintenance rather than formation, as myofibrils in skeletal muscle in fhod3a mutants appears normal at 2 dpf. We observe similar defects in sarcomere stability in Caenorhabditis elegans nematodes lacking their sole FHOD protein, FHOD-1. In FHOD-1-deficient worms, sarcomere Z-lines in body wall muscle are more dynamic, and are prone to severe deformation after muscle contraction, and dislodgement of sarcomeric actin filaments. An analysis of

mutants for worm profilin genes or FHOD-1 residues important for actin assembly suggest FHOD-1 promotes dense body stability through a canonical formin/profilin mechanism. Our results suggest a range of striated muscle types depend on FHOD proteins for normal myofibril stability, although the severity of FHOD-deficiency varies by muscle type.

B010/P0006

Formin N-terminal dimerization can impact F-actin assembly via polymer mechanics of the FH1 domain

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Many proteins contain intrinsically disordered regions (IDRs) that lack 3-dimensional structure. IDR behavior is poorly understood, leading to challenges for biochemical and computational analysis of IDR-containing proteins. Formins are a diverse set of homodimers containing an IDR — the FH1 domain — that facilitates polymerization of the cytoskeletal protein actin by increasing the local concentration of actin monomers. A standard model of formin-based actin polymerization involves a capture-and-deliver process. There is evidence that formin FH1 domains are dimerized on both ends, but much research uses formin constructs lacking the N-terminal dimerization site. Here, we ask: What happens when N-terminal dimerization is added to the standard model of formin-mediated actin assembly? We extend the standard kinetic model of FH1-mediated actin polymerization by incorporating a coarse-grain polymer model of FH1 dynamics, modeling the FH1 domain as a freely-jointed chain. We find that N-terminal dimerization can impact polymerization rates in four distinct ways: deceleration due to decreasing binding site accessibility, acceleration due to increased binding site accessibility, deceleration due to dampening of the increased local concentration effect, and acceleration due to enhancement of the increased local concentration effect. Which effect dominates depends on kinetic parameters and formin properties such as FH1 size and binding site location. Additionally, we demonstrate that our model can be fit to experimental data and used to make predictions for the effects of N-terminal dimerization on a variety of formin family members.

B018/P0014

Functional analysis of the cor-1 coronin gene in Caenorhabditis elegans striated muscle

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Actin filaments within a sarcomere, a basic contractile unit in striated muscles, are arranged in a highly ordered manner, and their proper assembly and maintenance are vital for contractile function. Although many actin-binding proteins are known to play various roles in the regulation of sarcomeric actin, the mechanism of sarcomere assembly and maintenance is still not well understood. The body wall muscle of the nematode Caenorhabditis elegans is striated muscle and has been used as a model to study the mechanism of sarcomere assembly. C. elegans has two coronin genes: cor-1 encoding typical coronin proteins and pod-1 encoding an ortholog of coronin 7 containing two tandem coronin sequences. Previous transcriptome studies have demonstrated that the cor-1 is predominantly expressed in the body wall muscle, whereas pod-1 is widely expressed in non-muscle tissues. Therefore, we hypothesize that cor-1 has an important function in the body wall muscle. Coronin is a conserved actin-binding protein that is typically involved in promoting actin filament dynamics in non-muscle cells, but its role in muscle cells is unknown. We tagged the endogenous cor-1 gene with green fluorescent protein (GFP) and found that GFP-COR-1 was expressed in the body wall muscle and

colocalized with actin filaments within sarcomeres, suggesting a role of cor-1 in the regulation of sarcomeric actin filaments. A homozygous deletion of cor-1 was lethal and caused developmental arrest at an early larval stage. Effects of the cor-1 deletion on the assembly of sarcomeric actin filaments are currently under investigation. The cor-1 gene produces at least four coronin isoforms by alternative splicing. As an initial step to understand biochemical properties of the coronin isoforms, we have produced bacterially expressed 4 isoforms (COR-1A, COR-1B, COR-1C, COR-1D) and demonstrated that each directly binds to actin filaments in vitro. In conclusion, these results strongly suggest that coronin is a novel regulator of sarcomeric actin in striated muscle.

B041/P0037

Functional characterization of novel SPTBN1 neurodevelopmental syndrome genetic variants

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βII-spectrin, encoded by SPTBN1, is a critical scaffolding protein within the neuronal cytoskeleton, where it interacts with F-actin to form submembrane lattices that support membrane integrity and organize transmembrane proteins. Heterozygous SPTBN1 variants have recently been linked to a neurodevelopmental syndrome characterized by developmental delay, intellectual disability, frequent seizures, muscle tone abnormalities, and behavioral manifestations such as ASD and ADHD. Many of these disease-associated variants cluster within the highly conserved calponin homology domain (CHD), which mediates F-actin binding and is notably intolerant to genetic variation in the general population. As the cohort of affected individuals expands, it is imperative to quickly assess the potential pathogenicity of newly reported variants and to identify their unique and convergent pathophysiological mechanisms. To investigate how CHD missense variants impact βII-spectrin function, I employed a cellular screening approach using tagged constructs in HEK293 cells. These analyses revealed variant-specific effects on protein localization and cytoskeletal organization, suggesting distinct pathogenic mechanisms within the same domain. Notably, a subset of variants led to the formation of prominent cytosolic aggregates, suggesting potential disruptions in protein folding or turnover. Other variants showed altered membrane localization and changes in cellular architecture, often accompanied with mislocalization of known βII-spectrin binding partners, indicating broader cytoskeletal disruption. I will discuss our efforts to define the structural basis of these phenotypes, including alterations in F-actin binding and to reverse protein aggregation using engineered disaggregases. Together, our findings uncover diverse cellular consequences of SPTBN1 missense variants and provide new insights into the molecular underpinnings of SPTBN1 syndrome.

B067/P0063

Functions of the Membrane-Binding Domain of CARMIL

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Actin polymerization is promoted at membranes by various membrane-associated proteins. One such membrane protein, CARMIL, contains an actin capping protein (CP)-binding domain that both targets CP to the membrane and activates CP allosterically by promoting dissociation of CP's stoichiometric inhibitor V-1. The CP-binding region (CBR) of CARMILs includes two motifs that contact CP, the CP-Interaction (CPI) motif and the CARMIL-Specific Interacting (CSI) motif, followed by a basic hydrophobic membrane-binding (BH or MB) domain. The MB domain is necessary for the function of CARMIL in cells, and it is sufficient on its own for

targeting to the plasma membrane. Relatively little is known about the mechanism and significance of the relationship of the MB domain to CP's actin capping activity and to the promotion of Arp2/3-mediated actin assembly. Using biochemical reconstitution, we find that the MB domain of CARMIL binds to lipid-coated beads, bringing the CPI and CSI motifs to the bead surface, and this activates CP and promotes Arp2/3-mediated actin assembly. When the MB domain of CARMIL is present as part of the CBR, we find partial competition between lipid-binding and CP-binding, consistent with the MB domain dynamically dissociating from the lipid once CP binds. We also report that the dynamic association with lipid via the MB domain, acting in tandem with CPI and CSI domains, further promotes the activation of CP. Thus, the CARMIL MB domain has multiple functions serving to promote actin assembly – targeting CARMIL to the plasma membrane, allowing active CP to leave the membrane, and promoting CP activation by dissociation from V-1.

B066/P0062

Host-cell invasion by Apicomplexans driven by cortical actin contraction

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Apicomplexans are a family of parasites that includes Plasmodium falciparum, which causes malaria, and Toxoplasma gondii, which causes toxoplasmosis. Their invasion into host cells involves the formation of a tight junction through which the parasite squeezes. During this process, an F-actin ring at the junction and a network of posteriorly localized F-actin are experimentally observed [1]. For T. gondii, the push-and-pull model hypothesizes that the contraction of the posterior actin network contributes to pushing the nucleus through the junction [1]. We quantify the forces that a cortical actin cytoskeleton of Apicomplexans can exert during host-cell invasion using computer simulations. We use Cytosim [2], a cytoskeleton simulation suite that simulates F-actin as semiflexible polymers using overdamped Langevin dynamics and incorporates interactions with myosin F and crosslinkers. A turnover of the F-actin filaments has been shown to lead to a pulsatile 2D network for planar networks [3]. We measure contractile forces for the actin ring and a 2D network on a sphere, and characterize the active tension and network dynamics. Increased filament turnover increases the network stability but reduces the active tension. Comparison of our in-silico predicted forces with values reported for Apicomplexan invasion hints that a cortical actin cytoskeleton with myosin F motors [4] can play a significant role in the invasion process. In combination with our recent findings on parasite deformability [5], this work provides a physical framework to explain how cortical cytoskeletal tension can drive host-cell invasion by Apicomplexan parasites. [1] M. Del Rosario et al., EMBO Rep 20, e48896 (2019). [2] F. Nedelec et al., New J. Phys. 9, 427 (2007). [3] J. M. Belmonte et al., Mol. Sys. Bio. 13, 941 (2017). [4] J. A. Kellermeier et al., MBoC 35, ar57 (2024). [5] N. Baruah et al., Biophys. J. 124, 740-752 (2025).

B005/P0001

INF2 regulates IP3R-mediated ER calcium release

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Timely and regulated actin filament turnover regulates various cellular functions including inter-organelle interaction and homeostasis. INF2 (Inverted formin 2) is a member of the formin family of actin assembly factors that promote both actin nucleation and filament elongation. In cells, INF2 exists in two distinct isomeric forms, INF2-caax and INF2-noncaax. INF2-caax localizes to Endoplasmic Reticulum (ER) while INF2-noncaax

exists in the cytosol. Gain-of-function dominant mutation of INF2 has been associated with FSGS and CMTD. Previous reports have shown that INF2-caax generated actin filaments on the ER acutely induces ER-mitochondrional contacts (ERMC) and facilitates ER to mitochondrial calcium transfer that promotes mitochondrial division (PMID: 29142021). While most of the cellular roles of INF2 have been attributed to mitochondrial dynamics including its ability to promote ER to mitochondrial calcium transfer, its role on ER physiology and dynamics have remained elusive. In the present study we provide evidence that INF2 regulates agonist-induced ER calcium release through IP3-receptors. Acute knockdown of total INF2 in HeLa cells results in reduced histamine-induced ER calcium release measured both through cytosolic and ER calcium probes. Interestingly INF2 depleted HeLa cells also show an increased frequency of cytosolic calcium oscillations upon histamine stimulation. SERCA inhibition showed comparable ER calcium in both WT and INF2-KO cells. Rescue experiments carried out on INF2-KO cells showed that both the ER-bound and cytosolic INF2 isoforms could significantly rescue histamine-induced ER calcium release suggesting ER-residency was not required for this function. Conversely, over-expression of INF2 leads to an increase in agonist-induced ER calcium release. Further we examined whether INF2 activity affects all the IP3R isoforms equally. To this end we show that carbachol stimulation in INF2 depleted (KD and KO) HEK-IP3R2 (R1/R3 DKO) and IP3R3 (R/R2 DKO) cells result in significantly reduced ER calcium release compared to the respective control cells. Like HeLa cells, both INF2 isoforms could rescue this phenotype in the KO cells. Investigating further we show that INF2 depletion does not affect the amount of IP3Rs but decreases the frequency of IP3R clusters in cells (HeLa and HEK) which are the business units of the molecule in effecting ER-calcium release. These results indicate that INF2 play a significant role in shaping IP3R-mediated ER calcium dynamics. Future experiments will unravel the specific mechanisms through which INF2 regulate agonist-mediated ER calcium release.

B038/P0034

Investigating a noncanonical role for cofilin in the rootlets of epithelial microvilli

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Transporting epithelial cells, such as intestinal enterocytes, form apically localized, membrane wrapped actin-based protrusions called microvilli, which are critical for nutrient absorption and pathogen protection. Microvilli are supported by a core bundle of parallel actin filaments (F-actin) oriented with barbed ends toward the distal tip. The basal end of the actin core forms a “rootlet” that is anchored in the terminal web (TW), a subapical meshwork of actin, intermediate filaments, and crosslinking proteins. In cell culture models, core bundles exhibit treadmilling driven in part by NM2C which localizes to the rootlets. In efforts to further define the molecular composition of the core bundle, we identified cofilin as a novel component of the microvillar rootlet. While cofilin is primarily thought of as an actin filament severing protein, previous studies also suggested that cofilin could saturate and stabilize F-actin bundles in response to increased concentration or cellular stress. To probe cofilin’s function in the rootlet, we used live cell imaging of a human intestinal cell culture model, CRISPR/Cas9-mediated endogenous protein tagging, drug treatments, overexpression of fluorescent proteins, and native tissue immunolabeling. In mouse intestinal tissue and an intestinal cell culture model, cofilin localized to microvillar rootlets along a segment basal to NM2C. Interestingly, in opposition to cofilin’s canonical severing role, cofilin activation increased cofilin localization to the TW and elongated microvillar rootlets. Additionally, overexpression of cofilin produced cofilin-saturated core bundles and rootlet elongation. Inhibition of NM2C increased cofilin localization in the TW and appeared to stabilize rootlets, further suggesting that cofilin is not acting solely as a severing protein, and that NM2C and cofilin competitively bind F-actin. Collectively, these data suggest an unconventional role for cofilin in regulating cytoskeletal architecture – likely switching between severing and stabilizing in a filament/bundle dependent manner, respectively, as well as spatially regulating actin-binding proteins.

B042/P0038

LifeAct-mRFP Affects the Actin-Rich Podocyte In Vivo Causing Injury.

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Podocytes, essential for kidney filtration, are actin-rich cells with a unique non-motile, octopus-like morphology. Podocyte injury leads to loss of the filtration barrier and cytoskeletal remodeling. LifeAct, a 17–amino acid peptide that labels filamentous actin (F-actin), enables visualization of actin dynamics in vivo. Transgenic LifeAct–monomeric red fluorescent protein (mRFP) mice, driven by the ubiquitous CAG promoter, progressively develop proteinuria with age, making them a useful model for studying podocyte injury. Podocyte injury in LifeAct-mRFP mice was assessed by urinary albumin-to-creatinine ratio, histological staining (H&E, PAS), ultrastructural analysis (TEM), and cytoskeletal imaging (immunofluorescence, confocal, and expansion super-resolution microscopy). Sarcomere-like structures (SLSs) were detected by alternating synaptopodin and myosin IIA staining. To accelerate injury, LifeAct-mRFP mice were crossed with Col4a3^{-}/$^{-}$ mice, a model of progressive glomerular disease. LifeAct-mRFP mice exhibited spontaneous podocyte injury characterized by progressive proteinuria, podocyte detachment, and loss. While myosin IIA levels remained unchanged, SLS organization was disrupted. Interestingly, podocytes exhibit ectopic expression of Tropomyosin 1 (Tpm1) and Tpm 4 isoforms that precedes the injury. LifeAct-mRFP; Col4a3^{-}/$^{-}$ mice developed a more severe phenotype than Col4a3^{-}/$^{-}$ controls. Whole-genome sequencing confirmed intronic integration of the LifeAct transgene without gene disruption. Although LifeAct-mRFP mice lack an overt phenotype at birth, they develop podocyte injury over time, underscoring the importance of finely tuned actin regulation in podocytes. These findings suggest that LifeAct disrupts cytoskeletal balance, leading to injury. Similar to effects observed in other systems (e.g., muscle in zebrafish and C. elegans), LifeAct expression in podocytes may impair SLS formation, weakening adhesion to the glomerular basement membrane and ultimately resulting in podocyte loss.

B058/P0054

LIM Domain Proteins link molecular and global tension by recognizing strained actin in adhesions

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Mechanotransduction is fundamental to cell signaling and depends on force-sensitive adhesion proteins. How these proteins differentiate and integrate their responses to tension remains an open question. We show mechanosensitive LIM domain proteins like zyxin detect global adhesion tension by recognizing strained actin within these structures. In sharp contrast, vinculin localization and intramolecular tension remain unchanged, despite vinculin's well-documented role in mechanotransduction. This reveals a stark disconnect between molecular tension and global tension in adhesions. We further show tension-dependent localization is specific to LIM domain proteins that recognize strained actin and extends to LIM proteins at cell-cell junctions, suggesting a common mechanotransduction mechanism. Finally, we show zyxin's tension-dependent adhesion localization stabilizes actin and recruits VASP to promote stress fiber polymerization, identical to its role in stress fiber repair. Our findings reveal a fundamental role for LIM domain protein force-sensing in adhesions and highlight the non-linear connection between molecular and global tension.

B039/P0035
LIMA1 stabilizes actin and supports intestinal tuft cell rootlet structure
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Tuft cells represent less than 1% of the intestinal epithelium yet play an important role in parasite sensing and barrier maintenance. Tuft cells are characterized by large apical protrusions supported by core actin bundles spanning over 7 mm in length and terminate above the nucleus. Despite the identification of actin-bundling proteins in the tuft cell, how these proteins support core bundle structure remains unknown. Actin cross-linking protein LIMA1 is uniquely localized to tuft cell rootlets. Previous studies point to actin-stabilizing functions of LIMA1 making it a strong candidate to drive tuft core actin bundle formation. This study leveraged cell culture and animal models to determine the impact of LIMA1 on actin architecture in cells and identify its contribution to the tuft in a LIMA1 knockout mouse model. We found that LIMA1 localizes to rootlets in both filopodia and microvilli, and expression of LIMA1 is sufficient to drive the formation of exaggerated filopodia that display a bifurcated morphology. Additionally, LIMA1 stabilizes actin in filopodia and protects against actin depolymerization. Remarkably, the loss of LIMA1 in mice compromised the integrity of tuft cell rootlets providing evidence that LIMA1 supports the integrity of rootlet actin architecture in tuft cells.

B028/P0024
Loss of actin-binding protein Profilin-1 leverages genomic instability to trigger anti-tumor immune response in breast cancer
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The role of actin cytoskeletal remodeling is being increasingly recognized in the context of immune surveillance and modulation of tumor progression. Reduced expression of actin-binding protein Profilin-1 (Pfn1) in tumor cells, a key regulator of actin cytoskeletal dynamics, is a feature of human breast cancer. The goal of this study was to investigate whether loss of Pfn1 in tumor cells has any impact on immune surveillance in breast cancer. We utilized an inducible CRISPR/Cas9 knockout (KO) model to first demonstrate that triggering Pfn1 depletion selectively in tumor cells promotes a highly immunogenic tumor microenvironment marked by a striking increase in tumor infiltration by $CD8^+$ T cells, leading to a robust tumor regression in an immunocompetent mouse model of breast cancer. Notably, tumor regression is not observed in immunodeficient hosts, indicating that it is an immune-dependent effect. Mechanistically, Pfn1-deficient breast cancer cells exhibit features of genomic instability (polyploidy, micronuclei, and DNA damage) and accumulation of cytosolic DNA, leading to the activation of nucleic acid–sensing cGAS-STING pathway and the downstream signaling consequences including type-I interferon response and transcriptional upregulation of major T-cell recruiting chemokines. Based on these findings, we conclude that the loss of Pfn1 in tumor cells leverages genomic instability to foster a T-cell supportive tumor microenvironment and drive an anti-tumor immune response in breast cancer. Therefore, our studies highlight potential opportunities in targeting Pfn1-driven pathways for immunotherapeutic gain for breast cancer in the future.

B023/P0019

Mechanism of APC-mediated branched actin assembly at the microtubule tip

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The branched actin filament network at the cell leading edge generates driving force for membrane protrusion during cell migration and extension of neuronal processes. On the other hand, microtubules largely control cell navigation, but the underlying mechanism remains to be fully understood. Previous study in the lab showed that branched actin can assemble at the microtubule tips in growth cones of cultured hippocampal neurons and that Adenomatous Polyposis Coli (APC) is essential for this process. These observations suggest that microtubules control cell navigation through local formation of branched actin network. Here, we explore the molecular mechanism of this APC-triggered assembly of branched actin networks. We found that knocking-down APC in Ref52 fibroblasts suppressed formation of cell processes and rendered cell morphology. Overexpression of APC constructs containing the N-terminal region of APC promoted process formation, suggesting a key role of the N-terminus of APC in process extension. On the other hand, APC-depleted Ref52 fibroblasts lost the fusiform shape and rounded up. Interestingly, N-APC can rescue APC-depleted Ref52 cells, whereas C-APC failed to rescue the phenotype, suggesting that a functional domain in N-APC is responsible for inducing processes, presumably through local actin polymerization. Given that the N-terminal Armadillo repeat domain of APC can activate the Cdc42 GEF Asef1, we hypothesize that the Asef1 - Cdc42 - N-WASP - Arp2/3 pathway downstream of APC nucleates branched actin filaments at the microtubule tip for growth cone navigation. In support of this hypothesis, we found that depletion of Asef1, Cdc42 or N-WASP, or pharmacological inhibition of Cdc42 or N-WASP, in rat hippocampal neurons closely mimicked the phenotype of the APC depletion characterized by nearly complete elimination of branched but not linear actin filaments in growth cones, as revealed by platinum replica electron microscopy. In conclusion, current results revealed that APC is responsible to induce branched actin formation via its armadillo repeats at the N-terminus of APC. This domain activates Asef1 and downstream effectors Cdc42 and N-WASP. In hippocampal neurons, APC and its downstream effectors are responsible for the formation of branched actin network at the tip of microtubule tips. This crosstalk between microtubule and actin in growth cones is critical for neurite outgrowth in early neuronal development.

B027/P0023

Mechanism of processive pointed-end elongation of actin filaments by Leiomodin2

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The actin cytoskeleton drives essential processes like cell migration and muscle contraction. While barbed-end polymerization is well-established, pointed-end elongation was long considered impossible in vivo. We demonstrate that Leiomodin 2 (Lmod2), localized to thin-filament pointed ends in striated muscle, functions as a processive actin polymerase. Single-molecule and single-filament imaging reveal that Lmod2 stably associates with pointed ends in vitro, enabling elongation even under profilin-rich, cytosol-like conditions that otherwise promote depolymerization of free pointed ends. Strikingly, Lmod2's activity persists in the presence of tropomyosin, confirming its physiological role. In mouse hearts, Lmod2 governs thin-filament length regulation, dependent on its WH2 domain for processivity and polymerization regulation. A human dilated cardiomyopathy-associated mutation ablates Lmod2's polymerase activity, directly linking this mechanism to

cardiac dysfunction. Our work establishes pointed-end polymerization as a novel cellular mechanism of actin assembly in eukaryotes and provides a mechanistic basis for Lmod2-related muscle diseases.

B032/P0028

Membrane composition controls cortical F-actin assembly and self-organized active Rho and F-actin patterning

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The animal cell cortex, comprised of the plasma membrane and the adjacent array of filamentous actin (F-actin), is critical for cell structure and function. Cortical F-actin generates forces that push and pull the membrane to drive changes in cell shape and support essential cell functions, like cell division. The cell cortex is dynamically patterned with waves of F-actin assembly in a phenomenon called cortical excitability. In developing Xenopus laevis embryos, cortical excitability is closely coupled to cytokinesis, the physical division of the cytoplasm at the end of the cell cycle. Cortical patterning is driven by an excitable circuit where the small GTPase Rho, in its active state, recruits its own activator, the RhoGEF Ect2, and promotes the downstream assembly of F-actin, which recruits a Rho inactivator, the RhoGAP RGA3/4. While the protein regulators of cortical patterning during cytokinesis have been identified, the role of the plasma membrane in regulating cortical F-actin assembly and dynamics is not known. Importantly, lipids within the plasma membrane partition into and out of the division plane, suggesting that membrane composition is important for successful cytokinesis. Here, we investigated how candidate phospholipids regulate cortical assembly and dynamics using in vitro reconstitution of an artificial cell cortex. The artificial cell cortex combines cytoplasmic extract from X. laevis eggs and supported lipid bilayers (SLBs) to reconstitute traveling waves and standing oscillations of active Rho activity and F-actin assembly with spatiotemporal dynamics that match cortical patterning characterized in vivo. Using this system, we found that cortical dynamics can be reconstituted using cytoplasmic extract that lacks intracellular organelles. We used this cytoplasm to investigate defined membrane compositions and understand the role of key lipid groups. We found that SLBs containing phosphatidylethanolamine (PE) dramatically alter traveling waves in the artificial cortex, supporting multiple rounds of high-amplitude, high-velocity wave fronts. We also found that PE SLBs support higher amplitude standing oscillations, suggesting that PE may alter the kinetics of Rho activation in vitro. We also find that SLBs containing phosphatidylinositol 4,5-bisphosphate (PIP2) support the initial assembly of cortical F-actin, and that metabolism of phosphatidylinositol (PI) is required for establishing the F-actin cortex. These findings indicate that membrane composition regulates the establishment of the cortical F-actin network and the dynamics of Rho activity and F-actin assembly in cortical patterning. This work also suggests that lipid partitioning during cytokinesis is important for regulating the activity of key cytokinetic proteins.

B064/P0060

Metabolic and mechanosensitive activation of AMPK leads to phosphorylation of specific downstream substrates

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AMP-activated protein kinase (AMPK) is a master regulator of cellular energy homeostasis. AMPK is activated by deprived cellular energy status, and it restores energy homeostasis by phosphorylating downstream substrates to promote catabolic pathways while inhibiting energy-consuming processes. Interestingly, recent studies provided evidence that AMPK can be also activated by changes in cell's mechanical microenvironment,

and that AMPK additionally phosphorylates cytoskeletal targets to induce formation of actomyosin stress fibers when cells are grown on a stiff substratum. To elucidate if the metabolic and mechanosensitive roles of AMPK are interwind or operate in parallel with each other, we compared the effects of changing matrix stiffness (0.5 kPa vs. 64 kPa) and metabolic status (cells grown on full media vs. acute glucose starvation) on the activation of AMPK and its selected downstream substrates: ACC and HSL, which are regulators of fatty acid synthesis and lipolysis, respectively, and regulatory myosin light-chain (MLC) and vasodilator-stimulated phosphoprotein (VASP), which control the actin cytoskeleton. Interestingly, we revealed that metabolic and mechanosensitive activations of AMPK selectively affected the phosphorylation of AMPK's downstream substrates. Increasing matrix stiffness elevated only MLC and VASP phosphorylation, whereas glucose starvation induced selective phosphorylation of ACC and HSL. We also provide evidence that mechanosensitive activation of AMPK is controlled by CaMKK2 kinase, whereas metabolic activation requires the LKB1 kinase. Moreover, we revealed that on stiff matrix a large fraction of phosphorylated AMPK co-localized with CaMKK2 in focal adhesions, but that phospho-AMPK was rapidly displaced from cell-matrix adhesions following glucose starvation. Our results provide evidence that, depending on the mechanism of activation, AMPK localizes to a specific cellular compartment to phosphorylate different downstream substrates.

B040/P0036

MRTF-dependent actin dynamics are essential for proliferation of adherent cells but dispensable for KRAS-transformed ones

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The MRTFs (Myocardin-Related Transcription Factors A/B) are SRF (Serum Response Factor) co-factors regulated by G-actin levels. They control the expression of cytoskeletal-related genes (actins, myosin), adhesion molecules (vinculin, integrins) and ECM components (collagen, fibronectin) and are essential for adhesion and motility. Our lab has recently shown that MRTF-A/B inactivation in MEFs and epithelial cells causes a proliferation arrest with features of both senescence (elevated SA-β-gal activity, SASP phenotype) and quiescence (reversibility upon MRTF-A re-expression, upregulation of p27, similarities with serum-starvation). In this study we aim to better understand the mechanisms linking MRTF to proliferation and the contexts where these are needed for cell cycle progression. First, we tested whether CKI upregulation might be the cause of the cell cycle arrest. Neither p27 inactivation, nor triple depletion of all CIP/KIP family members (p21, p27 and p57) rescued the proliferation defect upon tamoxifen addition. Thus, CDK-inhibitor upregulation must be a consequence, rather than the cause of the proliferation arrest. Since cytoskeletal integrity and contractility are crucial for proliferation of adherent cells, we hypothesised that the cytoskeletal defects in MRTF-null MEFs underlie the proliferation arrest. MRTF-A/B depletion leads to cytoskeletal defects relating to actin filament organisation, adhesion and migration. Interestingly, MRTF-dKO MEFs exerted less force to their substrate, implying that the cells are less contractile. Direct disruption of actomyosin dynamics in WT cells with ROCK (H1152) or Myosin (Blebbistatin) inhibitors phenocopied the MRTF-dKO, suggesting that the proliferation arrest upon MRTF inactivation reflects at least at part a contractility defect. Classical experiments have shown that oncogenic transformation is accompanied by relaxation of physical/adhesive requirements for proliferation, known as adhesion-independent growth. We selected transformed MEF clones expressing KRASG12V and investigated whether MRTF activity is necessary for proliferation of transformed cells. Our data show that in this oncogenic context, MRTF-A/B inactivation does not impact proliferation, despite persistent cytoskeletal gene downregulation. We are currently testing the possible signalling pathways contributing to this effect, as well as the types of adhesions involved in the KRAS-transformed cells. These results demonstrate that MRTF-dependent actin dynamics are essential for proliferation in normal adherent cells, but

dispensable in transformed ones. Future work will investigate the molecular basis underlying this differential requirement for MRTF-controlled cytoskeletal dynamics during proliferation.

B013/P0009
New roles for yeast Bud6 in reinforcing actin cable thickness and remodeling septins
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Budding yeast grow in a polarized manner by assembling a dynamic network of polarized actin cables, used by myosin V to transport secretory vesicles to the bud tip. Each cable is a bundle consisting of shorter, overlapping actin filaments polymerized by formins. The cables polymerized by formin Bnr1 (anchored to septins at the bud neck) are 'thicker' than cables polymerized by Bni1 in the bud, i.e., consist of more filaments in cross-section. It has remained a mystery how cable thickness is controlled in vivo. Here we describe a new role in this process for the polarity factor Bud6. Our previous studies focused on the C-terminal half of Bud6 (C-Bud6), and demonstrated that it binds to formins and G-actin, serving as an actin nucleation-promoting factor (NPF) in vitro and in vivo (Moseley et al., 2004; Graziano et al., 2011, 2013; Garabedian et al., 2018). Until now, the N-terminal half of Bud6 (N-Bud6) has never been implicated in actin regulation, but is required and sufficient for localization of Bud6 to polarity sites (bud tip and bud neck) (Jin and Amberg, 2000). We purified full-length Bud6 (FL-Bud6) and compared its activities to those of C-Bud6 and N-Bud6 in TIRF microscopy assays. Unexpectedly, we found that FL-Bud6 and N-Bud6 (but not C-Bud6) bundle actin filaments. Single molecule imaging further revealed that FL-Bud6, but not N-Bud6, is able recruit labeled formin molecules to the sides of actin bundles and stimulate formin-mediated actin polymerization to reinforce actin bundle thickness. Our dissection of N-Bud6 also revealed that a minimal fragment (1-145) is both required and sufficient for Bud6 localization to polarity sites. Moreover, single molecule TIRF imaging revealed that FL-Bud6 directly binds to septins, and that Bud6 works collaboratively the septin-binding protein Bni5 to remodel septin filaments. Together, these observations expand our understanding of the roles Bud6 plays in regulating the actin and septin cytoskeletons.

B059/P0055
Nuclear F-actin safeguards the mammalian oocyte genome
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Oocyte genome integrity is critical for healthy embryogenesis and reproductive success. The presence of unrepaired DNA lesion in oocytes generally blocks meiosis I completion and formation of fertilizable eggs. Fertilization of eggs with damaged DNA risks transmission of incorrect genetic information to the newly formed embryos. This can completely block embryonic development or be passed onto the offspring. Despite the clinical relevance of genome damage in gametes, DNA repair mechanisms that operate in oocytes and why they fail at advanced reproductive ages remain unclear. Here, we show that nuclear F-actin structures are required to maintain genomic integrity in mammalian oocytes. By combining advanced time-resolved protein degradation tools with quantitative microscopy assays, we demonstrate physiological DNA lesions occurring in the absence of nuclear F-actin are poorly recognized by the oocyte DNA repair machinery. Our 3D actin filament volume analyses revealed that experimentally inducing DNA double strand breaks dramatically increases nuclear F-actin assembly and induces extensive filament bundling. The cytoskeleton rearrangements occurring following DNA damage are accompanied by high chromatin mobility motorized by nuclear Myosin-

5b. Collectively our data define a new nuclear F-actin and myosin based mechanism of genome integrity facilitating efficient DNA damage recognition and repair in mammalian oocytes.

B060/P0056
Pannexin-1 is Recruited at Actin-Rich Bacterially-Generated Structures
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Pathogenic bacteria employ distinct strategies to control their host cells and often exploit the host cell's cytoskeleton for their purposes. One such strategy is employed by enteropathogenic Escherichia coli (EPEC), an extracellular pathogen that generates actin-rich pedestals within the host cell, but beneath the adherent bacteria. In contrast, Listeria monocytogenes, an invasive pathogen, hijacks host actin for its invasion, intracellular motility and spreading from cell-to-cell. Pannexin-1, a pore-forming transmembrane protein is primarily recognized for its role in forming channels and releasing ATP. Despite its role in channel formation, Pannexin-1 has also been shown co-precipitate with actin. Actin further contributes to Pannexin-1's stability at the plasma membrane and regulates channel activity. To examine Pannexin-1's role during EPEC and L. monocytogenes infections, immunostaining was performed. We found that Pannexin-1 localized to EPEC actin-rich pedestals and L. monocytogenes actin-rich clouds, but not to comet tails, sites of initial invasion or cell-to-cell spreading. These findings show that Pannexin-1 is a component of a subset of actin-rich structures generated by adherent and invasive bacteria and highlights a new aspect of its involvement in bacterial pathogenesis.

B034/P0030
Perturbing cortical excitability dynamics by altering membrane composition and membrane curvature in an artificial cortex
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Cortical excitability, a phenomenon in which the cell cortex is dynamically patterned with waves of F-actin assembly, has been described in a variety of animal model systems, including embryos of mammals, flies, frogs, echinoderms, and a variety of cultured cells. Cortical excitability is driven by dynamically patterned Rho family GTPases that, when activated, promote waves of cortical F-actin assembly that are present during cell cytokinesis. While the cortical F-actin network is closely linked with the plasma membrane, it is not known if membrane composition regulates dynamic cytokinetic patterning. Lipids partition within the plasma membrane during cytokinesis, and phospholipids play a key regulatory role in other excitable systems, suggesting a role for membrane-dependent regulation of cytokinesis. Here we use an artificial reconstituted cell cortex comprised of Xenopus egg extract and supported lipid bilayers (SLBs) to show that membrane composition regulates self-organized cortical patterning. We find that manipulating the levels of candidate lipids, including sphingomyelin and cholesterol, results in both increased amplitude of F-actin oscillations for cholesterol containing SLBs and increased amplitude of active Rho oscillations for sphingomyelin containing SLBs. We also find the temporal shift of active Rho and F-actin peaks decreased for sphingomyelin containing SLBs. Our data shows a quantitative change in the dynamics of standing oscillatory patterns of active Rho and F-actin for both sphingomyelin and cholesterol containing SLBs. Lastly, we find the rate of Rho activation and F-actin assembly on supported lipid bilayers slows on both sphingomyelin and cholesterol containing SLBs. These results show that changes to SLB composition with sphingomyelin and cholesterol alter the spatial and

temporal oscillations of active Rho and F-actin and as well as the rate of cortical Rho activation and F-actin assembly. We also investigated the role of membrane curvature in regulating cortical F-actin assembly, as cytokinesis represents a stage of extreme membrane curvature. Using glass beads to induce positive curvature, we find that curved SLBs increase membrane fluidity as assayed by photobleaching. We also find that F-actin and active Rho associate with curved membranes, and F-actin polymerizes perpendicular to curved membrane, in contrast to parallel assembly that occurs along planar SLBs. Our findings demonstrate that membrane composition and curvature directly regulate membrane fluidity, the assembly of cortical F-actin, as well as emergent active Rho and F-actin patterning.

B053/P0049

PES-8 Regulates Calcium Signaling and Cytoskeletal Organization in the C. elegans Spermatheca

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Proper regulation of contraction and relaxation in biological tubes is essential for organisms' function. In C. elegans, the spermatheca, a fertilization site composed of smooth muscle-like cells, undergoes repeated cycles of stretching and contraction as oocytes pass through. We previously showed that calcium (Ca^{2+}) oscillations driving spermathecal contraction are regulated by G-protein signaling to Protein Kinase A. However, the triggers of Ca^{2+} oscillations and the integration of mechanical and biochemical signals at the tissue level remain unclear. The Gαi/o subunit GOA-1 modulates spermathecal contractility by counteracting GSA-1/Gαs. To identify GOA-1 activators and effectors, we fused GOA-1 to the TurboID biotin ligase for in vivo proximity labeling. Mass spectrometry identified PES-8, a previously uncharacterized protein, as a candidate interactor. PES-8 is expressed in the spermatheca and localizes to the plasma membrane, the spermathecal-uterine valve, and the uterus. PES-8 contains a predicted extracellular zona pellucida-like (ZP) domain and an unstructured cytoplasmic tail, suggesting roles in both extracellular interactions and cytoplasmic signaling. Functional studies reveal that PES-8 is essential for spermathecal function and activity. Loss of PES-8 disrupts basal actomyosin fiber alignment, apical junctions, and focal adhesion protein organization, leading to disorganization of FLN-1/filamin, NMY-1/myosin, DEB-1/vinculin, and AJM-1/apical junction molecule. PES-8 depletion results in defective Ca^{2+} signaling during oocyte transit, and failed oocyte passage through the spermatheca. Together, these findings identify PES-8 as a key regulator of cytoskeletal organization and calcium-mediated contractility in the C. elegans spermatheca.

B054/P0050

Probing the molecular and biophysical features mediating bacterial-induced host cell fusion

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Across tissues, programmed cell-cell fusion confers novel function to multinucleate cells. Intracellular microbes co-opt these cell-cell fusion pathways to spread between host cells, offering a unique view into mammalian cell biology. Burkholderia thailandensis and other bacteria from the pseudomallei group of Burkholderia species, a group of Gram-negative, facultative intracellular microbes, spread by promoting host cell-cell fusion to form multinucleate giant cells. Burkholderia uses actin-based motility to generate force at the plasma membrane, extending bacteria-containing plasma-membrane protrusions into the neighboring cell where fusion takes place. However, it remains unclear what molecular and mechanical mechanisms mediate cell-cell fusion in physiological and pathogenic contexts. Here, we test whether and how the force balance between

the bacterium and the host cell at the cell-cell boundary impacts the efficiency of fusion and spread. We also seek to identify the molecules necessary and sufficient to trigger fusion following B. thailandensis infection. Live-cell imaging and comparative analysis of bacteria expressing actin-based motility effectors from different Burkholderia species reveal differences in protrusion extension dynamics that vary with actin-tail architecture, suggesting that bacterial force-generating capacity impacts cell-cell fusion dynamics. Ongoing experiments aim to probe the role of host cell cortical mechanics by measuring live protrusion extension dynamics and infection spread in host cells treated with blebbistatin to inhibit myosin and relax cortical tension. In parallel, we are testing putative bacterial fusogens VgrG5 and TagD5, characterizing their localization and activity and testing their role in a minimal synthetic cell fusion system. This work will provide a framework for how mammalian cell mechanics and molecular co-factors both promote and constrain cell-cell fusion and demonstrate how Burkholderia species hijack this underlying biology to mediate infections.

B035/P0031

Rapid filament turnover maintains cortical connectivity while allowing for actin cortex deformation and flow during polarization and cytokinesis

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Cells harness actomyosin contractility to drive rapid cellular deformations and intracellular flows during cell polarization and division. Cells control actin filament assembly and disassembly rates to sustain contractile network architectures while allowing for network deformation and remodeling. Understanding how a dynamic balance of assembly and disassembly tunes network architecture and contractility in vivo remains a challenge. Here, we combine quantitative measurements and manipulations of filament assembly and disassembly with live imaging of network contractility dynamics in the C. elegans zygote to identify co-dependencies between assembly rates, disassembly rates, and large-scale deformations of the cortical actin network. Combining TIRF microscopy with particle tracking of endogenously-tagged formin (CYK-1::GFP) dimers in the one-cell C. elegans embryo, we measure formin-mediated assembly rates of ~1.2 µm/sec, and find that strong depletion of profilin (PFN-1) reduces assembly rates 3-fold. Tracking single-molecule actin::GFP at the cortex, we measure turnover of cortical actin filaments and find that actin::GFP displays two rates of dissociation: an initial slow rate of disassembly (~10 sec) likely due to the dissociation of phosphate from ADP-actin and a later, rapid rate (~5 sec) likely due to cofilin-mediated severing and disassembly. Direct observations of endogenously-tagged Plastin/PLST-1::GFP, which decorates growing filament bundles, reveals that formin-assembled bundles undergo rapid disassembly from their pointed ends. Depleting zygotes of cofilin (UNC-60A) inhibits filament disassembly by more than 2-fold and also causes a reduction in filament assembly rates, indicating that filament disassembly and assembly are tightly coupled. Combining these assays with high resolution observations of cortical actomyosin dynamics, we find that strong reductions in either filament assembly or disassembly rates result in contractile instabilities and cortical collapse, but with distinct effects on actin cortex dynamics. Mild reductions in assembly speeds induce local contractile instabilities, followed by self-repair, but coherent global deformation and flows are maintained. At significantly reduced filament assembly speeds, cortical instabilities dominate and polarization and cytokinesis fail. Conversely, mild reductions in filament disassembly rates slow cortical flow speeds, without loss of cortical integrity, while strong reductions induce catastrophic cortical tears, which cannot self-repair. These data show that rapid turnover, involving coordinated assembly and disassembly, allows the cortex to maintain a connected architecture while undergoing rapid deformation and coherent flow.

B021/P0017
Reciprocal regulation between the actomyosin cytoskeleton and fibroblast plasticity
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Fibroblasts are crucial to normal tissue function and maintenance via extracellular matrix (ECM) deposition, cytokine signaling, and contractile remodeling. Fibroblasts exhibit a high level of plasticity and, upon injury, can differentiate into myofibroblasts. Myofibroblasts are highly contractile and important in wound healing and tissue repair. With injury resolution, myofibroblasts can dedifferentiate back to their native fibroblast state. Dysregulated fibroblast activation in chronic injury can lead to persistent myofibroblast differentiation, driving fibrotic disease through excessive ECM stiffening and sustained contractility. Myofibroblasts are characterized by high expression levels of smooth muscle actin (SMA) and smooth muscle myosin (SMII), but the role of these cytoskeletal proteins in driving myofibroblast differentiation is currently unclear. We hypothesized that overexpression of SMA and/or SMII in fibroblasts would be sufficient to induce differentiation into myofibroblasts, characterized by increased contractility and markers of differentiation. Primary human fibroblasts were transduced with SMA, SMII, or both SMA and SMII. Over the course of one week, we observed that SMII overexpression alone was sufficient to drive the gradual upregulation of SMA and transgelin, both markers of myofibroblast differentiation. Surprisingly, SMA overexpression alone was also sufficient to upregulate the expression of SMII and transgelin. These results suggest that increased SMA and/or SMII activity in fibroblasts alone may be sufficient to drive the differentiation of fibroblasts into myofibroblasts. To assess how these changes in gene expression altered the cells' behavior, we used traction force microscopy to quantify whole-cell contractility, a functional output of myofibroblast differentiation. Serum starvation supplemented with transforming growth factor beta (TGF-β) treatment served as a positive control for myofibroblast differentiation. Fibroblasts overexpressing SMA, SMII, and SMA+SMII all demonstrated significantly increased contractility compared with controls, and at similar levels to TGF-β–treated fibroblasts. Morphological changes consistent with myofibroblast differentiation were also observed, including the reorganization of actin stress fibers and cellular elongation. These results suggest SMA and SMII are not just markers, but are mechanistic drivers of myofibroblast differentiation. Future work will include RNA sequencing and cytokine profiling to define the downstream transcriptional and signaling changes that accompany actomyosin-induced differentiation.

B030/P0026
Regulation of cell motility and actin cytoskeleton structure by KDAC6
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Lysine deacetylase 6 (KDAC6 or HDAC6) has been associated with cell motility and deacetylation of cytoskeleton-related proteins. However, the contributions of each of the three functional domains of KDAC6, including two catalytic domains (CD), have not been established. To probe the functions of each domain, we created HT1080 cells containing genetically-encoded KDAC6 mutated to result in an inactive CD1, an inactive CD2, or a knockout of the entire protein. The resulting three cell lines were characterized using live cell imaging and fixed cell fluorescent staining to characterize their motility and distributions of cytoskeleton-related proteins compared to wild-type cells. Inactivation of CD1 resulted in cells that migrated faster as single cells

and in scratch assays, as well as more concentrated regions of cortical actin associated with leading edges. In contrast, inactivation of CD2 resulted in cells with slower migration, depleted cortical actin, and a much greater density of stress fibers. Furthermore, the CD2 mutation caused a loss of stress fiber alignment in many cells and a large increase in cell area. The knockout cell line presented most similar to wild-type cells, with the opposing effects of the CD1 and CD2 mutations apparently canceled out. The cortical localization of proteins associated with both KDAC6 and cortical actin structures, including HSP90 and cortactin, were reduced in all cell lines, with the strongest effects when CD2 was inactivated. Gene expression changes implicate additional actin-related proteins as mediators of these effects. These results establish a distinctive role for KDAC6 CD1 and suggest potential mechanisms linking KDAC6, the actin cytoskeleton, and cell motility that are independent of tubulin hyperacetylation.

B062/P0058

Reversing chirality of cortical flows leads to situs inversus nematodes

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Left–right (LR) asymmetry is a defining feature of animal body plans, yet the origins of its conserved handedness remain poorly understood. In C. elegans, LR axis specification is initiated by a chiral spindle skew that establishes a handed cell–cell contact pattern at the 6-cell stage — an arrangement that, when reversed, leads to situs inversus nematodes (Wood, 1991). We previously identified chiral actomyosin flows as the driver of the characteristic LR asymmetric spindle skew (Naganathan et.al., 2014). Here, we show that high expression of a particular Lifeact variant used to label F-actin reverses the handedness of cortical chiral flows in the ABa and ABp cells during LR symmetry breaking. Notably, this results in mirrored 6-cell stage contact patterns and situs inversus nematodes. Our findings establish chiral acto-myosin flows, driven by active torque generation, as instructive for LR body axis establishment in C. elegans.

B051/P0047

RIAM and Lamellipodin: Master Regulators of Cell Fusion and Actin Remodeling in Osteoclasts

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Bone undergoes continuous remodeling through the coordinated activity of osteoblasts, which form the bone matrix, and osteoclasts (OCs), multinucleated cells that resorb it. Disruption of this balance underlies major skeletal diseases such as osteoporosis and cancer-associated bone diseases. Current anti-osteolytic treatments inhibit bone resorption primarily by reducing OC viability. However, this strategy has the drawback of also impairing osteoblast function, since there is a tight dialogue between OCs and osteoblasts, essential for balanced bone remodeling. Instead, we envision specifically targeting OC fusion and activity, thereby reducing their bone resorption capacity, while preserving their viability and maintaining bone formation. OC formation and resorptive activity are tightly linked to the dynamics of actin-based structures, including tunneling nanotubes, podosomes, and the sealing zone. Here, we investigated the roles of RIAM and Lamellipodin: two Rap1 effectors that regulate Talin1-dependent integrin activation and actin remodeling, in OC biology. We found that Lamellipodin is essential for OC precursor fusion. Lamellipodin-deficient OCs exhibit greatly reduced

multinucleation, which correlates with a significant decrease in tunneling nanotube formation at early stages of fusion. Consequently, their bone resorption capacity is markedly impaired. In contrast, loss of RIAM affect neither OC fusion nor tunneling nanotube formation but significantly reduces the resorption efficiency of mature OCs. Interestingly, while podosome dynamics remained largely intact in both mutants, the organization of podosomes into a functional sealing zone in mature OCs is currently under investigation. In vivo analysis of total knockout mice shows that loss of RIAM does not alter steady-state bone mass, whereas Lamellipodin deficiency impairs OC number and activity, leading to detectable bone loss. These findings highlight the distinct and complementary roles of RIAM and Lamellipodin in controlling the cytoskeletal machinery underlying OC fusion and bone degradation. By specifically interfering with the RIAM/Lamellipodin –Talin1 axis, it may be possible to limit pathological bone resorption without compromising OC viability or osteoblast–OC crosstalk. This approach opens new therapeutic perspectives for the treatment of osteoporosis and cancer-associated bone diseases.

B069/P0065

Semi-automated Tracking of in vitro Actin Parameters

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Actin filament dynamics underlie key cellular processes, yet quantifying these behaviors produced in biomimetic assays visualized with fluorescence microscopy remains a major bottleneck due to the time, bias, and variability inherent to manual analyses. We developed a semi-automated image analysis pipeline that integrates the TSOAX image segmentation software with a custom interface for rapid identification, verification, and measurement of actin filaments imaged by in vitro Total Internal Reflection Fluorescence (TIRF) microscopy assays. Once actin filaments are segmented and tracked in each TIRF movie through time by TSOAX, several parameters related to actin polymerization and higher-order structures can be extracted, including filament counts (e.g., nucleation), elongation rates, bundling, and sites of filament branching. Our TIRF experiments spanned a range of actin assembly conditions, including concentration-dependent polymerization, reactions that accelerate or halt elongation, and those altering filament architecture by initiating filament bundling or branching. The semi-automated pipeline produced quantitative measurements that closely match published and manual analyses but require less time and reduce user bias. This adaptable computational approach resolves a key quantitative barrier in cytoskeleton research, expanding the experimental scale, reproducibility, and scope of in vitro actin studies, additional actin regulatory proteins, and potentially other cytoskeletal polymers.

B009/P0005

Somatic support cells non-autonomously regulate the germline cytoskeleton through secreted FGF

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Building gametes, egg and sperm, is a complex yet highly conserved process. From Hydra to humans, germ cells divide and differentiate as interconnected units, or cysts. Formation of germ cell cysts is critical for robust detection and elimination of genomic damage, ensuring fidelity of gametogenesis. In most cell types, mitosis culminates in completion of cytokinesis and abscission, or membrane severing, producing two independent daughter cells. By contrast, germ cell cysts execute incomplete cytokinesis. Here, cytokinesis machinery is

reorganized to build stable intercellular bridges termed ring canals (RCs) that allow for cytoplasmic sharing and maintain germ cell interconnectivity. While many RC proteins have been identified, it is still poorly understood how these proteins are regulated over several reiterative mitoses. By employing longitudinal live imaging of male germ cells ex vivo, I characterized the F-actin cytoskeleton over several rounds of mitosis and incomplete cytokinesis. I identify two spatiotemporally distinct F-actin polymerization events required for establishing RC F-actin: (1) Retention of contractile ring F-actin at newly generated RCs, and (2) de-novo re-polymerization of F-actin at pre-existing RCs. Furthermore, I find that F-actin is required for fidelity of incomplete cytokinesis. Failure to maintain RC F-actin permits progression of cytokinesis through abscission, compromising integrity of the germ cell cyst. Thus, regulation of RC F-actin is critical for germ cell differentiation and spermatogenesis. Excitingly, I find that associated somatic support cells non-autonomously promote RC F-actin maintenance through the conserved RTK, FGF. Through genetic knockdown of both germline FGFR (Drosophila: Heartless) and somatic FGF (Drosophila: Pyramus), I find that both ligand and receptor are required for promoting sustained polymerization of F-actin at the RC. Within the germline, I link FGFR activity to the cytoplasmic tyrosine kinase Src64, a previously identified intrinsic regulator of germ cell F-actin. Additionally, we have found that FGF signaling is attenuated following starvation to promote germ cell plasticity and replenish lost germline stem cells. Together, this data identifies a novel requirement for somatic cells in regulating germ cell incomplete cytokinesis through FGF signaling during both homeostasis and regeneration.

B014/P0010

Spef1 is a microvillar component that limits apical actomyosin contractility and preserves intestinal barrier function

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Epithelial sheet integrity is established by adherent contacts that form between cells, at the interface between their apical and basolateral domains. Although cell contacts are reinforced by actomyosin contractility, which generates tension that propagates across the apical surface. How epithelial cells tune tension to reinforce junctions without compromising their physical barrier properties remains unclear. Herein, we report that Sperm Flagellar 1 (Spef1) is a microvillar component enriched in the apical domain and terminal web of enterocytes that prevents actomyosin hypercontractility. Loss-of-function in Caco-2 BBE cells showed that Spef1 depletion induced invaginations of the apical domain at tricellular contacts, with a redistribution of the tricellular and tight junction components. These changes, which were paralleled by increased levels of NM2A across the apical surface, compromised barrier function. These findings highlight Spef1 as a microvillar resident that tunes actomyosin contractility across the apical surface, to a level appropriate for junctional reinforcement and maintenance of epithelial function.

B007/P0003

Structural Determination of Drosophila Tropomyosin with Novel Characteristics

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Actin is a highly conserved protein that organize into filaments as part of various morphologies in order to accommodate critical cellular functions such as migration, cytokinesis, and mechanosensing. Tropomyosins constitute a large, conserved family of alpha-helical coiled-coil proteins that regulate most if not all of these actin-mediated processes. In mammalian cells four tropomyosin genes code for upwards of 40 isoforms

through differential splicing. Although it is thought that the functions of different tropomyosin isoforms are non-redundant from one-another, their chemical and cell-distribution similarities makes it difficult to study their sequence to function relationship within the context of the cell. The Mullins Lab has characterized Drosophila Schneider 2 Cells as expressing only three isoforms of tropomyosin- making them a useful model system for studying tropomyosin functions. More recent biochemistry has elucidated novel behaviors of these tropomyosins in vitro. Here I present work on using cryo-electron microscopy in order to understand the structure of novel Drosophila tropomyosinsas and their contribution to unique functions.

B033/P0029
Structural mechanism of Lmod2-mediated pointed-end elongation
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The length of thin filaments, primarily composed of filamentous actin (F-actin), in striated muscle is tightly regulated to ensure optimal overlap with thick filaments, a key determinant of contraction strength and efficiency. Dysregulation of thin-filament length (TFL) is a major cause of cardiac and skeletal muscle diseases, most notably dilated cardiomyopathy and nemaline myopathy. In the sarcomere, the basic contractile unit of striated muscle, the pointed end of thin filaments serves as the primary site for actin subunit exchange. Despite this dynamic exchange, thin filaments display a uniform length across the sarcomere, suggesting that regulatory mechanisms mediated by actin-binding proteins (ABPs) maintain this uniformity. However, the underlying mechanisms remain poorly understood. Two ABPs, tropomodulin (Tmod) and tropomyosin (Tpm), regulate TFL by capping the pointed end. Recent studies in cell and animal models suggest that a Tmod homolog, leiomodin (Lmod), plays a role in thin filament pointed end elongation. However, Lmod-mediated elongation has not been demonstrated in vitro, and the underlying molecular mechanism remains unknown. Moreover, it is unclear how Tmod and Lmod, despite sharing similar domain architectures, perform distinct functions. To address these gaps, I have solved three preliminary cryo-electron microscopy (cryo-EM) structures of Lmod2 bound to the pointed end of F-actin in different configurations. These structures support a preliminary mechanism for Lmod-mediated elongation. Additionally, I have solved a preliminary cryo-EM structure of the Tmod-Tpm complex bound to the pointed end of F-actin. Comparison of these structures reveals differences in how Lmod and Tmod interact with pointed-end subunits, likely underlying the functional divergence in these proteins. Together, these structures support a model for Lmod-mediated elongation and provide novel insights into the mechanisms of TFL regulation.

B022/P0018
Systematic comparison of genetically encoded F-actin probes for visualizing actomyosin dynamics in an animal system in vivo
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Actomyosin networks drive essential processes throughout development. F-actin markers for visualizing actin dynamics have been systematically compared for live cells in culture (Belin et al., 2013, 2015), but not yet for animal models in vivo, to our knowledge. Importantly, we observed that popular strains for F-actin visualization exhibit fertility defects, embryonic lethality, slowed post-embryonic development, and abnormally

polarized probe localization. To advance quantitative analysis of actin dynamics in developmental and other in vivo animal contexts, we used CRISPR/Cas9 to generate single-copy genomic insertions of three widely-used genetically encoded actin probes: LifeAct, utrophin, and F-tractin. We designed these transgenes to be modular to accommodate different fluorescent proteins and different promoters for cell- or tissue-specific labeling. We are evaluating these probes across multiple embryonic and post-embryonic developmental contexts with distinct actomyosin architectures, including endoderm precursor internalization, cytokinetic ring closure, anchor cell invasion, sex myoblast cell migration, post-embryonic growth, and oogenesis. We have found that these strains are brighter reporters of F-actin than previous strains. The speed and fidelity of embryonic and post-embryonic development, and fertility of our new strains are indistinguishable from controls. Our comparative analysis is helping establish which probe(s) most faithfully report different actin structures and dynamics across cellular contexts without perturbing development, providing the C. elegans community and the broader cell biology field with validated tools and resources for investigating actomyosin-driven morphogenesis.

B020/P0016

Team Player or Wild Card? CED-2/CRKII's Divergent Role in Actin Regulation

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The coordinated activation and inhibition of F-actin supports the cell movements of morphogenesis. Among these cellular movements are the engulfment of dying cells during embryogenesis and the larval migration of the distal tip cell. Misregulation of proteins that promote F-actin has been implicated in many diseases, from cancers, to immune and neuronal disorders. We showed that epidermal morphogenesis in C. elegans depends on the Rho GTPase CED-10/Rac1, which promotes branched actin via the Arp2/3 Nucleation Promoting Factor, WAVE. To identify the GEF (Guanine-nucleotide Exchange Factor) activating CED-10 during embryonic migrations, we examined the canonical two-component GEF CED-5/CED-12 (DOCK/ELMO), which is required with CED-2/CRKII for corpse engulfment and distal tip cell migration. Surprisingly, while loss of CED-5/CED-12 (DOCK-180/ELMO) caused embryonic lethality from failed epidermal migrations, CED-5/CED-12 increased F-actin accumulation in migrating epidermal cells, the opposite expected for a CED-10/Rac1 GEF. A CRISPR mutation in a candidate GAP region we discovered in CED-12 preserved engulfment but caused failed migrations with elevated epidermal F-actin, indicating this GEF also harbors a region that promotes GAP-like inhibitory control of actin. In contrast to CED-5/CED-12, CED-2/CRKII supports local F-actin enrichment, and CED-2 mutants display reduced actin at leading epidermal cells and around apoptotic corpses. Notably, CED-2, CED-5, and CED-12 mutants share embryonic morphogenesis phenotypes, including short, rounded embryos, underscoring a common requirement for cytoskeletal control despite their divergent effects on actin levels. These data indicate that proteins once thought to operate as a single trimeric complex upstream of WAVE instead may diverge into distinct signaling pathways to control actin dynamics during epidermal morphogenesis. We are therefore investigating how this trimer diverges. Branched actin is driven by the NPFs WASP, WASH, WAVE, under the control of Rho GTPases (CDC-42, CED-10/Rac1) while linear actin requires RHO-1/RhoA. Our work shows both types or actin support the epidermal morphogenetic movements. There are competing models of Rho GTPase crosstalk, from mutual inhibition to collaboration. By analyzing how CED-2, CED-5, and CED-12 coordinate the regulation of different Rho GTPases, we seek to define how they cooperate or antagonize one another to pattern actin, and to map how CED-2, CED-5, and CED-12 interface with WASP/WASH/WAVE to coordinate the assembly of branched versus linear actin during embryonic epidermal migration.

B026/P0022

The Actomyosin Cortex Controls T-tubule Remodeling In Skeletal Muscle

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The actomyosin cortex is essential for the mechanical properties of the cell membrane and its ability to reshape. During development, muscle cells need to grow an intricate network of plasma membrane invaginations called t-tubules that are coupled to the sarcoplasmic reticulum forming triads. Although the importance of t-tubules for controlling calcium and muscle contraction is well established, and abnormalities are found in multiple muscle disorders, the mechanisms that regulate t-tubule growth during skeletal muscle development remain elusive. In this work we found that t-tubule growth requires membrane availability by disruption of the actomyosin cortex. We had previously implicated the cortical actin nucleator Arp2/3 complex, through its Arpc5 subunit, in the organization of triads by an unknown mechanism in vitro. By depleting Arpc5 in primary myofibers, we now found that Arpc5 limits t-tubule overgrowth by organizing the proper distribution of the actomyosin cortex. Loss of Arpc5 leads to an increase in membrane roughness, suggesting an excess of membrane availability for t-tubule growth. This Arpc5-dependent t-tubule overgrowth leads to clusters of enlarged t-tubules at the triad and to asynchronous myofiber contraction and irregular calcium dynamics, emphasizing the role of controlling the actomyosin cortex and membrane availability in forming functional triads. Finally, we employed a mouse model with targeted Arpc5 depletion during postnatal muscle development and observed that depleting Arpc5 leads to significant muscle fatigue alongside increased membrane roughness and enlarged t-tubules at the triads, reinforcing the critical role of Arpc5-driven actomyosin cortex establishment in triad formation and muscle function. We propose that the actomyosin cortex through Arpc5 gatekeeps membrane availability for t-tubule growth and that t-tubule overgrowth leads to dysfunction of triads and muscle cell contraction. These insights offer a potential pathophysiological mechanism and open avenues for therapeutic strategies aimed at normalizing t-tubule morphology and membrane dynamics in muscle disorders.

B071/P0067

The fate of pyruvate: metabolic regulation of cellular homeostasis

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Cell growth requires a delicate balance between energy production, biosynthesis, and redox homeostasis. Our study identifies pyruvate metabolism as a central decision point that integrates these demands. We demonstrate that the fate of pyruvate—whether directed toward mitochondrial oxidation or alternative metabolic pathways—directly influences the cellular redox potential. This control over NADH/NAD^+ balance, in turn, dictates the capacity of cells to sustain growth. By establishing a mechanistic link between pyruvate utilization and proliferation, our work advances understanding of how metabolic networks are wired to support cellular fitness. These findings unify previously separate views of pyruvate as a fuel, biosynthetic precursor, and redox regulator, thereby reframing it as a pivotal node in controlling size and growth. Because dysregulated growth underlies cancer, regenerative responses, and tissue degeneration, our results open new

avenues to manipulate pyruvate metabolism therapeutically. More broadly, this work illustrates how altering the flow of central metabolites can reprogram fundamental cell behaviors.

B075/P0071
The role of Importin alpha 2 in actin organization during Drosophila oogenesis and early embryogenesis
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Actin is essential for establishing and maintaining the integrity of all eukaryotic cells. For example, in Drosophila, proper regulation of actin is critical for the success of several developmental processes in both oogenesis and embryogenesis. We are characterizing how these events are controlled by a novel actin regulator, Importin alpha 2 (Impa2). Impa2 mediates the nuclear import of many cargoes, and was previously reported to also promote the correct organization of specialized actin-rich structures in ovaries called ring canals. Using an endogenously-tagged Impa2 line, we discovered that Impa2 localizes to ring canals, suggesting that it might control actin directly at this structure. In addition, we found Impa2 localizes to the actin-rich nurse cell periphery. In Impa2 null mutants, both ring canals and the periphery exhibit disorganized, excess actin. During early embryogenesis, actin rings also form during a critical developmental process called cellularization; Impa2 also localizes to this actin-rich structure. We have used western blotting, imaging, and proteomics to better understand Impa2 dynamics across this short developmental timeframe. Interestingly, around the time Impa2 is localized to these actin rings, it exists in a dephosphorylated form, suggesting the modification status of the protein may mediate its localization to these structures. Therefore, we are currently focused on how Impa2's change in phosphorylation status may affect protein-protein interactions that could then alter Impa2's role in actin organization across early Drosophila development.

B025/P0021
Tropomyosin depletion triggers cofilin binding and disassembly of actin filaments.
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Cells orchestrate the assembly of diverse actin filament (F-actin) networks from a shared cytoplasmic pool of monomeric actin (G-actin) and actin-binding proteins (ABPs). F-actin networks are crucial for fundamental cellular processes such as cytokinesis, motility, and endocytosis. Proper size and density of F-actin networks are essential for their function, yet the underlying molecular mechanisms that regulate their differential formation in a common cytoplasm remain unclear. Recent findings from our lab and others have revealed that cytoplasmic G-actin is often limited and becomes depleted as actin networks grow, thereby constraining the size and density of competing F-actin networks. Beyond G-actin, we hypothesize that some ABPs may be similarly limiting, with their binding kinetics diminishing as their cytoplasmic concentration is depleted. To test this hypothesis, we investigated the competition between two key ABPs: the F-actin stabilizer tropomyosin (TM), and the F-actin disassembler cofilin. TM preferentially binds to newly assembled F-actin (in the ADP-Pi state), while cofilin preferentially associates with and severs older filaments (in the ADP state). Using 3-color Total Internal Reflection Fluorescence (TIRF) microscopy, we examined the dynamic interactions between TM and cofilin on F-actin. When actin filaments are assembled with high concentrations of TM alone, filaments are rapidly and uniformly coated with TM. At lower TM concentrations, initial uniform decoration is followed by a sharp decrease of TM binding, likely due to two factors: (1) depletion of TM from the solution, and (2) its high

binding cooperativity. Notably, this depletion is driven by the 'sequestration' of TM as it binds to F-actin. In contrast, when cofilin is included alone, its binding is delayed until F-actin ages into the ADP state. Cofilin then induces filament severing, which generates an exponential increase in total actin filament number. When both TM and cofilin are present, we observe an intriguing competitive interplay. At high TM levels, F-actin remains coated, with cofilin binding restricted to the pointed ends of filaments. However, at lower TM concentrations, the initial TM coating slows as TM is depleted, generating unprotected filaments that allows cofilin to bind and sever. These findings highlight the critical role that limited pools of ABPs might play in regulating F-actin dynamics. The concept of regulation through ABP sequestration may be more prevalent than previously recognized and will need further investigation. Our study demonstrates how competition between ABPs, such as TM and cofilin, governs actin filament turnover, offering a framework to better understand the broader mechanisms of ABP competition in cytoskeletal regulation.

B061/P0057

Tropomyosin Tpm4.2 regulates the differential incorporation of myosin isoforms NM2A and NM2B into stress fibers

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Actin in mammalian cells forms either branched or unbranched filaments which are involved in many cellular processes. The unbranched filaments largely consist of co-polymers of actin and tropomyosin, and individual filaments are usually homopolymers of a specific tropomyosin isoform. Many of these unbranched filaments interact with myosin motors. Previous studies in U2OS cells have identified a role for the tropomyosin Tpm4.2 in the recruitment of the conventional myosin 2 motor to stress fibers. We have evaluated if Tpm4.2 discriminates between the two isoforms NM2A and NM2B in human fibroblasts, neuroblastoma and osteosarcoma cells. siRNA knock down of Tpm4.2 resulted in dispersal of NM2A in all 3 cell lines. The dispersal of NM2A was not due to loss of actin filaments since phalloidin staining was unchanged by siRNA treatment. Similarly, the dispersal of NM2A was not due to the lack of tropomyosin associated with the stress fibers since the organization of Tpm3.1 was unchanged. In contrast, the distribution of NM2B changed from dispersed to incorporated into stress fibers. This suggests that NM2A and NM2B are competing for inclusion into stress fibers and that the organization of Tpm4.2 can determine the outcome of this competition. TroBio Therapeutics Pty Ltd have recently developed compounds that target Tpm4.2 and we have tested the ability of the compound TBT-0418 to impact both the organization of this tropomyosin and NM2A. After 24 h exposure to this compound, Tpm4.2 was dispersed in all 3 cell lines. In addition, NM2A was also dispersed further supporting the role of Tpm4.2 in determining the incorporation of NM2A into stress fibers. As with the siRNA exposure, TBT-0418 treatment resulted in the replacement of NM2A by NM2B in stress fibers. The impact of TBT-0418 is reversible. Compound washout restored the association of Tpm4.2 with stress fibers within 4 h. We conclude that the association of NM2A and NM2B with stress fibers is regulated by the presence of Tpm4.2.

B019/P0015

Tuning polarized growth by altering the balance between actin polymerization and depolymerization

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The actin cytoskeleton is critical for cell polarity in many eukaryotes. In plants, specialized cell types, such as root hairs and pollen tubes, grow by secreting new cell wall material to the tip of the cell, and this process

depends on actin dynamics. How actin regulators integrate their functions and work together to support actin dynamics at the cell tip is still not well understood. In the moss Physcomitrium patens, loss of function of one class of actin nucleator, the class II formins, generated very small plants with spherical-shaped cells. In this mutant, the actin signal is diffuse throughout the cell, and at the cell tip, the actin is significantly diminished. To identify regulators of class II formin function, we performed a suppressor screen and found second-site mutations that partially suppressed the stunted phenotype of the formin loss-of-function line (Δfor1BCE2AB). The suppressors were larger than the parental line and exhibited clear polarized growth. Whole genome sequencing revealed that each of the four suppressors had a mutation that resulted in a change to a highly conserved amino acid in Actin Depolymerizing Factor (ADF)/cofilin. In fact, two of the suppressors contained the same mutation. To validate that these mutants were responsible for suppression, we used CRISPR-Cas9 to introduce each of the three point mutations found in the suppressors into the original parental line (Δfor1BCE2AB). We found that each point mutation partially suppressed Δfor1BCE2AB, resulting in larger plants with rod-shaped cells. The suppressed plants still grew significantly slower than wild type, and imaging of actin revealed reduced apical actin and the presence of subapical bundled actin. Introduction of each of the point mutations into wild-type resulted in severely stunted plants that grew slower than Δfor1BCE2AB and wild type, and had very large subapical actin bundles. Large actin bundles likely result from reduced actin severing and depolymerization, enabling filaments to persist long enough for actin bundling proteins to bind, suggesting that the point mutations reduce ADF/cofilin function. Based on these findings, we propose that weak actin severing activity partially rescues the lack of actin nucleation in Δfor1BCE2AB. Thus, the balance between polymerization and depolymerization of actin is critical for polarized cell expansion. Ongoing research aims to study whether introducing these ADF/cofilin point mutations into plants lacking the Arp2/3 complex, another actin nucleator, can also partially rescue.

B057/P0053

TXBP-3 regulates spermathecal contractility in C. elegans

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TXBP-3/TAX1BP3 is a small, highly conserved protein consisting of a single PDZ domain that may interfere with interactions between PDZ-containing proteins. The human ortholog is involved in various signaling pathways, impacting adhesion, migration, polarity, and cell proliferation. In C. elegans, TXBP-3 is expressed in multiple contractile tissues, including the pharynx, spermatheca, spermatheca-uterine valve, and rectal epithelium. We examined its role in the transit of oocytes through spermatheca, the site of fertilization, using live imaging, timing analysis, and confocal microscopy. Deletion of txbp-3 has a modest effect on oocyte transits, disrupts apical F-actin bundle organization throughout the spermathecal bag, and leads to reduced fertility. Overexpression of TXBP-3 also results in reduced fertility, hyper-constriction of the spermatheca and faster transit of oocytes. Oocyte transit is often accompanied by constriction and pinching of oocytes, which yields rounder embryos at expulsion. These data support a dosage-sensitive role for TXBP-3 in keeping spermathecal contractility within a functional range.

B072/P0068

Understanding How Actomyosin Dynamics Drive Apical Constriction by Systematically Tagging and Mapping Actin-Binding Proteins

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Apical constriction is a critical cell shape change that sculpts tissues during development. Apical constriction is driven by contraction of an apical actomyosin network anchored to adherens junctions, yet how this network is spatially organized and dynamically remodeled remains poorly understood. C. elegans gastrulation provides a genetically and optically tractable model to investigate this question, as apical constriction drives the internalization of endodermal precursor cells. Actin-binding proteins (ABPs) are key regulators of actomyosin network assembly and turnover. We hypothesize that distinct ABPs function at specific subcellular regions to dynamically organize the actomyosin network during apical constriction. To test this, we used CRISPR/Cas9 genome editing to endogenously tag nearly all ABPs expressed in early embryos. This allowed us to build a comprehensive spatial map of ABP localization in apically constricting cells and neighboring cells. Our analyses revealed that endodermal precursor cells apically constrict using a typical, mixed-polarity actin filament network. We also found that Rac signaling activates WAVE and Arp2/3 basolaterally, and that the basolateral Arp2/3 population is required for apical constriction. Our ongoing work extends these findings by identifying distinct subcellular hubs where specific ABPs colocalize. These hubs appear to concentrate different types of actin-regulatory activities at defined locations. Guided by this spatial map, we are combining RNAi, auxin-inducible degradation, and quantitative imaging to test the function of individual ABPs and ABP groups at specific locations. This systematic approach is revealing how cells partition actin-regulatory functions across space and time to assemble, stabilize, and remodel the actomyosin network in a precise and robust manner. Together, our work provides new insights into the spatial organization of actin regulation driving morphogenesis and establishes a framework for dissecting complex cytoskeletal organization in vivo.

B012/P0008

V-1/Myotrophin regulates filopodia versus lamellipodia formation by controlling Capping Protein availability.

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Capping Protein (CP) binds the barbed end (BE) of the actin filament with ~0.1 nM affinity to block further monomer addition and loss. Paradoxically, while CP halts actin filament growth, it potently promotes Arp2/3 complex-dependent branched actin network assembly (Akin and Mullins Cell 2008). Conversely, CP suppresses formin- and VASP-dependent linear actin filament assembly by competing with these BE polymerases for the BE. Consistently, CP depletion reduces the size of branched actin structures like lamellipodia and promotes the formation of linear actin structures like filopodia (Mejillano et al Cell 2004). In 2014, Fujiwara and colleagues proposed a regulatory cycle for CP driven by the proteins CARMIL and V-1/Myotrophin that controls CP's availability and site of action (PNAS 2014). This cycle, which was recently confirmed by biochemical reconstitution (Mooren et al CB 2024), posits that the majority of cellular CP is sequestered by V-1 and only becomes active for BE capping when liberated from V-1 by CARMIL. Liberation occurs at the plasma membrane, where CARMIL is recruited and activated to drive a complex exchange reaction that converts freely-diffusing CP: V-1 complexes into membrane-bound CARMIL: CP complexes that cap nascent branched filaments with ~50 nM affinity. Consistent with this model, the measured lifetime of CP on BEs near the plasma membrane (~2-15s) is much closer to that of the CARMIL: CP complex (~8s) than to that of CP alone (~1800s) (Fujiwara et al JBC 2010), and cells engineered to express a version of CP that cannot see CARMIL phenocopy CP deficient cells (Edwards et al NC 2016). Here we sought more support for this regulatory cycle by depleting cells of V-1, thereby releasing CP from sequestration. Consistent with the cycle, V-1 knockdown (KD) HeLa cells exhibit fewer and shorter filopodia, broader lamellipodia, and enhanced membrane ruffling. Importantly, these phenotypes are recapitulated in V-1 knockout HeLa cells and mouse embryonic fibroblasts. Ongoing rescue

experiments seek to prove that these phenotypes arise specifically from the loss of CP sequestration. Overall, our results are consistent with a central role for V-1 in controlling CP availability, with the differential effects of CP on the assembly of branched and linear actin filaments, and with the proposed CP regulatory cycle driven by CARMIL and V-1.

Cytoskeleton and Motility - Cell motility

B176/P0170
Data-driven discovery of neutrophil 3D motility morphodynamic cycle
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As first responders to sites of injury and infection, neutrophils are innate immune cells whose functional roles require efficient 3D directional motility through a wide variety of physical environments. In culture, neutrophils are able to crawl rapidly through loose collagen matrices, where their movement is associated with dramatic cell shape changes, driven by intracellular forces generated by the cells' actomyosin cytoskeleton. To gain insight into how cell shape changes contribute to neutrophil migration, we observed a large population of membrane-labeled dHL-60 neutrophil-like cells migrating through 3D collagen matrices in vitro at high spatial and temporal resolution, and analyzed their morphology. Starting from 3D morphological analysis based on spherical harmonics, we used principal component analysis (PCA) to determine the primary modes of neutrophil shape variation, and reconstructed human-interpretable visualizations of these modes. We sought to understand how morphological dynamics may relate to migration behavior by measuring transition rates between states in coarse-grained phase spaces (CGPSs) defined by pairs of PCs. We found the strongest evidence for directed flux in the CGPS defined by PC1, roughly corresponding to a cell's elongation along its trajectory, and PC7, representing whether the bulk of the volume is toward the front or rear of the migrating cell. To visualize the PC1-PC7 morphological cycle, we linearized the cycle and reconstructed average shapes at different angular positions around the origin of the flux in the CGPS. The morphological cycle has three main phases: first the cell makes a relatively thin front protrusion, then it elongates along its trajectory, and, finally, it shifts the bulk of its volume toward the front and retracts the rear. Notably the total cell volume also undergoes a substantial cyclic change of about 10%, with higher volume during the protrusion phase and lower volume during the retraction phase. Experimental perturbations that affect motility, including inhibition of key cytoskeletal factors and introduction of directional cues, alter quantitative features of the morphological cycle without substantially changing the progression of motile cells through the various shape transitions. Using long-term imaging of individual cells, we find that neutrophil motility can be compactly described as a finite state machine with just three major gait states (progressing forward through the morphological cycle, stalled, and regressing backward through the cycle), where the apparent gait transitions behave as expected for a memoryless stochastic process governing the underlying cell shape changes.

B182/P0176
Decoding Immunological States using Morphodynamic Analysis
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Macrophages are dynamic immune cells capable of adopting polarized states (M1 pro-inflammatory or M2 anti-inflammatory) that are central to immune regulation. While current approaches identify polarization through molecular markers, they lack behavioral insight into the changing phenotypes and movements, or

morphodynamics, that accompany functional shifts. We developed a time-resolved imaging pipeline to analyze macrophage population morphodynamics across polarization states, sexes, and circadian phases. By extracting shape and motion features such as cell area, circularity, and energy, we observe distinct cell states over time. Our results have shown that shape features, like major axis, and textural features, like homogeneity, sufficiently describe circadian oscillation. This immunological cell state insight may be leveraged for clinical treatments like increased vaccine efficiency or symptom management. Notably, we observed sex-specific morphodynamic profiles that varied across circadian time, with significant divergence at 28 hrs and 40 hrs. These findings align with reported sex-dependent circadian transcriptomic rhythms and highlight the role of temporal and sex-specific factors in immune behavior. Our results suggest that macrophage behavior integrates both intrinsic circadian regulation and sex-based signaling, providing a novel window into immune function through live-cell morphodynamic analysis

B183/P0177
Invasion of bacteria swimming upstream into microstructured devices
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Bacteria have the remarkable ability to swim upstream, called positive rheotaxis. This motion against flows can cause not only respiratory, gastrointestinal, and urinary tract infections, but also the contamination of medical devices and hospital equipment. However, it remains unknown how bacteria navigate upstream through these microstructured environments with narrow channels and wide cavities. Here, combining microbiology experiments and nanofabrication with mathematical modeling and computational fluid dynamics, we reveal how Escherichia coli invade such environments in four stages: The (I) break-out from colonized cavities against the current, (II) propagation upstream in narrow channels, (III) infiltration of new cavities, and (IV) colonization with biofilms under flow. Surprisingly, we find that wider channels with faster counterflows are actually more prone to invasion, but this can be inhibited effectively with sharp corner designs. Next, we explore the serial invasion of multiple cavities in a row. Rather than slowly colonizing the cavities sequentially from the downstream end, bacteria rapidly swim all the way upstream and form streamer-like bioaggregates, enabling them to take possession of the entire channel three times faster. These results shed new light on pathogen motility in host-relevant shear regimes and offer solutions that can be implemented directly in biomedical devices.

B186/P0180
Measurement of Cellular Traction Forces During Confined Migration
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Migrating cells in vivo encounter a variety of stiffnesses and geometries. Often these are small constrictions in which the cell must deform its nucleus to effectively transit. Previous studies suggest cells adopt a variety of mechanisms to transit confinements including pulling the nucleus through, pushing it from behind, or actively deforming the nucleus using cytoskeletal machinery. Discerning between these strategies is challenging as generating micron-scale micropatterned substrates at physiological stiffnesses is currently non-trivial and current techniques to measure the forces cells use often lack spatial resolution. To address this, we have developed a sacrificial micromolding technique using table sugar and commonly available rigid

polydimethylsiloxane (PDMS) molds that allows for the generation of high fidelity, user defined 3D geometries in compliant PDMS. Using these substrates, we imaged the migration of cells through compliant and rigid confinements. To our surprise, cells on compliant substrates exhibited significantly impaired nuclear transit and increased nuclear aspect ratio compared to cells migrating through rigid confinements. To examine this surprising result, we adapted a traction force microscopy (TFM) platform to measure the forces cells exert during confined migration. Using TFM and spinning disk microscopy, we have found mesenchymal cells transiting confinements exert strongly contractile paranuclear tractions rather than deforming their nucleus with reactive forces from the channel wall. Thus, at least for these geometries, rigidities, and cells, the nuclear deformation required to transit tight confinements likely results from cytoskeleton rearrangement and internal forces rather than pushing or dragging the nucleus through the confinement. Taken together, these results suggest a complex relationship between the generation of substrate deformations by the cell, the physical geometry of the confinement, and the rigidity of the substrate that plays a key role in the cells mechanical response and ultimately mechanism of overcoming restrictive pores.

B175/P0169

Mono-Ubiquitylation-Dependent Rap2 Activation Regulates Lamellipodia Dynamics During Cell Migration

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Cell migration is a complex process hallmarked by front-to-back cell polarity that is established by the highly dynamic actin cytoskeleton. Branched actin polymerization creates a lamellipodia at the leading edge of the cell, while the contractile acto-myosin cytoskeleton is present at the lagging edge. Rap2, a Ras GTPase family member, has previously been reported to localize to the lamellipodia as a result of Rab40/CRL5 E3 ubiquitin ligase induced mono-ubiquitylation. However, how Rap2 functions and how mono-ubiquitylation targets Rap2 to the lamellipodia remained unclear. Here, we demonstrate that Rap2 is recruited to retracting lamellipodia ruffles where it inhibits RhoA, likely through interactions with ARHGAP29, and regulates lamellipodia dynamics, thus facilitating cell migration. Furthermore, using a variety of genetic and pharmacological techniques, we show that mono-ubiquitylation is required for GEF-dependent Rap2 activation, a necessary step for Rap2 targeting to lamellipodia membrane. As such, we demonstrate how this unique mono-ubiquitylation and activation of Rap2 regulates lamellipodia actin dynamics during cell migration.

B185/P0179

NDR1/2 kinases regulate cell polarization and cell motility through Pard3-Cdc42 signaling in migrating fibroblasts

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Controlling cell polarity and the directionality of cell motility is critical for effective cell migration during wound healing. Conserved NDR (nuclear dbf2-related) kinase pathways have conserved roles in cell morphogenesis in eukaryotes. Further, in humans, disruption in cell polarity is a hallmark of various human diseases, including cancer and chronic wounds. In this study, we report that knockdown of NDR1/2 kinases results in dramatic changes in cell size, shape and the actin-cytoskeleton. Furthermore, we observed a reduction of cell migration persistency and impaired cell polarity after NDR kinase knockdown in wound healing assays. Mechanistically, we found that NDR1/2 kinases spatially and temporarily control the dynamics of Cdc42 GTPase. Decreased

NDR kinase levels lead to increased Cdc42 GTPase activity and the mis-localization of polarity protein Pard3. Further analysis reveals that NDR kinases phosphorylate Pard3 at Serine144. Consistent with NDR kinase promoting Pard3 function, overexpression of Pard3 partially rescues the wound healing defects induced by NDR depletion. This rescue is prevented if Serine 144 is mutated to Alanine. Finally, we demonstrated that knockdown of NDR1 dramatically impaires wound healing in an ex vivo human wound healing assay, underscoring the physiological relevance of this study. In conclusion, our data demonstrate that NDR kinases control cell migration persistency by modulating the Pard3-Cdc42 signaling axis in human fibroblasts. Since our lab previously reported that the fission yeast NDR kinase Orb6 controls Cdc42 GTPase, our findings in human fibroblasts highlight the conservation of NDR kinases control mechanisms across the evolution, from yeast to human cells. Understanding how NDR kinases orchestrate cytoskeletal dynamics, and cell polarity may provide new insights into the treatment of diseases characterized by impaired cell migration and tissue repair.

B184/P0178

Neutrophil motility is regulated by the light-dark cycle in zebrafish embryos

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Neutrophils are the immune system's first line of defense against pathogens. A key aspect of neutrophil function is rapid cell motility, allowing them to scan tissues for infections and respond quickly to wounds. Recent evidence has suggested that some aspects of neutrophil biology are under circadian control. For example, mouse neutrophils are more abundant in the bloodstream during the time of day that the animals are more active (active phase) compared to their rest phase. It is hypothesized that this adaptation enhances protection when exposure to pathogens is most likely. However, little is known about whether motility and other aspects of neutrophil behavior are also under circadian control. Zebrafish (Danio rerio) embryos are a powerful in-vivo model for studying neutrophil motility because their transparency facilitates direct microscopic observation of cell behavior in living animals. Here, we ask whether neutrophil recruitment and motility in response to tail-wound injuries are under circadian regulation in zebrafish. Zebrafish embryos with fluorescently labeled neutrophils and skin epithelial cells were raised under a light–dark cycle (LD), constant light (LL), or constant darkness (DD), and imaged at 4 days post-fertilization, 1 hour after lights-on at ZT1 (beginning of active phase) or 1 hour after light-off at ZT15 (beginning of rest phase). After tail-fin injury, embryos were imaged for 6 h, and neutrophils were tracked using Fiji's TrackMate. We found no differences in the number of neutrophils present in the skin of the tail-fin immediately after wounding as a function of light condition, and neutrophil counts in this region increased over time across all conditions. However, neutrophils imaged under LD conditions displayed significantly higher speeds compared to those in constant conditions (either LL or DD). At ZT1, animals raised in constant conditions exhibited an increase in neutrophil motility over time, likely due to wound-induced signaling, while those raised in an LD cycle showed consistently high neutrophil motility at all times after wounding. Our findings suggest that neutrophil motility in zebrafish is under circadian control, with zebrafish in LD conditions showing enhanced motility at the start of the active phase as compared to constant conditions. This supports the idea that the circadian rhythm primes the immune system by making neutrophils more motile during times of likely pathogen exposure. Ongoing analysis will clarify whether motility rhythms are endogenous or driven by external cues. This work shows the utility of the zebrafish model for examining how the circadian system influences immune function.

B179/P0173

Optogenetic Control of Microtubule Detyrosination Reveals Crosstalk with Actin and Vimentin Networks

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Microtubule detyrosination is an evolutionarily conserved post-translational modification catalyzed predominantly by Vasohibin 1 (VASH1) and Vasohibin 2 (VASH2) in mammals. This modification regulates diverse cellular processes, including mitosis, intracellular transport, and cell motility. Effective cell migration requires coordinated interactions across cytoskeletal networks, yet the specific role of detyrosination in microtubule crosstalk with actin and intermediate filaments remains poorly understood. Here, we show that U2OS cells lacking VASH1 or VASH2 display reduced lamellipodial protrusions and impaired actin and vimentin dynamics compared. We further find that cytoplasmic localization of VASH proteins is essential for efficient microtubule detyrosination. Building on this spatial regulatory principle, we engineered a suite of optogenetic tools, termed optoVASH, to precisely control microtubule detyrosination in live cells. OptoVASH uses a modified light-inducible nuclear export system (LEXY) with reduced basal activity, fused to the catalytic domains of VASH1 or VASH2. In dark, optoVASH localizes to the nucleus, and upon blue-light stimulation, it rapidly and reversibly translocates to the cytoplasm. In HeLa cells, light activation of optoVASH induced robust microtubule detyrosination, validating its functionality. Strikingly, optoVASH stimulation also triggered dynamic cell protrusions accompanied by remodeling of the actin and vimentin networks. These protrusions were abolished by Rac1 inhibition, establishing a functional link between microtubule detyrosination and actin dynamics. To summarize, we have developed a versatile tool for dynamic control of microtubule detyrosination and revealed a role for microtubule detyrosination in cytoskeletal crosstalk driving cell protrusions.

B180/P0174

S1P guides dendritic cell migration through S1PR3-Gai, and exhibits cytoskeletal requirements distinct from those used by T cells.

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Sphingosine-1-phosphate (S1P) is a lipid mediator essential for immune cell trafficking, yet its role in dendritic cell (DC) migration remains incompletely defined. We reconstituted S1P-induced chemotaxis of LPS-matured bone-marrow derived DCs (BMDCs) in vitro, and investigated receptor usage and downstream cytoskeletal regulatory pathways. Using transwell migration assays, we found that mature BMDCs migrated efficiently in response to S1P, and that this response could be blocked by the pan-S1P receptor antagonist FTY720. Using additional pharmacological inhibitors specific to S1P receptors 1-4 (S1PR5 is not expressed in DCs), we found that migration depended exclusively on S1PR3. S1PR1, the dominant receptor in T cells, did not play a role. DC migration was pertussis toxin–sensitive, confirming Gαi dependence, while Gαq blockade had no effect. Western blot analysis revealed that S1P induced phosphorylation of AKT and ERK; this biochemical response was also dependent on signaling through S1PR3 and Gαi. In T cells, our lab has found that whereas the chemokine CCL19 induces sustained Rac activation leading to lamellipodial motility, S1P induces sustained Rho activation and myosin light chain (MLC) phosphorylation leading to bleb-based motility. Motivated by these findings, we asked how mature BMDCs respond to CCL19 vs S1P. As in T cells, we found that CCL19 induced sustained Rac activation as measured by PAK phosphorylation, whereas S1P triggered sustained MLC

phosphorylation. Interestingly, however, S1P induced transient moesin dephosphorylation in T cells, but moesin became hyperphosphorylated in S1P-stimulated DCs. Consistent with this, we found that deletion of moesin, which inhibits S1P-dependent T cell migration, had no effect on DC migration. These findings are consistent with a model in which release of moesin-dependent membrane-cortex attachment is not required for DC motility. To define the cytoskeletal mechanisms required for DC migration, we tested inhibitors of actin polymerization, myosin contractility, and membrane-cortex attachment. Inhibitors of Arp2/3 (CK666), formins (SMIFH2), and ROCK (Y-27632) all impacted S1P-driven migration, whereas inhibition of ERM kinases (SLK/LOK) had little effect. Both S1P- and CCL19-induced migration were equally dependent on Arp2/3 and formins, suggesting convergence at the level of actin nucleation despite upstream differences. These results demonstrate that in DCs, as in T cells, S1P and CCL19 direct distinct chemotactic responses, but that cell-type-specific adaptations are also involved.

B181/P0175

S1P induces bleb-based T cell motility via S1PR1-dependent activation of Rho and WNK1

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In vivo, signaling by the chemokine CCL19 and its receptor CCR7 is associated with T cell retention in lymph nodes, while signaling by the lipid chemoattractant S1P via S1PR1 directs lymph node egress. Our lab recently discovered that naïve CD4+ T cells engage in distinct modes of motility in response to these two chemotactic signals. Whereas CCL19 activates lamellipodial motility, driven by actin polymerization at the leading edge, we found that S1P induces myosin contractility and bleb-based motility. To better understand the mechanisms controlling S1P responses in naïve T cells, we have explored the relevant signaling cascades downstream of S1PR1. Using G-LISA, transwell, and immunoblot assays, we show that S1P signals via S1PR1 and Gai. By combining G-LISA data with a live-cell Rac1 biosensor, we found that whereas CCL19 induced sustained Rac1 activation, S1P led to transient Rac1 activation followed by sustained RhoA activation. Analysis of the signaling pathways leading to myosin light chain phosphorylation revealed a role for RhoA and PLC. mTORC signaling was also required, consistent with a model in which Rac1-dependent actin polymerization drives increased membrane tension, which is sensed by mTORC2 to induce RhoA activation. Surprisingly, we found that myosin contractility was not required for S1P-dependent migration in transwells. This led us to investigate the involvement of WNK kinases, which are known to regulate membrane-cortex attachment (MCA) and protrusion formation by regulating ion and water influx. S1P treatment induced phosphorylation of ion channels by WNK1, and full inhibition of WNK kinases blocked the ability of S1P to initiate membrane protrusions and inhibited T cell migration. Partial inhibition of WNK1 inhibited blebs but did not inhibit actin-driven protrusions or transmigration. Interestingly, however, partial WNK inhibition together with inhibition of myosin regulatory pathways effectively impaired T cell migration and motility. These results indicate that water influx and myosin contractility work together to elevate intracellular pressure and drive bleb-based migration. Taken together, our work shows that S1P signaling initiates parallel RhoA- and WNK1-dependent cascades that coordinately control naïve T migration.

B177/P0171
The Physical Consequences of Sperm Gigantism
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The male fruit fly produces ~1.8 mm long sperm, thousands of which can be stored until mating in a ~200 µm sac, the seminal vesicle. The evolutionary pressures driving such extreme sperm (flagellar) lengths have long been investigated (the 'big sperm paradox'), the physical consequences of their gigantism within the confines of the animal's reproductive tract are unstudied. Through reconstructions of in vivo sperm morphologies from highly resolved three-dimensional electron microscopy (SBF-SEM) and rapid live imaging, we discovered that stored sperm are organized into a dense and highly aligned state. The packed flagella exhibit collective and persistent flows, with persistent and slow-moving topological defects; individual sperm, despite their extraordinary lengths, propagate rapidly through the flagellar 'material', moving in either direction along lanes formed by the flagellar backbone. How does the sperm's axoneme-powered wave-propagating activity lead to such individual and collective dynamics? We conceptualize the motion of individual sperm as being topologically confined to a reptation-like tube formed by its neighbors; sperm thus propagate by pushing off counter-propagating neighbors. We test the theoretical model's predictions through quantitative photomanipulation experiments and simulations of active elastic filaments. From this conception, we derive a continuum theory that produces an extensile material stress that can sustain an aligned flagellar material. What are the consequences of this mechanism of sperm motility? We propose that the resulting active stresses resulting from the sperm's counterpropagation lead to active stresses in the flagellar 'material' that keep the sperm in an unentangled, hence functional state. We demonstrate that these findings hold true in the female's storage organs, where sperm are also stored for prolonged periods of time prior to fertilization, and are extracted almost singly from this densely packed state. Our findings demonstrate the value of investigating beyond-the-canon sperm and establish giant sperm in their native three-dimensional microenvironment as a novel and physiologically relevant active matter system.

Cytoskeleton and Motility - Cilia and Flagella Dynamics

B171/P0165
A Brief Exposure to Rotenone Alters Cytoskeletal Dynamics, Induces Aberrant Primary Cilia Elongation, and Disrupts Sonic Hedgehog Signaling
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Rotenone, an environmental toxin and pesticide, is widely used to model Parkinson’s disease owing to its well-studied effect to inhibit mitochondrial complex I function and to increase Reactive Oxygen Species (ROS), thereby leading to dopaminergic neuron degeneration. Less appreciated is its effect on microtubules (MT) except suggesting that it promotes microtubule depolymerization raising the question of whether rotenone also impacts primary cilia (PC) microtubule-based signaling hubs, essential for neuronal function. Using hTERT-RPE1 cells, which assemble PC upon serum starvation, we discovered that brief exposure to rotenone (100 nM, 2-4 h) causes a striking elongation of PC. This was accompanied by hyperacetylation of cytoskeletal microtubules, a modification known to influence cilia assembly-disassembly. Depletion of α-TAT1, the α-tubulin acetyltransferase, eliminated microtubule acetylation without preventing ciliary elongation, suggesting that

rotenone drives PC extension through additional mechanisms. Supporting this, rotenone increases soluble tubulin pools, consistent with its effect to enhance MT depolymerization. Similarly, cilia elongation was also observed in serum-starved U-87MG glioblastoma cells, while enhanced MT acetylation was observed in SHSY-5Y neuroblastoma cells, highlighting a conserved response, although the latter does not form PC even after 48 h serum starvation. Importantly, changes in cellular ROS level does not alter rotenone-induced microtubule acetylation or PC elongation. We next asked whether elongated PC retain signaling competence. To investigate that, we checked Sonic Hedgehog (SHH) signaling which is exclusively transduced by PC during embryonic development and is critical for the maintenance of adult tissue homeostasis. Strikingly, although cilia were longer under rotenone treatment, SHH signaling was impaired: smoothened failed to distribute along the axoneme and remained restricted to the ciliary base, while SHH target genes (Gli1, Ptch1, Hhip) showed no significant increase in expression. Thus, our findings reveal that even brief, low-dose rotenone exposure induces aberrant elongation of PC while simultaneously disrupting their signaling function. We propose that such ciliary dysfunction represents an underappreciated mechanism, by which rotenone and potentially other environmental toxins contribute to the pathogenesis of neurodegenerative disease.

B161/P0155

Asymmetric Microtubule Polyglutamylation Influences Basal Body Stability

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Motile cilia are highly conserved, dynamic microtubule-based structures which generate fluid flow in a variety of cellular contexts through coordinated, asymmetric beating. At the base of each cilium is a basal body, a triplet microtubule-based structure which nucleates, stabilizes, and anchors the cilium to the cell cortex. As these structures are responsible for maintaining cilia during beating, it is essential they are effectively stabilized and maintained. Basal body microtubules are extensively post-translationally modified, particularly by tubulin polyglutamylation. In the ciliate Tetrahymena thermophila, we find that the initiating tubulin polyglutamylase, TTLL1, and the elongating polyglutamylase, TTLL9, localize to the basal body at distinct points of basal body assembly and maturation. Furthermore, expansion microscopy (ExM) coupled with structured illumination microscopy reveals that tubulin polyglutamylation is distinctly asymmetric, both radially and longitudinally, and localizes specifically to the C tubule of the microtubule triplet. Furthermore, electron microscopy reveals that basal bodies lacking this modification show loss of stability specifically at the C microtubule of the triplet. We propose that tubulin polyglutamylation, and the enzymes that promote this modification, are actively controlled under differential ciliary forces to modulate basal body structure and stability.

B166/P0160

Ciliary extracellular vesicles biogenesis as a homeostatic response to disrupted cilia trafficking and signaling.

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Beyond their signaling function, cilia are also prime site for the biogenesis of ciliary extracellular vesicles (cEVs). The biogenesis and functions of cEVs remain poorly understood. Using C. elegans ciliated neurons as a model, we previously suggested that cEVs help alleviate receptor accumulation when intraflagellar transport (IFT) is impaired. We identified and characterized the tetraspanin TSP-6 as a robust cEV marker, which enters and

diffuses within the ciliary membrane in an IFT-independent manner, allowing us to investigate how IFT influences the rate and location of cEV biogenesis. Although TSP-6 normally diffuses within the ciliary membrane, in IFT mutants it becomes retained and concentrated in specific regions. These regions then serve as sites of outward budding. We demonstrate that cEV release increases following acute sensory stimulation that transiently disrupts IFT, or in mutants with defective membrane protein trafficking but functional IFT. In these mutants, the buildup of membrane proteins coincides with the sites of cEV biogenesis. We propose that membrane receptor crowding directly regulates the location and rate of cEV biogenesis. This adaptive export mechanism would prevent pathological receptor accumulation and dynamically shape the ciliary receptor repertoire.

B165/P0159

Ciliary Signaling Coordinates Tissue-Resident Macrophage and Preadipocyte Communication in White Adipose Tissue

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The primary cilium is a sensory organelle protruding from the surface of most vertebrate cells. It senses environmental signals and transduces this information into a cellular response. Within the adipocyte linage, only adipocyte precursor cells (APCs) but not mature adipocytes display a primary cilium. APCs lacking a primary cilium do not differentiate and primary cilium dysfunction can cause white adipose tissue (WAT) expansion. Thus, primary cilia are key in controlling adipose tissue homeostasis. However, which extracellular signals are sensed by primary cilia in APCs, the downstream signaling pathways, and how this controls preadipocyte differentiation and function remains enigmatic. Tissue-resident macrophages that are formed during early embryonic development have important homeostatic functions in tissues. In WAT, adipose tissue-resident macrophages (ATMs) can be distinguished from monocyte-derived macrophages by expression of TIM4. Loss of TIM4+ ATMs prevents lipid accumulation in adipocytes and, in turn, WAT development. This is controlled by the growth factor PDGFcc, which is secreted by ATMs and has been proposed to act on adipocytes in a paracrine manner. The phenotype observed upon loss of TIM4+ ATMs is reminiscent of the phenotype upon loss of primary cilia in APCs. Strikingly, in fibroblasts, the receptor for PDGFcc, PDGFRa, has been localized to the primary cilium. We hypothesize that ATMs control WAT development and function via PDGFcc-dependent primary cilia signaling in APCs. Using 2D and 3D in-vitro and in-vivo models, we demonstrate that TIM4+ ATMs are already present in WAT before adipocyte differentiation and express PDGFcc. PDGFcc activates PDGFRα, which is localized to the primary cilium of APCs and activates ciliary ERK/AKT signaling. This promotes APC proliferation and differentiation. Loss of primary cilia in APCs abolishes the PDGFcc-dependent action on adipocytes, which is mirrored by loss PDGFRa in APC. Altogether, we demonstrate that the PDGFcc-dependent communication of ATMs to APCs via primary cilia is the key driver of WAT development.

B174/P0168

Convergence of Autism Proteins at the Cilium

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Hundreds of high confidence autism genes have been identified, yet the relevant molecular underpinnings are unclear. Autism commonly co-occurs with congenital heart disease, hydrocephalus, and blindness, all conditions that are caused by or occur in diseases caused by defective cilia. Cilia are membrane-bound organelles critical for neurogenesis, brain patterning, and neuronal activity—processes strongly implicated in autism. Although our recent functional genetics work has suggested that autism-associated genes may function at the cilium, the localization and role of autism proteins at the cilium has not been systematically investigated.To investigate the hypothesis that ciliary biology is relevant to autism biology, we first utilized a systems biological approach to determine the representation of high-confidence autism proteins and their interactors among empirically-derived cilia-related proteomes, identifying significant enrichment. We also observed that expression patterns of high-confidence autism genes tend to be significantly correlated with canonical cilia marker genes. Next, we conducted a localization screen of the 30 autism proteins with the strongest statistical association with autism and identified 12 that localize to both primary cilia on neurons and to motile cilia on epithelial cells. These proteins included those categorized as gene expression regulators and categorized as canonical neuronal communication proteins, suggesting that these proteins are pleiotropic and share a role at the cilium. Indeed, we observed that chromatin regulators CHD8, CHD2, and POGZ, as well as synaptic protein SYNGAP1, not only localized to cilia, but were required for their formation. Further, autism-associated patient missense variants in SYNGAP1 mislocalized the protein away from cilia, and that patients with pathogenic variants in these genes have cilia-related co-occurring conditions and biomarkers of disrupted ciliary function. In sum, we have identified convergence of autism proteins spanning disparate functional annotations at the cilium, providing new mechanistic insights into autism, establishing the relevance of ciliary biology, and generating additional avenues to explore for therapeutic potential in treating impairing co-occurring conditions.

B160/P0154
CP110 uncapping defects in Trisomy 21 impact primary ciliogenesis
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Primary cilia are microtubule-based extracellular signaling structures crucial for development and tissue homeostasis. Defects in primary ciliogenesis can lead to ciliopathies. Similarly, Trisomy 21, the genetic cause of Down syndrome, also causes defects in cilia formation and function. Increased Pericentrin (PCNT), a chromosome 21 resident gene that encodes a centrosome protein that is elevated in Trisomy 21, is sufficient to disrupt primary cilia formation and function. Elevated PCNT forms cytoplasmic aggregates along microtubules and at their ends, delaying primary ciliogenesis through an unknown mechanism. Primary cilia assemble from centrosomes, consisting of mother and daughter centrioles surrounded by pericentriolar material. The mother centriole's appendage structures recruit factors essential for primary cilia assembly, including the machinery for centriolar cap removal. This machinery includes CP110-specific E3 ubiquitin ligases that trigger degradation and cap removal. Centriole uncapping, facilitated by removing CP110 and CEP97 proteins, controls primary cilia formation. We demonstrate that elevated PCNT in Trisomy 21 disrupts mother centriole uncapping, and CP110 depletion rescues the delayed cilia formation in Trisomy 21 without affecting PCNT levels. Dynein-dependent transport of uncapping machinery along microtubules is necessary for mother centriole uncapping. Elevation of PCNT aggregates in Trisomy 21 likely impede efficient trafficking of this machinery to the centrosome. We observed slower cytoplasmic PCNT aggregate movement and reduced centrosomal levels of cap-specific E3 ubiquitin ligases HERC2 and MIB1 in Trisomy 21. Consistent with the centrosomal reduction of HERC2 and

MIB1, we also observed reduced ubiquitination of CP110 in Trisomy 21. PCM1, a centriolar satellite protein required for uncapping and ciliogenesis, mediates trafficking of MIB1 and HERC2 to the centrosome. PCM1 accumulates with PCNT aggregates around centrosomes in Trisomy 21. This suggests that sequestering of PCM1 around centrosomes may disrupt trafficking of HERC2 and MIB1, decreasing CP110 ubiquitination, uncapping, and subsequent ciliation. Consistent with this model, reduction of PCNT aggregates in Trisomy 21 rescues uncapping and centrosomal localization of HERC2 and MIB1. In summary, this work explores trafficking-dependent mechanisms causing centriolar uncapping defects in Trisomy 21.

B172/P0166

Dissecting the gene expression regulation of ciliary length in Chlamydomonas reinhardtii

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Cilia are conserved, microtubule-based organelles vital for motility, sensation, and signaling. Defects in cilia result in a range of disorders termed ciliopathies, including polycystic kidney disease, retinal degeneration, and primary ciliary dyskinesia. Some of these conditions involve cilia of aberrant length, which affects both the motility and signaling of cilia. Our goal is to identify the signaling pathways that link cilia length to the expression of genes encoding ciliary building blocks and regulating ciliogenesis. To this end, we used Chlamydomonas reinhardtii, a unicellular green alga, as a model system to study how cells sense the presence and length of cilia. A luciferase reporter strain was used to monitor cilia length-induced changes in gene expression in real-time. A collection of insertional mutants in the reporter strain was generated and screened for altered luciferase activity, indicating defects in ciliary gene regulation. Whole-genome sequencing identified the disrupted genes encoding flagellar-associated proteins, transcription factors, and methylases/demethylases. We identified several mutants with defects in the expression of genes required for cilia assembly. In an additional approach, we integrated RNA-Seq data from classical cilia length mutants, cells undergoing cilia regeneration induced by a pH shock, and cells treated with cilia length-altering drugs. Comparative analysis revealed a subset of genes encoding intraflagellar transport proteins, signaling proteins and flagellar-associated proteins that were consistently upregulated in both long-flagella mutants and regenerating cells. These genes likely represent a core module regulating cilia length. By integrating real-time reporter activity, whole-genome mutational analysis, and comparative transcriptomics across genetic and drug-induced flagellar length variants, we provide a comprehensive framework to dissect the interplay between ciliary length and nuclear gene expression. The key genes reported in our research offer targets for functional studies of cilia length regulation. The data will advance our basic understanding of cilia biology and possibly also have implications for identifying therapeutic targets in human ciliopathies.

B154/P0148

Dynamics and underlying mechanism of chemotaxis in the green algae model system Chlamydomonas reinhardtii

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Chemotaxis is a directed migration in response to chemical cues, which allows cells to adapt to changing nutrient conditions as well as more complex processes such as intercellular signaling mediated by pheromones and peptides. The mechanisms of chemotaxis from signaling reception to transduction are well established in most model bacteria and eukaryotic organisms. However, these components are relatively unknown in the

green algae, and specifically in the model organism Chlamydomonas reinhardtii. The C. reinhardtii genome is missing several components with consistent homology to known systems such as the bacteria chemotaxis methyl-accepting chemotaxis proteins (MCPs). A previous screen of chemotaxis revealed several strains deficient in chemotaxis towards ammonium, which are further investigated here1. On such strain is a knockout of the membrane-localized protein COP5/HKR1, which does not exhibit chemotaxis toward ammonium, despite maintaining otherwise normal motility and photobehavior. COP5/HKR1 is a membrane-bound protein that encodes an eight transmembrane helix rhodopsin, histidine kinase, response regulator and motifs associated with protein-protein interaction. Microscopic analysis at the single cell level revealed that wild-type cells change the ciliary beat pattern when swimming in an ammonium gradient, while the COP5/HKR1 disrupted strain does not. We also describe a COP5/HKR1-independent negative response that occurs at higher concentration gradients of ammonium (<100 mM of NH4+). The second finding validated here is the role of the calcium voltage-gated channel CAV2 in Chemotaxis. In wild-type strains CAV2 mediates calcium influx into cilia2. The role of calcium and CAV2 was confirmed by showing that exposure to the calcium chelator EGTA reduced chemotaxis. Microscopic analysis further shows the effect of calcium influx on ciliary beat patterns during chemotaxis. These findings reveal two key components which are required for chemotaxis signaling in green algae and provides preliminary insights into similarities and divergences between chemotaxis in green algae and other model systems.

B158/P0152

Elucidating the in situ spatial organization of intraflagellar transport (IFT) at the flagellar base in Trypanosoma brucei

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Cilia, or flagella, are evolutionarily conserved cellular appendages that fulfill crucial sensory and motility functions for many organisms. Their assembly is powered by intraflagellar transport (IFT), a system of train-like protein complexes that move bidirectionally along the axonemal microtubule doublets (MTDs). In Chlamydomonas reinhardtii, IFT has been studied extensively, with anterograde trains shown to dock along all nine MTDs and travel exclusively on the B-tubule. The other powerful model organism in which IFT has been visualized by light and electron microscopy is Trypanosoma brucei, a protozoan parasite that causes deadly trypanosomiasis in mammals. In contrast, IFT is restricted to a subset of MTDs in T. brucei, suggesting selective regulation at an early transport stage. The axoneme of the trypanosome flagellum is further distinguished by the paraflagellar rod (PFR), an extra-axonemal structure critical for motility, which contributes to the asymmetric distribution of IFT. Despite progress, how trains assemble, dock, and initiate movement at the base remains poorly understood. Recent cryo-Focused Ion Beam (cryo-FIB) and cryo-electron tomography (cryo-ET) studies in Chlamydomonas revealed the transition zone as the central hub for IFT train assembly. High-resolution microscopy in T. brucei suggested that IFT restriction begins at the proximal axoneme, near the site of PFR attachment, but the spatial organization of train assembly and docking across the basal pool, transition zone, and proximal axoneme has remained unresolved. Here, using cryo-FIB-milling and cryo-ET, we identified two distinct stages of anterograde IFT landing within the transition zone of T. brucei: initial landing and complete landing. We discovered that anterograde IFT trains associate with both A- and B-tubules of the MTDs, in contrast to their exclusive binding to the B-tubule observed in Chlamydomonas. Due to the labor intensity of FIB-milling and difficulty to capture all nine MTDs of the transition zone with precise depth control, we genetically engineered enucleated T. brucei “zoids”, which are thin enough for direct cryo-ET imaging. This system revealed alternating patterns of IFT train docking at the initial landing stage. Strikingly, these patterns

persisted even after RNAi depletion of IFT46, a protein essential for anterograde transport, suggesting that regulatory mechanisms governing train assembly and positioning may differ. These findings provide the first in situ structural evidence of IFT train docking in T. brucei and uncover a new regulation at the transition zone. By combining genetic engineering with cryo-ET, this work establishes a framework for dissecting conserved and parasite-specific mechanisms of ciliary assembly.

B153/P0147

ESCRT-0 Condensation and Smoothened Clustering drive Extracellular Vesicle Budding from Cilia

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The central function of primary cilia is to transduce signals by dynamically concentrating signaling receptors and regulators. Proteins exit cilia by retrieval and ectocytosis. Ciliary ectocytosis has been implicated in both the disposal of excess ciliary material and cell-cell communication. However, how ciliary proteins are selected for inclusion into budding ciliary EVs remains an open question. Mechanistic understanding of cargo packaging will provide critical insights into the physiological and pathological roles of ciliary EVs. Here, we leverage quantitative mass spectrometry to identify the proteome of cilia-derived extracellular vesicles and uncover the ciliary GPCR Smoothened (SMO) as the major cargo and the ubiquitin reader ESCRT-0 (HRS/STAM) as a candidate sorting machinery. Notably, 2% of the total cellular HRS/STAM and 5% of SMO are packaged into EVs via cilia. We show that K63-linked ubiquitin chains serve as the sorting signal for SMO, as SMO is highly K63-ubiquitinated in EVs, and targeting a K63-specific deubiquitinase to cilia abolishes SMO secretion. While ESCRT-III is known to mediate almost all instances of reverse-topology budding events in cells, functional analysis of the full ESCRT machinery shows that the ESCRT-0 complex—rather than other ESCRT components, including the ESCRT-III remodeling enzyme VPS4—is specifically required for packaging SMO into ciliary EV. Instead, ESCRT-0 undergoes condensation within cilia and is co-packaged with SMO into EVs. Importantly, condensation of ESCRT-0 is essential for SMO secretion into EVs. Furthermore, FRAP analyses show that SMO clusters at the ciliary tip in an UbK63-dependent manner, and optogenetic induction of SMO clustering is sufficient to trigger EV budding from cilia. Together, our findings suggest that ESCRT-0 condensation and SMO clustering cooperate to drive reverse-topology budding of ciliary EVs independently of ESCRT-III.

B162/P0156

Exploring Roles of the Kinesin-8 KLP-13/KIF19A in Ciliary Integrity and Specialization in C. elegans Sensory Neurons

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Cilia are microtubule-based organelles present on most non-dividing cells. Defects in cilia formation or function contribute to syndromic diseases termed ciliopathies. Ciliopathies have a range of symptoms including neurodegeneration, which may be caused by defects in microtubule-based processes. Microtubules incur tubulin post-translational modifications (PTMs) that regulate many functional aspects of cilia. One such tubulin PTM that is enriched in cilia is polyglutamylation, which is deposited by tubulin tyrosine ligase (TTL) enzymes

and removed by cytosolic carboxypeptidase (CCP) enzymes. In humans, mutations in CCP1 that cause microtubule hyperglutamylation cause infantile-onset neurodegeneration. In C. elegans, mutations in the CCP1 homolog, ccpp-1, cause defects of the B-tubules in the ciliary microtubule doublet region and progressive ciliary degeneration. ccpp-1 ciliary defects are partially suppressed by loss of the NIMA-related kinase, NEKL-4 (Power et al 2020). Hyperactivation of NEKL-4 by overexpression or deletion of the PEST proteolytic degradation domain causes a similar progressive ciliary degeneration phenotype and disrupts microtubule ultrastructure, suggesting that NEKL-4 activity negatively regulates ciliary stability (Power et al 2024). Consistent with this hypothesis, the human NEKL-4 ortholog is NEK10, the mutation of which causes bronchiectasis- a ciliopathy- and defects in airway mucociliary clearance (Chivukula et al 2020). To understand the mechanism of NEKL-4 action, we took a candidate gene approach for suppression of the nekl-4(PESTΔ) ciliary degeneration phenotype and identified a predicted loss-of-function mutation in the kinesin-8 KLP-13. Kinesin-8 motors are unique in that they possess both plus-end directed motor and microtubule depolymerizing activity. In yeast and mice, kinesin-8 motors are primarily known to regulate microtubule dynamics during cell division. The mammalian ortholog of KLP-13, KIF19A, localizes to ciliary tips and negatively regulates cilia length, likely due to its depolymerizing function (Wang et al 2016; Arora et al 2020). In C. elegans, ectopically-expressed KLP-13 localizes to phasmid ciliary tips and negatively regulates ADL cilia length (Park et al 2021). Here, we aim to determine KLP-13 function in cilia and its relationship to the Tubulin Code. We show that klp-13 is expressed in ciliated neurons and endogenous KLP-13 localizes to ciliary tips in the nose and tail of the worm. Loss of KLP-13 function may also influence the spatial distribution of ciliary glutamylation. We are testing the hypothesis that KLP-13 regulates ciliary integrity through interaction with the Tubulin Code using molecular, cell biological, and behavioral assays.

B163/P0157

Flagellar Length Regulation in Chlamydomonas: An Experimental Test of the Kinesin Diffusion Model

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Cells control the size of their organelles to play a fundamental role in maintaining cellular homeostasis, ensuring proper function, and adapting to various environmental and physiological conditions. However, the mechanisms behind organelle size control remain largely unknown, partly due to the complexity of many organelles, which makes these mechanisms challenging to study. Eukaryotic cilia and flagella, being microtubule-based organelles with relatively simple structures, serve as ideal models for studying size regulation mechanisms. Several theoretical models have been proposed to understand the mechanism of flagellar length control. In this study, we experimentally tested the diffusion model using Chlamydomonas. According to the diffusion model, a signaling molecule is released at the tip of the flagellum and diffuses back to its base. This model hinges on the diffusion gradient of a signal molecule produced at the flagellar tip and consumed in the cytoplasm. We focused on kinesin as the signaling molecule. Kinesin drives anterograde intraflagellar transport (IFT) from the flagellar base to the tip but diffuses back to the base. To experimentally test the kinesin diffusion model, we reduced the diffusion rate of kinesin by trapping it on axonemes using a chemically induced dimerization system. Despite reducing the kinesin diffusion rate, the length of the flagella and the IFT behavior were mostly unchanged. This observation does not support the diffusion model based on kinesin diffusion.

B152/P0146
GSTA2 and the Glutathione System in Airway Motile Cilia Disease
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Motile cilia are microtube-organized subcellular machinery organelle capable of synchronized beating to generate fluid flow circulation over epithelia or facilitate locomotion of single cells and organisms. Dysfunction of motile cilia are associated with diverse pathological consequences that include respiratory diseases, situs-inversus, hydrocephalus and infertility, collectively called primary ciliary dyskinesia (PCD). Our single-cell RNA-Seq analysis revealed that PCD multiciliated cells have upregulated expression of glutathione-S-transferases GSTA2 and GSTA1, as well as NRF2 target genes. Endogenous GSTA2 and GFP-tagged GSTA2 showed that GSTA2 is uniquely localized to the axonemal cilia and apical surfaces of multiciliated cells. Loss of GSTA2 using CRISPR guided GSTA2 knock-out in multiciliated airway cells revealed that GSTA2 are crucial for proper functional and survival of airway cells. Traditional GFP-tagged immunoprecipitation and mass-spectrometry as well as proximity labelling proteomics analysis demonstrated that GSTA2 is associated with IFT proteins, known structural ciliary proteins, as well as novel ciliary proteins

B168/P0162
Impaired ciliogenesis drives centriole clustering and loss but does not impact meiotic chromosome pairing during murine spermatogenesis
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Cilia are ubiquitous, microtubule-based organelles essential for numerous biological processes such as environmental sensing and motility. Genetic defects in their assembly and function cause ciliopathies, a group of multisystem disorders often associated with infertility. In zebrafish oogenesis, zygotene primary cilia are required for meiotic bouquet formation and homologous chromosome pairing. The distal appendage protein CEP164 plays a key role in ciliogenesis by mediating basal body docking to the plasma membrane. Although CEP164 is dispensable for sperm flagellar assembly and fertility in Drosophila, its role in mammalian spermatogenesis remains unknown. To investigate this, we generated a mouse model lacking CEP164 in premeiotic male germ cells (Stra8-iCre;CEP164fl/fl, hereafter cKO). We found that CEP164 localizes to the mother centriole/basal body throughout spermatogenesis, and cKO males are infertile. Consistent with its essential role in ciliogenesis, cKO testes lacked zygotene cilia and sperm flagella. Nevertheless, analysis of meiotic markers uncovered no major defects in chromosome pairing or DNA double-strand break repair, and spermatogenesis progressed normally to the round spermatid stage. Examination of cKO round spermatids by immunofluorescence and electron microscopy revealed basal body docking defects. Notably, supernumerary centrioles clustered in cKO spermatids, were eliminated via residual bodies, and were accompanied by an increase in acentriolar spermatids. Live-cell imaging of centriole behavior in round spermatids prepared from control and cKO mice expressing EGFP-Centrin1 demonstrated that centrioles are highly mobile and dynamic in the absence of CEP164, and multiple centriole pairs from neighboring cells frequently associate through intercellular bridges, forming aggregates. Together, these findings establish that CEP164 is essential for basal body docking, centriole immobilization, ciliogenesis, and fertility, whereas zygotene cilia are dispensable for meiotic chromosome pairing and DNA repair during mammalian spermatogenesis.

B159/P0153
Importin a Regulates Ciliogenesis and Cilia Length with Implications for Xenopus Ciliopathies
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Cilia are microtubule-based organelles essential for a wide range of biological processes ranging from facilitating fluid flow to transducing developmental and growth signals. Defects in cilia structure or function can lead to ciliopathies. Ciliogenesis and cilia length regulation depend on protein transport to the ciliary base, but the underlying molecular mechanisms remain unclear. Here we identify that the nuclear adapter protein, importin α, has conserved localization in human epithelial primary cilia and Xenopus laevis (X. laevis) epidermal multiciliated cells. We find that importin α regulates both ciliogenesis and cilia length maintenance, dependent on its localization to the membrane via palmitoylation or in the cytoplasm when not palmitoylated. Upon screening for nuclear-localization signal containing ciliary proteins, we identified Cep78, Arl13b, and Cep164 as potential importin α cargos. We subsequently found that these proteins directly bind importin α, providing mechanistic insight into their localization to the cilium. Furthermore, we found that disruption of importin α palmitoylation in X. laevis resulted in a renal ciliopathy phenotype which was rescued by forced membrane localization of importin α via farnesylation. These findings reveal a previously unrecognized role for importin α in cilia biology and advances our understanding of ciliopathy etiology.

B164/P0158
Investigating the role of TLE4 in motile cilia in Schmidtea mediterranea and primary cilia in human hTERT-RPE1 cells
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Transducin-like enhancer of split 4 (TLE4) is a transcriptional co-repressor known to modulate gene expression by interacting with other transcription factors through its carboxy-terminal WD40 repeat domain. TLE4 is most highly expressed in bone marrow and testis, particularly in late and early spermatids. While TLE4 is known to contribute to hematopoiesis and kidney morphogenesis, its involvement in cilia biology remains unexplored. This study investigates the role of TLE4 in regulating both motile and primary cilia using Schmidtea mediterranea and mammalian cell models. In Schmidtea mediterranea, RNA interference (RNAi) was used to knock down the TLE4 homolog, resulting in a 26% reduction in gliding velocity and a 41% decrease in motile cilia length. To assess its role in primary cilia, TLE4 was silenced in human hTERT-RPE1 cells using self-delivering antisense oligonucleotides (sd-ASOs), a third-generation gene silencing method that induces RNase H mediated degradation of target mRNA. Immunofluorescence has been employed to quantify cilia length and number. These results may suggest that TLE4 plays a role in ciliogenesis and microtubule organization, providing new insights into how transcriptional co-repressors contribute to ciliopathies.

B167/P0161
Linking centrin architecture with its diverse functions in ciliogenesis, motility, and behavior
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Centrin is a conserved calcium-binding contractile protein found at centrioles and basal bodies across eukaryotes, where it provides structural integrity and is required for ciliogenesis. In free-living unicellular eukaryotes, centrin also forms filamentous networks that mediate calcium-dependent cellular contractions underlying the behavioral responses of these cells. Here, we investigate these dual roles in the biciliated alga Chlamydomonas reinhardtii, which contains centrin both at the basal bodies and in networks. Using previously isolated centrin mutants, we show that mutations in centrin result in defects in cilia number and length, coupled with impaired regeneration and elongation, consistent with disrupted interactions between centrin and core centriolar proteins. Centrin also organizes into bundle-like structures at distal striated fibers that connect basal bodies. Using expansion microscopy, we show that centrin mutants exhibit abnormally wide distal striated fibers. These structural defects lead to lack of cilia coordination, leading to impaired cell swimming. Centrin also forms the nuclear basal body connector (NBBC), linking basal bodies to the nucleus via filaments and branched perinuclear networks. We observe that the NBBC undergoes calcium-induced contraction which ultimately results in the cells shedding their cilia, a typical behavioral response of the cells to stress. Altogether, our work demonstrates that centrin performs distinct, essential functions at basal bodies and filamentous networks within the same cell, coordinating ciliogenesis, ciliary motility, and calcium-dependent behavioral responses.

B155/P0149

Lithium treatment in Chlamydomonas reveals hidden length capacity in long-flagellar mutants

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The flagella of the unicellular algae Chlamydomonas reinhardtii are a genetically and experimentally tractable system for uncovering general principles of organelle size control. Whereas the suite of flagellar proteins and the kinetics of regeneration are well characterized, the identity of the molecular “sensor” of length remains elusive. It is known that new transcription and translation is necessary to regenerate flagella following deflagellation and replenish the cytoplasmic pool of tubulin in preparation for the next deflagellation event. However, lithium treatment mobilizes an otherwise inaccessible precursor pool through an unknown mechanism. Lithium exhibits a dose-dependent effect on flagellar length: intermediate concentrations (~50 mM) induce a ~50% increase in flagellar length, whereas higher concentrations (>125 mM) trigger deflagellation. However, the effects of lithium on length regulation in flagellar length mutants and paralyzed flagella mutants remain underexplored. Preliminary results show that lithium elongates flagella in long-flagella (lf4) mutants by 50% or more even though lf4 was previously thought to be at length capacity. Whereas lithium is known to affect flagellar/ciliary motility in chlamydomonas and other flagellated cell types such as, mammalian and echinoderm sperm, our results suggest that lithium treatment can uncover a novel mechanism of flagellar length control.

B157/P0151

Mouse Knockout of Ciliary Gene Togaram1 Disrupts Embryonic Development and Cerebral Cortex Neurogenesis

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The primary cilium is a solitary, microtubule-based sensory organelle that protrudes from the cell surface. This organelle functions as the cell's antenna to sense and transmit extracellular cues. A variety of signaling receptors and pathways concentrate in the primary cilium of different cell types. Mutations in genes encoding ciliary proteins can cause a class of disorders known as ciliopathies. Joubert Syndrome (JS) is an autosomal recessive ciliopathy that causes neurodevelopmental abnormalities and one of its causative genes is Togaram1. Studies in model systems and in vitro have suggested that Togaram1 regulates the growth and stability of ciliary microtubules via its four tumor overexpressed gene (TOG) domains. These data have led us to hypothesize that Togaram1 has a role in primary cilia formation and function, thus influencing mammalian body and brain development. To investigate how Togaram1 causes JS phenotypes, we obtained a Togaram1 knockout mouse line. Here we show that Togaram1 loss leads to gross phenotypic changes and cellular level defects in mice. Our analyses show that Togaram1 knockouts are embryonically lethal with craniofacial and body abnormalities, such as microphthalmia, polydactyly, and heart laterality defects. Additionally, fewer and shorter primary cilia are found on mouse embryonic fibroblasts (MEFs) derived from Togaram1 knockout embryos. The cerebral cortices of Togaram1 knockouts are affected with altered size, thickness, and dorsal and ventral structural abnormalities. We find changes to mitotic index and neurogenesis within Togaram1 knockout cortices. These data led us to investigate how Togaram1 affects primary cilia of cortical neural stem cells (NSCs). NSCs in knockout embryos have abnormal Arl13b localization and changes to ciliary marker composition. Furthermore, NSC cilia have altered Shh activity as measured by Gli3 levels on immunoblots. Our study will further look at the role of Togaram1 in mammalian brain development and through this study, we aim to improve understanding of how primary cilia influence cortical development.

B151/P0145

Proteomic Analysis of Human Primary Cilia by High-precision Microscopy-guided Photo-biotinylation

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Obtaining a high-sensitivity high-specificity proteome of primary cilia remains challenging. Optoproteomics, where a microscopy system performs image-guided automated photo-biotinylation and the subsequent pulldown and mass spectrometry assays reveal the in situ proteome, can address this challenge. We used automated image processing to recognize the locations of primary cilia from human RPE-1 cells one field of view (FOV) at a time for thousands of FOVs and directed a two-photon illumination to trigger site-specific photochemical reaction at the precision of 300 nm. A high-speed mechatronic control is implemented to coordinate imaging, pattern generation, targeted illumination, and FOV movement, allowing for the rapid biotinylation of millions of ROI spots within hours. The top identified proteins encompassed key ciliary trafficking components and those involved in structural support and cellular organization. Gene ontology (GO) enrichment analysis highlighted the significant association of high-ranking proteins with critical ciliary processes such as assembly, transportation, and signaling, particularly including proteins involved in intraciliary transport. A group of novel protein contents were identified, providing testable hypotheses for their roles in primary cilia. These findings underscore the high sensitivity and high specificity of targeted photolabeling and proteomic analysis in unraveling the network of proteins essential for ciliary function and structure, and the technology can be readily applied to primary cilia from tissue samples.

B169/P0163
Regulation of basal body and cilia homeostasis in multiciliated cells
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Multiciliated cells (MCC) contain hundreds of motile cilia that generate directional fluid flow essential for airway, brain, and reproductive functions. The half-life of MCC is long, roughly 6-12 months, dictating the need for strict maintenance programs to preserve cilia ultrastructure, length, and motility. Importantly, loss of cilia maintenance occurs in motile ciliopathy patients with acquired and genetic airways diseases, highlighting the essential role of this homeostatic program in human physiology. While mechanisms of cilia assembly are widely studied, the cellular processes for maintenance of cilia in mature MCC remain unclear. Here, we define a key role for basal bodies (BB) in the regulation of cilia homeostasis. We show that in airway tissues from patients with asthma and COPD, there is loss of ciliary homeostasis that surprisingly features loss of BB proteins from their normal positions, and their translocation to the cytoplasm. These BB-associated proteins accumulate in unique cytoplasmic sites called the Fibrogranular Materials that contain autophagy and proteosome components. We observed similar effects on BB proteins upon loss of cilia maintenance in a mouse model of asthma, and in human airway epithelial cells treated with inflammatory stimuli that induce loss of cilia homeostasis. To further investigate the role of BB in regulation of cilia homeostasis, we conditionally deleted ciliopathy gene Cep120, which is essential for BB function, in adult mice with fully developed MCC. Using expansion microscopy, we show that Cep120 is enriched at the distal end of BB where attachment to the apical cell membrane occurs. Cep120 loss in mature MCC in vitro altered BB composition including loss of luminal proteins, distal appendages, basal feet, and rootlets. Moreover, we observed changes in the apical cytoskeletal networks causing loss of BB anchoring, defective cilia ultrastructure and ultimately disassembly of cilia, similar to the human disease tissues. Finally, we found that the changes in BB morphology and function led to loss of cilia homeostasis in mouse airways and brain in vivo. Collectively, our results indicate that BB act as multifunctional hubs to regulate the assembly and turnover of protein complexes essential for motile cilia homeostasis in mature MCC, and that these BB functions are interrupted in acquired airway ciliopathies.

B156/P0150
Regulation of radial spoke transport by the IFT adapter ARMC2
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Cilia assembly requires protein delivery by intraflagellar transport (IFT). How IFT binds to its diverse cargoes and how these interactions are regulated remains largely unknown. Here, we analyzed ARMC2, an IFT adapter that connects the multi-subunit radial spoke precursor complex (RSPC) to IFT trains. Mutations in ARMC2 cause paralyzed cilia lacking radial spokes in Chlamydomonas and male infertility and reduced lung function in mammals. Using Chlamydomonas, we show that the N-terminal intrinsically disordered region of ARMC2 (ARMC2IDR) binds to IFT, presumably via the IFT70/IFT52 complex. The C-terminal armadillo repeat region (ARMC2ARM) binds the RSPC, via the N-terminal IDR of its RSP3 subunit. While rarely observed in full-length cilia, IFT of ARMC2 is upregulated in growing cilia. Deletion of the ARM2ARM and expression of the ARM domain-binding RSP3IDR peptides increases ARMC2 traffic in full-length cilia. Thus, the ARMC2ARM has a dual function in RSPC cargo binding and suppressing ARMC2-IFT interactions. We propose that autoinhibitory

interactions between the ARM and the IDR to prevent ARMC2 interactions with both IFT and the RSPC cargo. Interaction between the ARM and IDR domains is supported by yeast two hybrid assays and structure guided mutations, which interrupt ARM-IDR binding and increase ARMC2 transport by IFT. IFT of ARMC2 was also strikingly increased in mutants lacking the cilia length-restricting kinases LF4/MOK and LF5/CDKL5, indicating that these kinases are required, direct or indirectly, to suppress ARMC2-IFT interactions. We propose that the frequency of radial spoke traffic is regulated by the activity of ARMC2, which is largely autoinhibited in inactive in full-length cilia and activated in short cilia, allowing its binding to IFT trains and its RSPC cargo.

B173/P0167

The role of ARL13B in WWP1 ciliary localization

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The Hedgehog (Hh) signaling pathway in vertebrates regulates essential processes such as patterning and cellular proliferation. When Hh signaling is disrupted, it can cause developmental defects, and when Hh signaling is hyperactive, it can lead to cancer. In vertebrates, the Hh pathway relies on primary cilia, tiny cellular protrusions that act as signaling hubs. Pathway components dynamically localize in and out of the cilium, including Smoothened (SMO), the primary transducer of the Hh pathway. When the Hh ligand is absent and the pathway is inactive, SMO is marked with K63-linked ubiquitin chains and removed from cilia, making it undetectable. SMO ubiquitination is mediated by the E3 ligase WWP1. Here, we investigate the role of ARL13B, a regulatory GTPase, in WWP1 localization. SMO accumulates in the cilia of Arl13b-null cells, but the mechanism of this accumulation is unknown. Furthermore, SMO constructs tagged with ubiquitin (SMO-UB) are undetectable in Arl13b-null cilia. Therefore, we hypothesized that ARL13B controls SMO ubiquitination by regulating the localization of WWP1. We expressed WWP1-FLAG in wild-type and Arl13b-null mouse embryonic fibroblasts (MEFs). In the absence of the Hh ligand, we found WWP1-FLAG localizes to the cilium in wild-type MEFs as expected from published work. However, we observed a possible reduction of ciliary WWP1-FLAG localization in Arl13b-null MEFs. We will follow this up in future work and will investigate whether rescuing the ciliary localization of WWP1 rescues the SMO phenotype of Arl13b-null mutants.

B170/P0164

γ-Tubulin23C And Centrosomin (Cnn) plays a synergistic crucial role in maintaining the olfactory cilia structure, thus function in Drosophila

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Cilia are involved in a plethora of motility- and sensory-related functions. Ciliary defects cause several ciliopathies, some of which are late-onset, suggesting that cilia are actively maintained. The ciliary base is involved in templating the cilium and organising the cytoskeleton. Though the ciliary base's role in ciliogenesis was quite well-explored, its role in cilium maintenance remained unknown. By using multidisciplinary techniques on the long-lived ciliated cells in Drosophila, we showed that while several core centriole and peri-centriolar proteins continue to localise to the ciliary base, others disappear from the ciliary base after ciliogenesis is complete. We further demonstrated that some of the ciliary base proteins that persist in the fully assembled cilia are also dynamic, suggesting that the ciliary base structure may be more dynamic than previously believed. Subsequently, using conditional knockdown experiments, we identified that at least two

pericentriolar proteins, such as **γ**-tubulin and Cnn, are critical for ciliary ultrastructure integrity. We then demonstrated that these two proteins also interact in ciliated cells, and the simultaneous loss of these proteins results in a synergistic increase in the defects observed in adult olfactory cilia. Finally, we described a novel process for how olfactory cilia maintenance is deregulated in neurons with the conditional loss of these two centrosomal components. Altogether, our findings elucidate the roles of two key pericentriolar proteins in regulating the stability of the ciliary base, thereby affecting the ciliary shaft, in long-lived metazoan cilia involved in olfactory reception.

Cytoskeleton and Motility - Intermediate filaments and septins

B146/P0140
Bni1-Boi2 Interplay Regulates Septin Localization during Yeast Mating
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Septins, first discovered in Saccharomyces cerevisiae, are crucial for cellular morphogenesis, especially during the mating response. In S. cerevisiae, mating is triggered by pheromone signaling, which activates Cdc42 polarization and leads to the formation of a mating projection called a shmoo. Depending on pheromone concentration, yeast cells either form a tight shmoo at high doses or elongate toward the gradient at lower doses. Both morphological outcomes depend on proper septin localization at the periphery of the polarity site, and any mislocalization results in abnormal cell shape. Despite their central role, the mechanisms regulating septin localization during this non-mitotic process remain poorly understood. Bem One Interacting protein2 (Boi2), part of the Cdc42 polarity machinery, functions in cytokinesis, exocytosis, and the NoCut pathway alongside septins during the cell cycle. Boi2 also interacts with the formin Bni1, which drives actin cable formation. Since Boi2 and Bni1 engage with polarity components during mitosis—many of which are reused during mating—we hypothesized they collectively contribute in septin localization during the pheromone process. We found that Boi2 localization is pheromone dose-dependent: at high doses, it localizes to the base of the projection (like septins), while at intermediate doses, it localizes to the tip. Boi2 also colocalizes with septins, supporting its role in septin positioning. In boi2Δ cells, septins were mislocalized near the polar cap, and cell morphology was abnormal, confirming Boi2's importance. Given Bni1's role in gradient tracking—a septin-involved process—we deleted BNI1 and observed mislocalized septins, and the overall septin structures were significantly reduced compared to wild-type cells, further implicating Bni1 in septin regulation. To probe their interplay, we examined localization of each protein in the absence of the other. In bni1Δ cells, Boi2 localized to the tip; in boi2Δ cells, Bni1 appeared in multiple foci, suggesting Boi2 promotes Bni1 polarization. To better understand how Boi2 and Bni1 contribute to septin localization, we examined the exocyst components Exo70 and Exo84, which interact with Boi2 and mediate exocytosis. In boi2Δ cells, these exocyst components appeared in scattered foci instead of a single polarized spot at the cell tip indicating that Boi2 organizes both Bni1 and the exocyst. In conclusion, our results suggest that septin localization during yeast mating is regulated through an interplay between the polarisome (Bni1), the exocyst complex (Exo70/Exo84), and Boi2.

B145/P0139
Cdc42EP3-bound septin scaffolds promote actin polymerization
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Septins are cytoskeletal filament-forming proteins that typically associate with membranes and perform critical functions in a variety of cellular processes. Septins colocalize with multiple actin and microtubule structures, yet our understanding of the ways that septins contribute mechanistically to actin- and microtubule-based functions is incomplete. The Cdc42 effector protein Cdc42EP3 (also known as BORG2) promotes septin localization to actin structures in vivo, but little else is known about how Cdc42EP3 influences the interactions of septins and F-actin. Here, using purified components, we show that Cdc42EP3 binds directly to septins, actin filaments and actin monomers. Moreover, septin-bound Cdc42EP3 accelerates actin filament polymerization, primarily through accelerated filament nucleation. Thus, Cdc42EP3 is not merely a factor that crosslinks septins and F-actin, but one that promotes the formation of actin polymers along septin scaffolds.

B148/P0142

Keratin filament and cell-cell junction cooperation during developmental fate decisions

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Epithelia are tissues composed of sheets of polarized cells whose strong cell–cell interactions form selectively permeable barriers throughout the body. A defining feature of vertebrate epithelia is the expression of keratins, a large, diverse family of intermediate filament–forming cytoskeletal proteins that impart critical mechanical integrity to epithelia through their coupling to cell-cell and cell-matrix adhesion complexes. Epithelial cells sense the mechanical properties of their cellular environment and integrate this information to control fate decisions (proliferation, differentiation, cell death). Epithelial mechanosensing is mediated by a complex network of cell-cell junctions, cytoskeletal filaments (including keratins), and signaling pathways that regulate cytoskeletal dynamics, transcription, and ultimately cell behavior. In the mammalian epidermis, differential expression of keratins with distinct biochemical properties was shown to regulate cell identity through a feedforward loop with the transcriptional co-activator YAP1. Using a Xenopus laevis (African clawed frog) embryo model, this project aims to build on these findings by, (1) contextualizing the keratin filament network within the broader epithelial cytoskeleton and cell-cell adhesion complexes and (2) examining the role of keratins during embryonic cell fate specification. In humans, the patterned expression of the large keratin gene family (N=54 in humans, N=45 in Xenopus laevis) has produced a sophisticated ‘keratin code’ linking specific keratins to discrete developmental, tissue, and pathological states, yet their roles in non-mammalian vertebrates like Xenopus laevis remain poorly understood. Through analysis of publicly available RNA-seq datasets and additional computational analyses, we have identified putative Xenopus laevis orthologs of human keratins as candidates for mechanistic manipulation. Using microinjection of mRNA encoding fluorescently-tagged proteins, we have successfully imaged candidate keratins at multiple developmental stages. Preliminary results suggest keratin-dependent effects on specification of post-mitotic, differentiated cell types during ectodermal stratification. Ongoing work seeks to quantify how differential keratin expression influences embryonic keratin network dynamics and cell fate, and to test whether this developmental mechanism also contributes to tumor initiation using a Xenopus laevis tumor model. By combining live imaging, computational analysis, and tumor modeling, this project will elucidate how keratin networks with differential biomechanical properties intersect with cell-cell adhesions, actomyosin dynamics, and transcriptional effectors to coordinate epithelial cell fate.

B142/P0136
Keratin filaments and soluble vimentin interact with isoform specificity to organize cell signaling
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The keratin intermediate filament (IF) cytoskeleton provides crucial mechanical support for epithelial tissues, but the large number of keratin genes and their context-dependent expression patterns suggest additional non-mechanical roles. How individual IF proteins might perform different non-mechanical functions while maintaining the canonical IF mechanical scaffold has not been clear. We recently found that changing keratin expression during skin wound healing triggers a myosin-activating signaling circuit, potentiating wound closure without compromising mechanical stability (Nanes et al., Dev Cell, 2024). We now report that this signal transduction pathway relies on non-filamentous small oligomers of vimentin, the principal IF protein in mesenchymal cells, to shuttle regulatory kinases into transient signaling complexes with myosin organized on keratin 6A-containing filaments. These results highlight two mechanisms by which IF proteins differentially influence cell signaling and cell mechanics. First, through isoform specific interactions, changing keratin expression modulates recruitment of different proteins to the IF cytoskeleton, increasing the likelihood that these proteins interact. This allows the IF cytoskeleton to spatially and temporally organize signal transduction, effectively functioning as a membraneless organelle. Second, largely overlooked non-filamentous pools of even lowly expressed IF proteins can function as signaling shuttles or adapters. Context-dependent cell and tissue regulation, rather than simple mechanics, may therefore explain the size of the IF protein family.

B141/P0135
Leveraging imaging mass cytometry to quantify the contributions of intermediate filaments to sensory neuron development
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Intermediate filaments (IFs) constitute a highly conserved superfamily of ubiquitous, cell-specific cytoskeletal proteins, with approximately 70 genes encoded in mammals. In neurons, up to ten different IFs are involved in many essential processes, like developmental axon outgrowth, axon caliber/integrity, and injury signaling. Remarkably, while all neurons express IFs, different types of neurons express different combinations of IFs; these highly regulated expression patterns (“IF codes”) imply specific roles for each IF that are yet to be uncovered. One subtype, peripherin, is expressed primarily in the peripheral nervous system and up-regulated coincidently with developmental axon outgrowth and during axon regeneration after injury. Mutations in peripherin are linked to several degenerative nerve diseases, such as peripheral neuropathies and amyotrophic lateral sclerosis. Still, how peripherin contributes to normal neuronal physiology and pathophysiology is unknown. We hypothesize that peripherin is a necessary component of IF networks of developing DRG neurons, specifically of nociceptive neurons. Here, we utilize imaging mass cytometry, a multiplexed spatial proteomics technique with subcellular (one square micron) resolution, to quantify the relative expression of eight IF proteins and define “IF codes”, cell type-specific IF protein expression patterns, across development within wildtype and peripherin knockout murine DRG neurons. Results: Using the neurotrophin receptors TrkA, TrkB, TrkC, and Ret, we track IF expression in four different DRG neuron subpopulations across all ages. With

additional, well-established cell identity markers, we further characterize eight adult DRG neuron subpopulations: TrkA+ (peptidergic nociceptors, 'temp-chem' nociceptors), TrkB+ (Aδ LTMRs, Aβ RA-LTMRs), TrkC+ (proprioceptors, Aβ SA-LTMRs), and Ret+ (non-peptidergic nociceptors, C-LTMRs). First, we show that the expression of neurofilaments L and M, α-internexin, and peripherin is broad across immature (E15) DRG neuron populations but becomes restricted to specific subpopulations by adulthood. Next, we confirm that peripherin appears to be largely dispensable for DRG cell type specification and show that global knockout of peripherin alone does not appear to alter adult DRG cell type percentages or properties. Finally, we provide evidence that all peripherin knockout neurons maintain a competent IF network into adulthood, likely due to their redundant expression of either α-internexin or neurofilament L. In conclusion, we have defined “IF codes” throughout development of the murine DRG and determined how the loss of peripherin affects these codes. Future studies will define “IF codes” in injured wildtype and peripherin knockout DRG neurons.

B144/P0138

Mechanically-Induced Septin Networks Protect Nuclear Integrity During Confinement

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Physiological environments are crowded and cells are constantly under tension from internal and external forces. The cytoskeleton allows cells to maintain their shape in these conditions. While mechanical contributions of actin, microtubules, and intermediate filaments have been explored in detail, the role of the septin cytoskeleton is less clear. Here we investigate the role of septins in cell mechanics. Using SEPT2-Halo knock-in fibroblasts, we observed septins preferentially co-localizing with actin filaments under the nucleus and moving with it during migration. Cell enucleation resulted in viable cytoplasts that lacked central actin-septin networks. Surprisingly, replacing the nucleus with a glass bead (8-12 um) was sufficient to partially recover this septin network. As these results suggest that nuclear mechanical forces stimulate septin network assembly we used a dynamic confinement system to apply a compressive force to cells. During confinement septins accumulated specifically under the nucleus, translocating from other regions. In comparison, actin and myosin intensity increased uniformly across the ventral surface. To test whether internally generated forces were sufficient to induce septin accumulation, we measured septin recruitment during migration through a microfluidic device. Septins consistently assembled at the cortex where the nucleus was pressed against the chamber walls. To better define the nanoscale organization of septin and actin at the cortex, we used iPALM. We determined that septin filaments are specifically positioned between the actin stress fibers and the membrane. This suggests septins are responding to mechanical forces on the cortex to dynamically reinforce these regions of the actin cytoskeleton. To investigate this hypothesis, we tested whether loss of septins impaired transwell migration or ability to withstand compression. Knockdown of SEPT7 resulted in greater nuclear deformation after migrating through a transwell and increased nuclear rupture following compression. We propose a model whereby septins are acting as a dynamic shock absorber to protect the cortex, and thereby the nucleus and other organelles, from excessive strain. This highlights a novel mechanosensing role for septins in the cytoskeleton, and illustrates how septins contribute to maintaining cell shape and migration through complex environments.

B149/P0143
Motile peripheral vimentin intermediate filament plays a role in cell migration and polarity
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Vimentin intermediate filaments (VIFs), together with microtubules and actin, are essential cytoskeletal elements that drive cell migration and establish polarity. The microtubule motor kinesin-1 actively transports VIFs to the cell periphery, and we demonstrate that vimentin is motile throughout the cell. In migrating cells, VIFs accumulate at the rear edge, where they align with focal adhesions (FAs). To determine whether vimentin dynamics is functionally important, we analyzed cells lacking either kinesin-1 or vimentin. Vimentin-null cells exhibit impaired directional migration, heightened FA turnover, and loss of FA polarity. In kinesin-1–null cells, VIFs collapse into a perinuclear bundle, depleting the periphery of vimentin and recapitulating the FA defects observed in vimentin-null cells. Rescue with non-filamentous vimentin mutant (Y117L) fails to restore adhesion patterning or polarity, underscoring the necessity of filamentous VIFs. Finally, using optogenetic and genetic tools to acutely remodel VIF assembly in kinesin-1–null cells, we show that dynamic control of filamentous vimentin immediately alters FA distribution. These findings support a model in which kinesin-1–mediated transport of peripheral VIFs directs FA organization and thereby establishes cell polarity.

B150/P0144
Septin crosstalk with microtubules and actin filaments is regulated by phosphorylation of septin 9 by the glycogen synthase kinase 3 beta.
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Septins are a family of hetero-oligomeric GTP-binding proteins that contribute to diverse cellular processes including morphogenesis, cell division, migration, and intracellular trafficking. Septins assemble into higher-order polymers and filamentous structures that associate, and crosstalk functionally with both actin filaments, and microtubules (MTs). How septin crosstalk with actin filaments and microtubules is regulated is poorly understood. Here, we show that phosphorylation of septin 9 (Sept9) by GSK3b shifts septins from microtubules to the actin cytoskeleton, a regulation that is critical for neuronal axon generation and the establishment of neuronal polarity. Phosphoproteomic studies had previously identified two serine residues in the N-terminal microtubule- and actin-binding domain of Sept9 as phospho-targets of GSK3β. Using in vitro phosphorylation assays, we found that recombinant Sept9 is directly phophorylated by GSK3β. To assess the functional consequences, we generated phosphomimetic (A-to-E) and phosphonull (S-to-A) mutants of Sept9, and expressed them in MDCK cells while knocking down endogenous Sept9. Microtubule regrowth experiments after nocodazole washout showed that phosphomimetic mutants reduce localization to centrosomal MTs and impair MT growth, whereas phosphonull mutants promote MT regrowth relative to wild type. Interestingly, phosphonull variants displayed decreased co-localization with actin filaments in both MDCK and U2OS cells, while phosphomimetic mutants exhibited enhanced actin association. In vitro binding assays also showed that recombinant phosphomimetic Sept9, and Sept2/6/7/9 complexes, which contained phosphomimetic Sept9, had diminished MT binding and bundling activity. In MDCK cells, inhibition of GSK3β with SB216763 reduced endogenous Sept9–actin association, and shifted Sept9 localization toward centrosomal MTs in nocodazole wash-out assays. In primary rat hippocampal neurons, Sept9 localization was elevated on microtubules with

inactivated pSer9-GSK3β. Notably, expression of the phophomimetic Sept9 mutants decreased asymmetric neurite growth, impairing the development of neuronal polarity. Collectively, these results indicate that septin crosstalk with microtubules and actin is regulated by Sept9 phosphorylation by GSK3beta, which may integrate various signaling pathway outputs into the function of septins in microtubule or actin driven processes.

B143/P0137

Septins dampen actomyosin forces during confined cell migration, protecting the nucleus from tensile damage.

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Septins associate with actomyosin filaments, but their functions in mechanotransduction are poorly understood. Here, we propose that actin-associated septins dampen the tensile forces transmitted along the actomyosin cytoskeleton, protecting actin filament integrity and the nucleus from mechanical stress damage. Using a confined 3D transmigration assay, which requires nuclear threading through micron scale wide pores, we show that zyxin levels increase along actin cables with decreasing pore diameters, indicative of higher tensile forces and strain-induced damage. Super-resolution imaging of the perinuclear cytoplasm inside the limiting pore revealed an array of contractile actomyosin cables, which associate with septin 9 (SEPT9) enriched septin filaments. SEPT9 knock-down by CRISPR/Cas9 resulted in actin cable bending, and increased zyxin. Consistent with increased mechanical strain and damage on actin filaments, SEPT9_i1 knock-out also enhanced actin stress fiber bending and breakage in mouse embryonic fibroblasts (MEFs) after hyper-activation of actomyosin contractility with calyculin A in 2D cell cultures. Time lapse imaging showed that over 80% of actin breaks take place on septin-free actin segments. Moreover, septin-free actin filaments retract significantly faster than septin-coated actin after breakage. In 3D transmigration assays of SEPT9-depleted cells, enhanced tensile forces and stress damage correlated with increased nuclear DNA damage (double stranded DNA breaks), micronuclei, nuclear blebs and cell death. To test whether SEPT9_i1 is both necessary and sufficient in protecting the nucleus from damage during confined migration, we over-expressed GFP-SEPT9_i1 in SEPT9 knock out MEFs migrating through 3 micron-wide pores. We found that GFP_SEPT9_i1 rescued DNA damage, micronuclei and nuclear blebs. Collectively, these data suggest that septins relieve tensile forces in the actomyosin network, protecting the nucleus from lethal levels of genotoxic damage.

B147/P0141

Septins Mediate Cell-Cycle-Dependent Exclusion of the Cdc42 GAP Rga4 from Growth Sites in Fission Yeast

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The Rho-GTPase Cdc42 is highly conserved amongst eukaryotes and establishes sites of polarized growth. Schizosaccharomyces pombe employs a simple yet highly conserved Cdc42 regulatory system to maintain a bipolar rod-like growth pattern and to coordinate arrest of this growth during mitosis. Rga4 is a potent inhibitor of Cdc42 and is confined to the sides of the cell cortex in a punctate pattern during interphase, where it inhibits Cdc42 activity everywhere but the growing cell ends. During mitosis, Rga4 becomes more dynamic and homogeneous along the entirety of the cortex, blocking growth until division completes. We investigated septins as potential regulators of Rga4 localization. These filament-forming cytoskeletal proteins share a punctate cortical localization pattern with Rga4 during interphase, before receding to form a ring at the cell middle during mitosis. In cells with septin deletion or mislocalization, Rga4 becomes highly mobile, homogeneously distributed, with increased levels in the cytoplasm regardless of cell-cycle stage. Curiously,

Rga4 does not appear to directly interact with septins. However, in the absence of septins on the cortex, we see enhanced localization of PI(4,5)P2 in the plasma membrane. In agreement, we find that cells with a defect in PI(4,5)P2 synthesis show enhanced Rga4 localization to the cortex compared to the cytoplasm. This suggests that the septins may indirectly regulate Rga4 localization via lipid organization. The septin mutants with enhanced Rga4 levels at the cell ends show a disruption of Cdc42 activation dynamics, increased monopolarity and changes in cell morphology. The septin cytoskeleton plays an important role in cytokinesis. Here we show that in interphase these proteins display a distinct cortical localization pattern that regulates Rga4 distribution along the cortex. We propose that septins regulate Rga4 localization and dynamics in a cell-cycle-dependent manner by confining Rga4 to the cell sides during interphase, during mitosis the septin filaments recede toward the division plane thus freeing Rga4 to spread out along the cell cortex. Our findings show how the cell cycle regulates cell polarity via the septin cytoskeleton-dependent membrane lipid organization.

Cytoskeleton and Motility - Microtubule Organization and Dynamics

B088/P0084
A mechano-chemical model for γTuRC-mediated microtubule nucleation
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Nucleation by the γ-tubulin ring complex (γTuRC) is the primary mechanism for microtubule (MT) multiplication in cells, yet its mechanism remains poorly understood—the prevailing template activation model cannot explain key experimental observations. We present a structure-based mechano-chemical model that integrates MT dynamic instability with a two-step open-to-closed transition of γTuRC. Closure resembles the MT sheet-to-tube transition, but with cone rather than tube geometry. The first step requires straightening of γ-tubulin complex proteins (GCPs), enabling lateral bonding between α,β-tubulin subunits bound to γ-tubulins, which supports sheet growth from γTuRC, suppressing catastrophe and promoting persistent elongation. The second step involves lateral rotation of GCPs and a bent-to-straight transition of γ-tubulins, driving sheet-to-tube conversion and yielding longer, more stable MTs and ensuring efficient nucleation. Simulations reproduce all recent in vitro results on γTuRC-mediated nucleation and reveal the underlying mechanism as an ordered sequence of conformational changes in GCPs and α, β, γ-tubulins.

B082/P0078
Arabidopsis AUGMIN8 contains two independent microtubule association domains
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Plant cells form a cortical microtubule network nucleated by γ-tubulin ring complexes primarily through microtubule-dependent microtubule nucleation (MDMN). The cortical array reorganizes in response to developmental and environmental cues to form patterns that influence cellulose patterning in the primary cell wall. The molecular mechanisms underlying formation of these microtubule array patterns are largely unknown. In animal cells, the hetero-octameric Augmin complex facilitates MDMN by associating γ-tubulin ring complexes to existing microtubules, creating parallel branched microtubules in mitotic spindles. Acentrosomal plant cells also use the Augmin complex (AUGMIN) to create mitotic spindles. Flowering plants encode a family of nine AUGMIN8 paralogs, identified as the QWRF gene family, several of which are expressed in non-mitotic cells. AUGMIN8 is the key linker protein connecting the AUGMIN complex to the parent microtubule. The AUGMIN8 paralogs are distinguished by an unstructured N-terminal half encoded in a single 5’ exon while

maintaining conserved C-terminal helices encoded by multiple short exons. Protein sequence alignments indicate conservation across animals and plants of the C-terminal helices known to associate AUGMIN8 with other AUGMIN subunits. We hypothesize that the QWRF paralogs form interchangeable AUGMIN microtubule binding subunits that confer specific roles to the AUGMIN complex in mitotic and non-mitotic microtubule arrays. The paralogs' distinct N-terminal elaborations may influence the geometry of AUGMIN's association with microtubules, thus specifying the direction and angle of MDMN. We determined the sites of cortical microtubule interaction for QWRF1,2,4, and 8 using transient expression of fluorescently tagged constructs in the heterologous Nicotiana benthamiana system. We observed that QWRF8 and QWRF4 associate with non-mitotic, cortical microtubules as distributed puncta where QWRF8 shows evidence for two independent sites of microtubule association. Sequence comparisons and in vivo assay with homologous fragments from QWRF1, 2, 4, and 5 define a shared N-terminal conserved microtubule association domain. We identify protein regions leading to formation of microtubule associated 'QWRF bodies' potentially linked to discontinuous localization on microtubules. We additionally identify the 'QWRF' protein motif as a conserved domain associating the AUGMIN8 paralogs to AUGMIN6, part of the larger AUGMIN complex. Future work will address the AUGMIN8 paralogs' roles in creating/maintaining cortical microtubule arrays in Arabidopsis.

B081/P0077

Asymmetric C-terminal Tail Dynamics and Interactions in the Microtubule Lattice Revealed by All-Atom Molecular Dynamics Simulations

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Microtubules (MTs) are dynamic cytoskeletal polymers assembled from α- and β-tubulin dimers that play central roles in cell structure, motility, and division. Since their discovery in the 1960s, extensive research has elucidated MT assembly, stability, and dynamic behavior, but the structural and functional roles of the flexible C-terminal tails (CTTs) of tubulin remain less understood. These highly dynamic, negatively charged regions influence the interactions of motor proteins and microtubule-associated proteins (MAPs) with the MT surface, yet their conformational landscapes within the lattice are largely inaccessible to experimental methods due to their intrinsic disorder. To address this gap, we performed microsecond all-atom molecular dynamics simulations of 14-protofilament MTs in the compacted GDP-bound state. Our analyses show that the CTTs of both α- and β-tubulin preferentially engage the offset "back" tubulin neighbor through high-frequency electrostatic contacts. Specifically, an arginine on β-tubulin and a lysine on α-tubulin form persistent salt bridges with acidic residues at the base of the opposing CTTs. These asymmetric tail–neighbor interactions stabilize local lattice environments and may influence protofilament bending during depolymerization. Collectively, our findings provide an atomistic view of how CTT-mediated contacts contribute to microtubule regulation.

B085/P0081

Axonal cytoskeletal bulk flow in vivo during early zebrafish development

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Axonal cytoskeletal bulk flow is an emerging transport mechanism that drives the coordinated movement of cytoskeletal structures, such as microtubules (MTs), actin filaments, and associated proteins, along the axon.

Unlike motor-driven cargo transport along individual filaments, bulk flow involves large-scale translocation of the cytoskeletal network itself. This large-scale translocation mechanism has been underexplored with respect to its abundance, properties, and functions. While bulk flow has been primarily characterized in cultured neurons, its presence and regulation in vivo remain largely unknown. Moreover, whether differences exist between bulk flow dynamics in peripheral (PNS) and central nervous system (CNS) neurons is unclear. To address these questions, we investigated cytoskeletal bulk flow in vivo using zebrafish larvae expressing fluorescently tagged cytoskeletal marker proteins in specific neuronal populations, including Rohon-Beard and dorsal root ganglion (DRG) neurons. Live imaging on DRG neurons revealed that in peripheral axons, anterograde and retrograde MT bulk flow rates were approximately 144 μm/hr and –154 μm/hr, respectively, whereas in central axons the corresponding rates were 114 μm/hr and –144 μm/hr. This is the first evidence of cytoskeletal bulk flow in both directions in both central and peripheral axons of the same PNS neuron in vivo. Our work aims to determine whether the differences in bulk cytoskeletal flow contribute to typical elongation rates and regenerative capacities, providing new insight into how cytoskeletal dynamics shape neuronal development and repair.

B103/P0099

Chlamydia uses a single inclusion membrane protein to control the host microtubule and actin cytoskeleton

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Chlamydia trachomatis is an obligate intracellular bacterium that has developed sophisticated mechanisms to survive inside its infectious compartment, the inclusion. Notably, Chlamydia weaves an extensive network of microtubules (MTs) and actin filaments around its inclusion to maximize its intracellular survival. The formation of these cytoskeletal scaffolds is temporally regulated. MT scaffolds form first, after which they are post-translationally modified (PTM). These PTM-MT scaffolds are critical for interactions between the inclusion and host organelles from which Chlamydia co-opts much-needed nutrients. Later, during infection, actin scaffolds form and are subsequently stabilized to reinforce the growing inclusion membrane. Without actin scaffolds, the inclusion prematurely ruptures, which is detrimental for Chlamydia. Despite the global health and economic burden caused by this sexually transmitted pathogen, little is known about how these cytoskeletal scaffolds are integrated into an increasingly complex virulence system. Chlamydia expresses an effector protein on the inclusion membrane called InaC, which we have demonstrated is a master regulator of the host cytoskeleton. InaC co-opts the host GTPases ARFs 1 & 4 and RhoA to form and stabilize MT and actin scaffolds. Crosstalk between these GTPases allows for the temporal coordination of MT and actin scaffolds around the inclusion. Ultimately, InaC orchestrates the formation of these cytoskeletal scaffolds using distinct functional domains. By understanding how Chlamydia hijacks complex networks, we will uncover how this clinically significant pathogen parasitizes its host and reveal novel cellular signaling pathways.

B086/P0082

Comparative Biophysics of the Tau Family Microtubule Binding Domain

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The tau family is a group of microtubule (MT)-associated proteins (MAPs) found across the tree of life. Its members are defined by a shared microtubule-binding domain (MTBD), which consist of a tandemly repeated

~29-32 amino acid sequence. These charged, unstructured repeats have several conserved sequence features, including a KXGS motif. How and why a diverse group of proteins in a variety of species uses a shared motif to bind MTs, and how this binding is tuned to suit their different needs, is not well-understood. We aim to understand how tau family MTBD sequence properties are distributed across the tree of life and to identify how both conserved and variable features affect MT binding. To explore how the conserved serine residue in the KXGS motif of this domain affects MT binding, we designed and purified two constructs containing three repeats based on the human MAPT MTBD region: one with wild-type serine residues, and one with alanines. We compared MT-binding affinity in two assays: surface-immobilized, taxol-stabilized bovine brain MTs and dynamic interphase MTs in Xenopus laevis egg extract. The extract system is conceptually equivalent to expression in living cells, but allows precise titration of the concentration of the added construct. In both systems, the serine construct displayed higher MT affinity than the alanine one, consistent with AlphaFold predictions that the serine forms a hydrogen bond with α-tubulin. Interestingly, the apparent affinity difference was greater in extract than in vitro, which we plan to explore further. To identify additional features of interest, we gathered over 30,000 MTBD sequences from the PFAM database and our own HMMER searches. We have begun analyzing these sequences to reveal shared and divergent sequence properties across taxa, which will guide the design of new constructs. For example, we observed that vertebrate and invertebrate repeats are typically 31 and 30 amino acids long, respectively, and designed synthetic constructs to test the effects of repeat length on MT-binding. Preliminary experimental results suggest that 31-residue repeats have a higher binding affinity for vertebrate MTs than the 30-mers, suggesting that MTBD repeat length may be adapted to taxon-specific MT lattice features. In summary, this workflow allows us to identify important sequence features and test their functional contributions and offers a new approach to biophysical characterization of this important domain.

B091/P0087

Determining the mechanistic roles of tubulin glutamylation in the regulation of neuronal functions

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The microtubule cytoskeleton plays crucial roles in regulating a vast range of biological processes in different cell types. In neurons, microtubules are essential for intracellular transport over long distances. Posttranslational modifications (PTMs) of tubulin, which are enriched on neuronal microtubules, are expected to dynamically regulate their specific cellular functions. The PTM polyglutamylation has been shown to control the binding of some microtubule interactors and affect neuronal transport of different cargos. Abnormally increased polyglutamylation causes neurodegeneration in mice and humans. How polyglutamylation of neuronal microtubules translates into such drastic physiological effects is, however, not understood. We thus aim at determining how polyglutamylation affects physiological functions driven by microtubule-based transport in neurons. We obtain primary neurons with different levels and patterns of tubulin polyglutamylation from knockout mouse models. Using super-resolution microscopy, we determine how changes in this PTM affect cargo delivery to their target sites, as well as their ensuing functions. We further investigate how polyglutamylation regulates the intracellular dynamics of microtubule-associated proteins (MAPs) and whether changes in their dynamics affect cargo transport. Using fluorescence decay after photoactivation we investigate the molecular dynamics of MAPs in neurons with altered tubulin polyglutamylation. We found that the MAP Tau, which is linked to a range of neurodegenerative conditions, changes its interaction with microtubules in neuronal axons if polyglutamylation is altered. We thus start to understand how tubulin PTMs contribute to the regulation of cytoskeletal processes in vivo. Our results might provide a mechanistic rationale for early alterations of microtubule functions in neurodegeneration.

B098/P0094

Discovery and characterization of novel TOG domain-containing proteins using C. elegans

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Microtubules are essential for segregating DNA, scaffolding signaling and organelle architecture, and serving as the tracks for cargo trafficking. Microtubule associated proteins (MAPs) functionalize the microtubule cytoskeleton to perform its various functions. Many MAPs possess one or more canonical microtubule-binding domains that can appear multiple times in an organism's proteome, and throughout phylogeny. One microtubule binding domain is named TOG, named for its initial characterization in a "tumor overexpressed gene," is a solenoid shaped domain typically with six HEAT repeats. One class of TOG-domain containing proteins contains vertebrate XMAP215 and budding yeast Stu2, which act as microtubule polymerases. C. elegans is a powerful model animal for the in vivo study of conserved proteins. C. elegans, with its relatively small proteome, has one known XMAP215 family protein, ZYG-9, which is essential for embryonic development. To identify additional TOG domain containing proteins, we searched the C. elegans genome for sequences predicted to encode TOG domains and identified two uncharacterized genes, F54A3.2 and T08D2.8. Unlike ZYG-9, which has three TOG domains, F54A3.2 has two TOG domains, and T08D2.8 only has one. Using CRISPR-Cas9 mediated genome editing, we deleted these genes and replaced the open reading frames with the gene encoding mNeonGreen, to assess the null phenotype and expression pattern. F54A3.2 was expressed in a variety of cells and tissues across larval development. In adult animals, F54A3.2 was most prominently expressed in the hypodermis, sperm, oogenic germline, and intestine. In adult worms, T08D2.8 was expressed in hypodermis, germline, intestine, and for a small window of developmental time, in a subset of the vulval precursor epithelial cells. We also knocked in mNeonGreen to these novel proteins' endogenous loci and found that fluorescently tagged proteins localized diffusely within the cells mentioned above. Both F54A3.2 null and T08D2.8 null animals laid an abnormally high number of unfertilized oocytes. These findings support the prediction made by AlphaFold that the novel TOG domain-containing proteins bind soluble tubulin, and that this is important for sperm function. In parallel ongoing work, we are characterizing the effects of depletion of a large panel of microtubule associated proteins from the spermatogenic and oogenic germlines to generate an unbiased and holistic picture of microtubule dynamics and regulation in a single large, complex cell in the context of an intact animal.

B089/P0085

Dynamic remodeling of centrioles and the microtubule cytoskeleton in the lifecycle of fungi with motile cilia

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Cells reorganize in space and time to move and divide – complex behaviors driven by their internal cytoskeleton. As cells transition between these states, their cytoskeletal structures are remodeled to meet new needs. For example, in many organisms, microtubule-based centrioles transit from serving as the ciliary base to become the center of the centrosome. While the molecular components and general principles of cytoskeletal assembly are well studied, less is known about cytoskeletal remodeling to support different cell states and functions and how remodeling pathways emerge and change in evolution. To study remodeling of

the microtubule cytoskeleton in an evolutionary context, we use the chytrid fungus, Rhizoclosmatium globosum, that retains centrioles and cilia lost in most other fungal lineages. Chytrids undergo extensive reorganization of their microtubule cytoskeleton as they grow from a motile zoospore with a cilium to a sessile multinucleated coenocyte in which new zoospores are built. Thus, they provide an ideal system in which to study remodeling of the cytoskeleton as they shift between having motile cilia and centrosomes. We use evolutionary comparison, RNA sequencing, and expansion microscopy to understand this reorganization and further develop this organism as a model for evolutionary cell biology. We find that when motile zoospores transition to sessile sporangia, cilia are retracted into the cytoplasm and degraded, while centrioles detach from the ciliary axoneme yet persist. During the mitotic cycles, short centrioles are associated with a centrosome-like microtubule-organizing center (MTOC) and a dense microtubule array at the spindle pole. After the mitotic cycles, centrioles elongate and form cilia, driven by transcription of genes associated with centriole maturation and ciliogenesis, and microtubule bundles are reorganized. Thus, in chytrids structural remodeling of the centriole is temporally coupled to specific changes in cytoskeletal organization over the coenocytic life cycle.

B083/P0079

How augmin establishes the angle of the microtubule branch site

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How microtubules (MTs) are generated in the proper orientation is essential to understanding how the cytoskeleton organizes a cell and MT-dependent events such as cell division. In the spindle, most MTs are generated through the branching MT nucleation pathway. In this pathway, new MTs are nucleated from the side of existing MTs and oriented at a shallow angle by the branching factor augmin, ensuring that both MTs have the same polarity. Yet, how augmin binds MTs and sets the branch angle has remained unclear. Here, we report the cryo-electron microscopy structure of an augmin subcomplex on the MT. This structure resembles that of NDC80 bound to the MT, with the conserved CH domain of augmin's Haus6 subunit directly proximal to the MT lattice. We find that the Haus6 CH domain is a bona fide MT binding site that increases augmin's affinity for the MT and helps establish branch angle. A second binding site, located in the disordered N-terminus of Haus8, also establishes branch angle,. Thus, we find that augmin regulates MT branching using two domains, each tuned to modulate MT affinity and MT branch angle. This work expands our mechanistic understanding of branching MT nucleation and thus spindle formation.

B107/P0103

Insights into microtubule dynamics regulation by glutamylation from mechanistic studies of tubulin code erasers

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Microtubule function is modulated by the tubulin code, diverse posttranslational modifications that are altered dynamically by writer and eraser enzymes. Glutamylation, the addition of branched glutamate chains, is the most evolutionarily widespread tubulin modification. It is introduced by the tubulin tyrosine ligase-like

enzymes (TTLLs) and erased by the cytosolic carboxypeptidases (CCPs). Glutamylation marks stable microtubules, such as those in axons and axonemes, and its homeostasis, achieved through the balance of writers and erasers, is critical for normal physiology. Disruption of this balance by mutations in CCP1 and CCP5 leads to human pathologies. Despite its importance, how glutamylation affects intrinsic microtubule dynamics remained unclear. By generating tubulin with short or long glutamate chains, we showed that glutamylation is a negative regulator of microtubule growth. Thus, the stabilizing effect observed in cells should come from effectors. We found that CCP1 preferentially deglutamylates soluble tubulin, in contrast to the TTLLs that prefer modifying microtubules. CCP5, by contrast, showed similar activity on soluble tubulin versus microtubules, while it is inactive on α tubulin and a major β tubulin isotype in the brain. Cryo-EM structures of CCP5 in complex with the microtubule, and X-ray structures in complex with transition-state substrate analogues, explain tubulin/microtubule recognition and reveal that CCP5 deforms the tubulin main chain into a unique turn that enables branch glutamate recognition. CCP5 binding of the sequences flanking the branch point primarily through peptide backbone atoms enables processing of diverse tubulin isotypes with glutamate branches at different positions, as well as non-tubulin substrates. Our work provides the first atomistic view into glutamate branch recognition and resolution, and sheds light on homeostasis of the tubulin glutamylation syntax and its effects on microtubule dynamics.

B084/P0080

Investigating how the microtubule associated protein TPX2 contributes to spindle architecture and the fidelity of chromosome segregation

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The dynamic, microtubule-based spindle is required for the accurate distribution of chromosomes to two daughter cells during cell division. Spindle size and architecture vary across cell types and species; however, the molecular mechanisms underlying this diversity and their effects on the fidelity of chromosome segregation are poorly understood. The protein TPX2, a key regulator of spindle size, microtubule nucleation, and organization, is present in all metazoans, but its domain structure and composition are not highly conserved. In this study, I am purifying wild-type and chimeric TPX2 proteins from two species with distinct spindle morphologies and adding them to Xenopus egg extracts, which reconstitute microtubule nucleation and spindle assembly in vitro. Using fluorescence microscopy, I am evaluating the role of the long, unstructured N-terminus of sea urchin TPX2 in branching microtubule nucleation and whether this domain facilitates co-condensation with tubulin as a mechanism to regulate microtubule nucleation. Additionally, I will compare spindle morphologies and the fidelity of chromosome segregation during anaphase. These experiments will provide new insights into the contribution of specific TPX2 domains to spindle diversity and their functional roles in cell division.

B097/P0093

Investigating the Role of Kif2c in Transforming the Microtubule Cytoskeleton at the Oocyte-to-Embryo Transition

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Microtubule (MT) aster assembly has long been studied in taxol-treated cells or cell-free extracts exposed to nonhydrolyzable RanGTP, but a recent report described the assembly of short-lived MT asters at the cortex of

maturing zebrafish oocytes. These asters are distinct from the meiotic spindle and may contribute to the exocytosis of cortical granules at egg activation, but more studies are needed to fully understand their regulation and function. Here, we provide further evidence of cortical maturation aster assembly in zebrafish oocytes and identify a novel regulator, Kif2c, a kinesin-13 family MT depolymerase also known as mitotic centromere-associated kinesin (MCAK). Kif2c has previously been shown to contribute to mitotic spindle assembly, chromosome alignment, as well as pole-focusing in Ran-dependent aster assembly. Our studies identify a novel, maternal-specific function of Kif2c as a regulator of transient cortical asters during oocyte maturation. Using live imaging of MT dynamics, followed by 3D segmentation algorithms to quantify aster number and MT length, we have shown that oocytes lacking kif2c display aberrant cortical MT aster assembly during maturation. Furthermore, we found that rather than MT asters disassembling before egg activation, they instead persist into the egg and elongate in the embryo, which contains disorganized chromosomes and fails to complete the first cell division. The rapid polymerization of MTs in persisting asters during egg activation either indicates that Kif2c is required for the depolymerization of MTs observed in wild-type activating eggs or that the activating egg environment is especially permissive of MT polymerization, despite the calcium flux that drives egg activation. Our preliminary results also suggest that Kif2c localizes to cortical aster centers during oocyte maturation, similar to its localization in Ran asters. Thus, the acentrosomal maturing oocyte represents a special case of noncanonical MT organization, and zebrafish represents a powerful tool to uncover novel functions of Kif2c. Together with cutting edge live imaging and chemical dimerization tools, our studies will reveal novel mechanisms governing cytoskeletal transformations at the oocyte-to-embryo transition.

B099/P0095

Jupiter 2 (JPT2) is a microtubule associated protein that compensates for loss of EB

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Microtubules are polar cytoskeletal polymers composed of α/β-tubulin subunits that assemble in a head-to-tail fashion to form protofilaments which then laterally associate to form tubes. Tubulin dimers that are added to growing plus ends exist in the expanded GTP-bound state whereas tubulin subunits within the microtubule lattice are primarily in a compacted GDP-bound state. Emerging data in the field indicate that microtubule associated proteins (MAPs) can expand or compact the lattice and thereby regulate the formation of tubulin post-translational modifications (PTMs). End-binding (EB) proteins recognize and are sensitive to the nucleotide state at growing microtubule ends and promote microtubule lattice compaction. Although much has been done to understand the role of EBs at microtubule plus ends, the roles of EBs in regulating the tubulin code are largely unknown. To address this, we utilized two EB knockout (KO) systems. We found that the levels of microtubule detyrosination, a prominent microtubule PTM, are significantly increased in cells that are acutely disrupted for EBs but are unaffected in cells that have been propagated for many generations in the absence of EBs (i.e., a stable knock-out cell line). We hypothesized that the difference is due to a compensation mechanism wherein protein levels of lattice-regulating MAPs change in response to loss of EBs. Using quantitative mass spectrometry, we identified Jupiter Microtubule Associated Homolog 2 (JPT2) as a protein that is upregulated in long-term EB KO cells. We show that JPT2 overexpression in short-term EB KO cells reduces microtubule detyrosination, suggesting that it is a MAP capable of regulating lattice conformation. In cells, JPT2 is rapidly evicted from the lattice upon Taxol-induced lattice expansion, suggesting that JPT2 prefers a compacted lattice. To further characterize JPT2's microtubule binding, we used in vitro reconstitution assays and found that JPT2 binds to microtubules in a GDP-Pi state (GTPγS microtubules and growing plus ends) with higher affinity than the GDP lattice. The localization of JPT2 at plus ends is maintained in the presence and

absence of EB1, indicating that JPT2 functions independently of EBs at plus ends. Together, these results reveal JPT2 as a MAP sensitive to the microtubule lattice state and provide valuable new insights into how microtubule plus end proteins regulate the tubulin code.

B100/P0096
Kinesin-14 HSET functions in condensate assembly
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Human kinesin-14 HSET (KIFC1) is a minus-end–directed motor protein that crosslinks and slides microtubules (MTs). Unlike most kinesin with plus-end–directionality, HSET moves toward MT minus ends, organizing MTs and focusing spindle poles during mitosis. HSET's centrosome clustering ability is particularly important in cells with supernumerary centrosomes, it is a critical process to promote accurate chromosome segregation and increase the survival of cancer cells with chromosome instability and centrosome amplification. Beyond functioning on centrosomes, HSET also organizes bridging fibers and mediates MT-MT sliding, highlighting its broader role in spindle mechanics. Its elevated expression in various tumors also underscores its potential as a therapeutic target. However, its function in spindle assembly remains incompletely understood. Building on our previous work showing that HSET participates in bridging fibers to regulate MT sliding and chromosome segregation, we further investigated its impact on microtubule-associated proteins (MAPs). In this study, we found that HSET overexpression alters the localization of key MAPs, including NuMA. Overexpression of HSET impairs NuMA condensate formation during prometaphase, causing more NuMA to localize along bundled MTs rather than forming phase-separated condensates at centrosomes during metaphase. This led us to hypothesize that HSET may regulate the condensate assembly of NuMA. To test this, we performed in vitro condensate formation assays using co-pelleting and fluorescence imaging with quantitative analyses. The results confirmed that HSET modulates NuMA droplet formation. Furthermore, adding pre-assembled MTs demonstrated that HSET interacts with NuMA and recruits it along MTs, reducing its phase separation. Collectively, our findings reveal a functional link between HSET and NuMA and a mechanism by which HSET regulates mitotic spindle architecture and the spatial organization of other MAPs.

B080/P0076
Microtubule Lattice Conformations Promote Molecular and Morphological Neuron Polarization
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Emerging evidence suggests microtubule (MT) networks are not simply structural, they act as platforms for modifications that convey molecular information through a tubulin code. Tubulin post-translational modifications (PTMs) and microtubule associated proteins (MAPs) that bind MTs constitute two forms of molecular information that have been shown to regulate cell processes. In addition, conformational changes in tubulin subunits that expand or compact the MT lattice have recently emerged as another layer of MT based molecular information. Previous studies have established a positive relationship between the expanded MT lattice conformation and the activity of MAPs enriched in the axon including Kinesin-1. Tubulin PTMs elevated in the axon include tubulin detyrosination and acetylation. Recent studies have demonstrated the activity of MAPs that detyrosinate tubulin increase when interacting with the expanded MT lattice conformation. These observations provide insight into how MAP activity may be regulated but they do not answer how key MAPs, like Kinesin-1, are initially polarized in cells. In this study we investigate the role of MT lattice conformation

states in the context of neuron polarization by axonogenesis. I hypothesize that a region rich in expanded MT lattice acts as a local cue for polarization of the axon MT network. My preliminary evidence shows that increasing the abundance of expanded MT lattice conformations in neurons promotes the polarized morphogenesis of the nascent axon and a global accumulation of PTMs and MAPs that are normally restricted to the axon. These changes are in tandem with an increase in MAP2. MAP2 has been shown to promote and stabilize compacted MT lattice states. This suggests that cells may sense the abundance of expanded MT lattice states and respond by deploying a counteracting MAP. Based on our results, we propose a model for axon formation that involves positive feedback between lattice expansion, Kinesin-1, and tubulin PTM writers, coupled with negative feedback to limit accumulation of the expanded state. Our work provides some of the first evidence that cortical neurons precisely regulate MT conformations to maintain the polarization of specialized axonal and somatodendritic cytoskeletal networks during morphogenesis.

B079/P0075

Modeling large cytoskeletal structures reveals how unstable systems robustly partition embryonic cytoplasm

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Embryos reliably partition their cytoplasm into cells using large cytoskeletal structures. Yet the very structures responsible for this process harbor an intrinsic organizational instability. To uncover the rules governing this instability, we developed both continuum and high-performance agent-based simulations that reproduce the experimental microtubule density profiles observed from large cytoskeletal structures. By fitting these profiles, we extracted otherwise inaccessible microscopic and phenomenological parameters, identifying a competition between microtubule orientation-dependent inhibition, autocatalytic nucleation, and nucleation from organizing centers that determines both the stability and the instability onset timescale. Guided by these predictions, we experimentally perturbed nucleation pathways and pushed biological systems from stable to unstable regimes. Strikingly, some organisms rely on the very conditions that promote instability to efficiently sense the geometry of their space. However, when the cell cycle is faster than instability growth, cells tame this innate volatility. Together, our results show that the interplay between physical instabilities and biological clocks underlies strategies for rapid and robust spatial organization of biological structures.

B102/P0098

Modeling Microtubule Severing as a Dynamic Competition between Damage Propagation and Repair

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Microtubules are dynamic polymers of tubulin heterodimers that play essential roles in intracellular transport and cell division, while providing structural support. Their functions depend on continuous remodeling, which is tightly regulated by microtubule-associated proteins. Among these are severing enzymes, which remove tubulin dimers from the microtubule lattice and generate localized damage. Recent experiments have shown that such damage does not always lead to severing: it can be repaired by the incorporation of free tubulin, suggesting that severing is governed by a balance between damage propagation and repair. However, the underlying dynamics of the process remain poorly understood. To address this, we developed a stochastic computational model to study how severing protein–induced damage propagates on the microtubule lattice. We model microtubule lattice as a two-dimensional spatiotemporal grid, where severing results from

competition between damage spread and tubulin-induced repair on the lattice. We compute severing probability and severing time across a range of tubulin concentrations and find that our model reproduces trends observed in recent experiments: severing probability decreases with increasing tubulin, while severing times broaden. Importantly, the model also provides new mechanistic insights by characterizing the size of repair patches composed of newly incorporated tubulin and identifying initial damage configurations that enhance both the likelihood and speed of severing. These results provide a quantitative framework to design targeted experiments to study damage propagation on a microtubule lattice. More broadly, our approach can be extended to dynamic microtubules to investigate how severing contributes to cytoskeletal remodeling in living cells.

B101/P0097

Nucleo-cytoskeleton connections during myofiber development and disease

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Proper nuclear positioning is critical for several cellular functions. In skeletal muscle cells, that contain hundreds of nuclei, the precise spatial distribution of nuclei is essential for muscle function. During differentiation, nuclei accretion is achieved through myoblast fusion. Initially, the nuclei cluster at the center of the developing myofiber. Subsequently, nuclei redistribute along the myofiber via microtubule-mediated transport before migrating to the cell periphery, where they become anchored. Mispositioned nuclei are a hallmark of several neuromuscular diseases, including centronuclear myopathies, although the molecular mechanisms involved remain poorly understood. In this study, we investigate the role of MT–nucleus connections during skeletal muscle development, and how they are disrupted in models of centronuclear myopathies. We found that several centrosomal proteins, including Akap9, Pcm1, and Pericentrin, relocate to the nuclear envelope during muscle differentiation. Our data indicate that Akap9 depletion blocks nuclear translocation to the periphery, while PCM1 depletion has no effect on this process. This difference is mediated by the role of Akap9 in microtubule nucleation, as Akap9 knockdown impairs MT nucleation, whereas PCM1 knockdown does not. Given that nuclear positioning can influence transcriptional profiles, we performed mRNA sequencing of centrally and peripherally located nuclei in differentiating myofibers. We observed a downregulation of Akap9 in peripheral nuclei, which may prevent further MT-directed movement once anchoring occurs. In cellular models of centronuclear myopathy, we observed nuclear clustering and impaired nuclear spreading, a phenotype consistent with defects in Akap9-mediated MT nucleation. Although no changes in Akap9 gene expression have been reported in CNM patients, we investigated whether its localization might be altered. Our preliminary data show that in control myofibers, Akap9 localizes prominently at the nuclear envelope. However, this perinuclear localization is diminished in cellular models of the disease, suggesting that Akap9-mediated myonuclear distribution might be disrupted. Together, our findings highlight a critical role for Akap9-mediated microtubule–nucleus connection in myonuclear positioning in both normal muscle development and muscle disease.

B096/P0092

Oocyte-derived microtubule projections: a novel putative signaling organelle in oocyte development

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Sexual reproduction requires the production of egg cell precursors called oocytes. Oocytes develop in the ovary in follicles, where they are enveloped by somatic follicle cells which support their development. However, oocytes and follicle cells are separated by thick extracellular layers. How do these cells communicate across such a barrier? Communication is canonically considered to be mediated by actin-based structures called transzonal projections (TZPs) that extend from follicle cells toward oocytes. However, in prophase I-arrested oocytes in the sea star Patiria miniata, we observe thin, microtubule-based projections (which we call OMPs, or oocyte-derived microtubule projections) that emanate from the oocyte surface toward the follicle cell, in the opposite direction as TZPs. To our knowledge, such structures, which are structurally distinct from both cilia and microvilli, have not been seen before in any animal oocyte. We hypothesize that OMPs may be important for oocyte-follicle cell communication. Using time lapse imaging of live oocytes at high spatial and temporal resolution, we show that OMPs contain microtubules, mainly lack actin, are dynamic on the order of minutes, and can contact follicle cells. In contrast to cilia, OMPs do not contain acetylated tubulin, but some OMPs do contain components of the intraflagellar transport complex. We have investigated OMPs under conditions where oocytes lack follicle cells and where follicles or whole ovary explants are treated with developmental signaling inhibitors and see some changes in their properties. OMPs appear only when oocytes are fully grown and are lost during the process of meiotic maturation, suggesting that they play a specific developmental role. We have also compared gene expression between early-stage and fully-grown oocytes to determine differences that may relate to OMP formation or function. We propose that OMPs are likely dynamic signaling organelles that are important for oocyte-follicle cell communication during oocyte development.

B106/P0102

Phospho-Regulated Intracellular Localization of Vasohibins Regulate Microtubule Detyrosination

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Background: Microtubule detyrosination is an evolutionarily conserved post-translational modification that stabilizes microtubules and is implicated in various physiological and pathological events. In vertebrates, microtubule detyrosination is predominantly catalyzed by Vasohibin 1 (VASH1) and Vasohibin 2 (VASH2). While the functional effects of microtubule detyrosination are being increasingly studied, the molecular signaling pathways that regulate VASH1 and VASH2 remain poorly understood. Results: We have utilized computational sequence analyses and mutational analyses to identify and characterize a conserved signal motif in the intrinsically disordered VASH1 and VASH2 C-termini that mediate their intracellular distribution. This signal motif consists of a nuclear localization sequence (NLS) flanked by putative phosphorylation sites. Using live cell microscopy, we observed that VASH1 and VASH2 shuttle between the nucleus and cytosol but are mainly cytosolic due to basal inhibition of these NLS. Nuclear localized VASH1 or VASH2 catalytic domains did not induce microtubule detyrosination, suggesting that cytosolic localization of vasohibins is critical for their function. We further show that the VASH2 NLS is phospho-inhibited by cyclin dependent kinases. Conclusions: Our findings establish a novel regulatory role of the intrinsically disordered C-termini of VASH1 and VASH2 in their function through modulation of their intracellular localization downstream of kinase and phosphatase action.

B076/P0072
Posttranslational microtubule modification alters podocalyxin-trafficking in epithelial cells
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Epithelial polarization is characterized by separation of the plasma membrane into an apical and a basolateral membrane domain. This morphology is verified by cytoskeletal organization that stabilizes the cellular architecture and provides specific tracks for correct polarized cargo delivery. Here, we studied effects of tubulin (de-) tyrosination on epithelial polarization and apical trafficking of the membrane protein podocalyxin/gp135. Therefore, tubulin tyrosine ligase (TTL), the enzyme that adds tyrosine to the carboxy terminus of detyrosinated α-tubulin, was knocked out or overexpressed in MDCK cells. TTL-knockout alters podocalyxin-expression and -glycosylation, which was compensated by overexpression or rescue of TTL. Moreover, intracellular interaction of podocalyxin with ezrin was reduced in the absence of TTL. This suggests that posttranslational microtubule-modification can modulate maturation and function of the glycoprotein. We used the SNAP-tag system to examine membrane delivery of podocalyxin and found atypical spreading of the newly synthesized glycoprotein all over the apical membrane and an altered subapical architecture of microtubules in cells with an elevated content of detyrosinated α-tubulin. Our studies suggest that intracellular trafficking of podocalyxin can be controlled by TTL-dependent posttranslational modification of microtubules in polarized epithelial cells.

B077/P0073
Readers of a tubulin conformational code
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Microtubules, dynamic polymers of αβ-tubulin heterodimers, are essential for myriad cellular functions. Recent advances in structural biology revealed that tubulin undergoes conformational changes upon GTP hydrolysis, transitioning from an "expanded" GTP-bound state to a "compacted" GDP-bound state. While tubulin isotypes and post-translational modifications (PTMs) are well-established components of the "tubulin code" that regulates microtubule-associated protein (MAP) recruitment, the role of tubulin conformation within the cell is not understood. We hypothesized that the conformational plasticity of tubulin within the microtubule lattice is a third, unrecognized layer of the tubulin code. As a first step to test this possibility, we sought to identify MAPs that selectively bind microtubules with compacted versus expanded lattice conformations. To this end, we assembled microtubules using two distinct microtubule-stabilizing agents, Taxol and Peloruside A, to generate microtubules with different lattice states. Taxol expands the microtubule lattice, whereas Peloruside A stabilizes the microtubule lattice in a compacted state. We assembled microtubules in HeLa cell lysate with each drug and performed quantitative mass spectrometry analysis on the microtubule pellets. This approach revealed MAPs that bind to microtubules in a manner that is sensitive to microtubule lattice conformation. Pursuit of these proteins confirm that a subset of MAPs do indeed selectively bind microtubules in a lattice conformation-dependent manner in cells. Our findings offer novel insights into how the tubulin conformational code can selectively recruit specific MAPs, thereby diversifying microtubule function and properties within the cell.

B090/P0086

Reduced tubulin polyglutamylation affects neuronal function and causes late-onset neurodegeneration

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Microtubules (MTs) are essential for neuronal functions. The strong enrichment of posttranslational modifications (PTMs) on neuronal MTs suggests they are critical for their regulation. One PTM, polyglutamylation, was shown to control axonal transport, microtubule-associated proteins (MAPs) binding to MTs, and to induce early-onset neurodegeneration in both mice and humans when excessively accumulated in neurons. To investigate the physiological roles of polyglutamylation, we developed mouse models with reduced polyglutamylation. Unlike mice with excessive polyglutamylation, these mice exhibited no signs of early-onset neurodegeneration. By contrast, magnetic resonance imaging and immunohistochemical analyses revealed progressive atrophy of the olfactory bulb and frontal cortex accompanied by reduced numbers of neurons. Moreover, biolistics-based analysis revealed progressive reduction of dendritic spine density, which correlates with the observed deficits in olfaction, memory, and learning. We have previously shown that polyglutamylation modulates MT interaction with Tau, a MAP implicated in neurodegenerative diseases, such as Alzheimer disease and other tauopathies. Decreasing polyglutamylation in a mouse model for tauopathy accelerated the demise of these mice, demonstrating a functional link between MT polyglutamylation and tauopathies. Given that perturbed interactions between Tau and MTs are considered as early events in tauopathies, our findings suggest that polyglutamylation could play a role in the pathogenesis of these diseases. We will investigate this by quantifying polyglutamylation levels in patient samples. Collectively, our findings establish MT polyglutamylation as a crucial cytoskeletal regulator of neuronal survival and function. Dysregulation of this PTM may contribute to the pathogenesis of late-onset neurodegenerative diseases, highlighting its potential as a therapeutic target.

B109/P0105

Severing Without Power: UNC-45A Prevents Microtubule Lattice Repair and Drives Neurite Dystrophy

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Microtubule severing enzymes regulate cytoskeletal dynamics and cellular architecture. While their roles in pathology have been proposed, they remain underexplored. To date, mechanistic understanding of severing centers on AAA+ ATPase enzymes—katanin, spastin, and fidgetin—which hydrolyze ATP to extract tubulin subunits from the microtubule lattice, altering its integrity (Roll-Mecak and Vale, Nature, 2008; Vemu et al., Science, 2018; Zehr et al., Dev Cell, 2020). Recently, we identified UNC-45A as a novel severing protein that, unlike canonical enzymes, lacks an ATPase domain yet severs microtubules (Hoshino et al., STAR Protoc., 2025; Hoshino et al., J Biol Chem, 2023; Habicht, Mooneyham, Hoshino, et al., J Cell Sci, 2021, Editor's pick and First-Author Interview; Mooneyham. et al., Mol Cancer Res, 2019, Cover Art; Habicht, et al., Cancer Biol Ther, 2019). This discovery reveals a previously unrecognized, energy-independent mechanism of microtubule severing and

represents a paradigm-shifting advance in cytoskeletal regulation. Using in vitro reconstitution, electron microscopy, high-resolution imaging, and analysis of Alzheimer's disease (AD) brain specimens, we investigated ATP-independent UNC-45A severing and its relevance to AD. UNC-45A exists in an equilibrium of oligomeric species and engages microtubules in its oligomeric state. EM reveals these oligomers form compact architectures distinct from the linear arrangement seen in C. elegans UNC-45 (Gazda et al., Cell, 2013) and exhibit bent conformations. UNC-45A preferentially targets structurally damaged microtubule regions, occupying otherwise repairable defects and amplifying deformation to trigger severing. In neurons, UNC-45A localizes to axonal swellings with compromised microtubules and exacerbates this pathology. It is also enriched in dystrophic neurites near amyloid deposits in both mouse models and human AD brains. Together, these findings define a novel, damage-responsive, ATP-independent severing mechanism that links defect recognition to repair inhibition. This previously unrecognized mode of microtubule quality control may be broadly relevant to pathological conditions marked by microtubule loss and elevated stress—such as oxidative damage in AD and taxol-induced neuropathies. Importantly, UNC-45A may have a functional advantage in cells with low ATP, as seen in age-related diseases. Part of the work described here has been previously made available as preprint (doi: https://doi.org/10.1101/2025.08.26.671351).

B111/P1801

Structural Basis of Tubulin Post Translational Modification Recognition by EML Proteins

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Post-translational modifications (PTMs) of microtubule (MT) C-terminal tails modulate MT functions. A family of highly conserved MT binding proteins, the Echinoderm Microtubule-associated protein-Like (EML) family, regulates neuronal outgrowth and mitosis and are readers of MT PTMs. However, resolving the structural basis by which PTM reader proteins recognize PTMs has been challenging due to the intrinsic flexibility of the MT C-terminal tails. Using cryo-electron microscopy and single-molecule imaging, we uncovered the mechanism by which EML proteins recognize PTMs on the tubulin tail. Remarkably, our structures reveal the entire alpha-tubulin tail in a fully ordered conformation, enclosed within a beta-propeller domain of the EML protein. High-resolution structural analysis shows that this interaction is mediated by a unique tyrosine recognition motif, coupled with simultaneous binding across multiple tubulin dimers along the MT lattice. Furthermore, our structures of EML proteins from different species demonstrate that this motif is evolutionarily conserved and essential for their robust MT binding. Through single molecule imaging, we found that EML proteins preferentially associate with the GTP-like MT lattice and promote MT catastrophe events, in contrast to the stabilizing role typically attributed to many MT binding proteins. Together, our findings define a novel tubulin tyrosine recognition motif that is conserved within EML proteins, revealing the structural mechanism of how they bind to MTs and affect their dynamics.

B104/P0100

Structure-based mechano-chemical model reveals how microtubules nucleate from stabilized seeds

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Microtubule (MT) nucleation is essential for organizing and regulating the cellular MT network. Nucleation from stabilized MT templates is a fundamental mechanism, yet its underlying process remains poorly understood. Here, we present a physics-based, structure-resolved mechano-chemical model that, unlike the

prevailing critical nucleus framework, fully integrates MT dynamic instability into the nucleation process. In our model, growth, catastrophe, and shortening emerge from non-equilibrium conformational transitions of tubulin subunits and their interfaces. This framework quantitatively reproduces key experimental observations—including the high critical concentration, concentration-dependent delay time, and regulation by catastrophe-modulating proteins—within a unified mechanistic picture. The rate-limiting step is not forming a special nucleus, but elongating a nascent MT beyond a detectable length without undergoing catastrophe. The observed delay arises from repeated cycles of growth, catastrophe, and regrowth, while persistent elongation at subcritical tubulin concentrations is enabled by memory of plus-end geometries inherited from earlier high-concentration growth.

B108/P0104

Tau-Mediated Microtubule Organization Impacts Neurite Outgrowth and Axonal Transport in Dorsal Root Ganglion (DRG) Neurons

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In axons from neurons, microtubules form a dynamic and spatially regulated network that is crucial for structural integrity, regeneration, and long-distance transport. In peripheral neurons such as DRGs, the microtubule-associated protein - tau is expressed in different isoforms, including a particularly long isoform called “big tau”. However, how tau affects microtubule organization, supports neurite outgrowth, and influences axonal transport remains unclear. We used DNA-PAINT, a super-resolution microscopy technique, followed by algorithmic image analysis to investigate microtubule organization in cultured DRG neurons from one and two-year-old control and tau-knockout (TauKO) mice. We found that in TauKO neurons, mean microtubule length was decreased in an age-dependent manner by 30 – 50% compared to controls. In addition, we observed that microtubule straightness was significantly reduced in DRG neurons from TauKO mice at both ages. To determine the functional consequences of these structural alterations, we examined axonal regeneration and mitochondrial transport in TauKO and control DRG cultures. Surprisingly, TauKO neurons exhibited significantly longer axonal processes, indicating increased axonal sprouting and less dynamic mitochondrial movement. To investigate the proteome changes caused by the absence of tau, we performed mass spectrometry of cultures prepared from one-year old mice. We did not observe differential expression of other microtubule-associated proteins, but identified the kinesin motor Kif1b as significantly upregulated in TauKO DRG neurons. Our study demonstrates that DNA-PAINT followed by algorithmic image analysis is a powerful approach for quantitatively assessing the organization of densely packed microtubule in cultured peripheral neurons. The data suggests that big tau is a regulator of microtubule architecture and axonal regeneration in DRG neurons and influences mitochondrial transport.

B095/P0091

TDP-43 Regulates Microtubule Dynamics

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Microtubules are dynamic polymers essential for cellular organization and function, yet how their regulation intersects with disease-associated proteins remains unclear. TransActive Response DNA-Binding Protein 43 kDa (TDP-43) is best known for its roles in nuclear RNA metabolism and pathological aggregation in neurodegenerative disorders, like amyotrophic lateral sclerosis (ALS). TDP-43 notably shares features with

microtubule-associated proteins including the ability to form liquid-liquid phase separated biomolecular condensates. Here we identify a direct role for TDP-43 in regulating microtubule dynamics. Using purified proteins, multi-wavelength TIRF microscopy, and biochemical assays we show that TDP-43 binds both tubulin dimers (kD = 102 ± 14 nM) and polymerized microtubules via a multifaceted mechanism that can promote microtubule bundling while also sequestering tubulin or microtubules within condensates. Functional truncation mutants mapped the TDP-43-tubulin interaction to the protein's N-terminus, independent of its canonical phase-separating domain. In Neuroblastoma-2A cells, TDP-43 depletion or overexpression altered microtubule organization, while Taxol treatment promoted significantly enlarged cytoplasmic TDP-43 condensates, linking cytoskeletal regulation to disease-relevant mislocalization. Moreover, TDP-43 condensation levels vary throughout mitosis, where the protein associates with spindle microtubules in prometaphase. Together, these findings establish TDP-43 as a previously unrecognized regulator of microtubule dynamics and suggest that cytoskeletal disruption may be a key contributor to its role in neurodegenerative disease.

B092/P0088

The Relationship Between EB Comets and Microtubule Growth Reveals Differential Regulation of the Tubulin GTP Hydrolysis Rate Throughout the Cell Cycle

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Microtubules are dynamic cytoskeletal polymers essential for cell division, cell motility, and intracellular transport. Microtubule dynamics are characterized by dynamic instability—the ability of microtubules to switch between phases of growth and shrinkage. Dynamic instability is explained by the GTP-cap model, suggesting that a 'cap' of GTP-bound tubulin subunits at the growing microtubule end has a stabilizing effect, protecting against microtubule catastrophe—the switch from microtubule growth to shrinkage. Notably, microtubule end-binding EB proteins recognize the nucleotide state of tubulin, and their comet-like localization at growing microtubule ends can be used as a proxy for the GTP-cap. Our previous work directly comparing EB comets in vitro with those in interphase LLC-PK1 cells revealed that, despite the presence of numerous regulatory proteins in cells, the relationship between GTP-cap size and microtubule growth rate is the same as in vitro. Whether this relationship changes in other cellular contexts, such as mitosis, when cells modulate microtubule dynamics to rapidly restructure the microtubule network, is not known. Here, we use high spatiotemporal resolution live-cell imaging to directly determine the relationship between EB comet size and microtubule growth throughout the cell cycle. Our data reveal that although EB comet size scales linearly with microtubule growth rate in all investigated cell cycle phases, the size of EB comets at matched growth rates is reduced as cells progress through mitosis. We find that EB comet size reaches its lowest point during anaphase before resetting once two daughter cells are formed. Taken together, our results demonstrate that the dependence of EB comet size on microtubule growth rate is modulated as cells progress through the cell cycle, suggesting that tubulin GTP hydrolysis kinetics are differentially regulated as a means of altering microtubule dynamics throughout the cell cycle.

B094/P0090

The testis-enriched tubulin isotype TUBA3D alters the intrinsic properties of microtubules

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Microtubules are dynamic polymers essential for numerous fundamental cell processes, while also serving tissue-specific roles. Eukaryotes generally encode many isotypes of the microtubule (MT) building blocks alpha and beta tubulin, and these isotypes differ in sequence and expression across tissues, indicating MT composition and properties could be tissue-specific. For example, testis-enriched tubulin isotypes have recurrently evolved across animals, including insects and mammals. The sequence divergence of testis-enriched isotypes from ubiquitously expressed isotypes suggests distinct MT properties and/or regulation. Here, we investigated whether the testis-enriched human alpha tubulin TUBA3D alters MT dynamics and/or structure. We determined that TUBA3D evolved at least 160 million years ago, and its sequence and testis expression are largely retained across mammals, suggesting a well-conserved role in sperm development. Indeed, TUBA3D is the predominant alpha tubulin in structures unique to the testis, including the sperm flagellum and the manchette, a transient MT structure that helps shape the sperm head. To test whether TUBA3D confers unique MT dynamics and structure, we expressed and purified one of two sequence variants of recombinant TUBA3D in complex with β-tubulin TUBB4B, the predominant beta-tubulin isotype in the testis. We measured the dynamic properties of TUBA3D in vitro and discovered that TUBA3D-TUBB4B microtubules exhibit slow growth rates with a drastic reduction in catastrophe frequency relative to ubiquitously expressed α-tubulin TUBA1B-TUBB4B, suggesting that TUBA3D enhances MT stability. To assess the mechanical properties of TUBA3D microtubules, we performed a microtubule gliding assay to measure the persistence length of isotype-pure microtubules. Our measurements show that TUBA3D-TUBB4B microtubules are roughly an order of magnitude stiffer than TUBA1B-TUBB4B microtubules in vitro. To begin understanding the basis of these intrinsic differences, we used Cryo-EM to resolve high-resolution MT structures, and our preliminary structures indicate that TUBA3D-TUBB4B protofilaments are nearly identical to those composed of TUBA1B-TUBB4B, indicating that the sequence divergence in TUBA3D significantly alters microtubule intrinsic properties without striking changes in polymerization interfaces. We are extending our analyses to the second variant of TUBA3D, which differs by one residue, to test if it shares these unique properties. We hypothesize that MT structures in the testis, including the sperm flagellum and manchette, require less dynamic and/or stiffer MTs for their assembly or function.

B105/P0101

Tubulin autoregulation tunes microtubule dynamics to support multicellular architecture and viability

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Alpha- and beta-tubulin heterodimers dynamically assemble into microtubules, key cytoskeletal elements involved in intracellular trafficking, cell adhesion and division. The availability of free tubulins regulates the synthesis of new subunits. In response to excessive soluble αβ-tubulins, tetratricopeptide protein 5 (TTC5) selectively recognizes nascent tubulins at the ribosome, recruiting downstream effectors that degrade their encoding messenger RNAs, in a process known as tubulin autoregulation. Despite its well-characterized molecular framework, the biological relevance of this regulatory pathway remains unknown. Here, using human 3D cellular models, advanced optics, and genetic perturbation of tubulin biosynthesis, we reveal that loss of TTC5-dependent tubulin autoregulation elevates soluble tubulin levels, inducing microtubule hyperstability, and disrupting cytoskeletal organization. These defects impair the localization of adhesion molecules at cell-cell junctions and extracellular matrix interfaces, compromising tissue architecture and reducing overall cell viability. Our findings establish tubulin autoregulation as a critical mechanism that tunes microtubule dynamics to sustain cellular integrity and tissue homeostasis.

B087/P0083
Tubulin isotype composition as a regulator of microtubule post translational modification state
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Microtubules are cytoskeletal polymers composed of αβ-tubulin heterodimers which form distinct network architectures essential for cell-type specific functions. For a structure so ubiquitous to fulfill such distinct roles suggests an intricately regulated system at play. Indeed, emerging evidence has identified molecular-level differences between microtubule networks in different cell types, and these appear to be generated by developmental programs of gene expression and post-translational modification (PTM). α and β tubulins are encoded by families of different genes known as isotypes, which vary in the amino acid sequence of their carboxy terminal tails (CTTs). In addition, expression of these isotypes differs according cell type and developmental stage, with some prominently noted in specific tissues; The β-tubulin isotype TUBB3, for example, is primarily expressed in neurons and comprises ~25% of β-tubulin in the mature brain. While TUBB3 has become a neuronal cell marker, its specific role as a component of neuronal microtubules has yet to be understood. TUBB3 knockout studies, however, have shown that a deficit in TUBB3 results in an altered composition of PTMs in neuronal tissues, markedly glutamylation and acetylation. This suggests that the tubulin isotype blend itself may regulate the PTM state, altering microtubule dynamics and interactions. To investigate this, we have designed a TUBB3 knockout cell line from the immortalized murine neuronal CAD cell line. These TUBB3 knockout cells have also been designed for doxycycline-inducible TUBB3 expression, allowing for tunable expression to evaluate changes in PTMs with variable microtubule isotype compositions. We find that a TUBB3 knockout changes levels of microtubule polyglutamylation and acetylation. Loss of TUBB3 in these cells increases polyglutamylation during its process of morphological polarization, and reduces acetylation. Both of these PTMs influence microtubule characteristics and activities, such as microtubule flexibility, trafficking, and severing. This raises the question of whether local isotype composition regulates regional PTMs within a cell or cell structure, further refining context-specific microtubule interactions and dynamics.

B110/P1800
Tubulin isotype functions evaluated by single gene knockouts in zebrafish
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Microtubules are non-covalent, polar, protein polymers that are essential to many cellular processes, including development and maintenance of asymmetry, directional intracellular transport, cell migration, mitosis, and others. Microtubules are composed of heterodimers of a- and ab tubulins, each of which is coded by a family of genes (isotypes) that can be expressed in a tissue- and developmentally-specific manner, although some show nearly ubiquitous expression. In order to investigate the developmental roles of different isotypes in the zebrafish Danio rerio, we produced CRISPR-Cas9 mediated knockouts (crispants) of individual tubulin genes (11 of 21) at the one-cell stage after fertilization, and then followed development for 7 days. Targeting of one gene (tubb2a) produced no readily detectable phenotype. By contrast, loss of each of the other ten genes led to visible phenotypes that were: distinctive by tubulin isotype, often not highly penetrant, and occurred from head to tail, including a number of distinctive tail morphologies. Early death was rare in crispants of all isotypes, but cumulative mortality ranged from 5% - 60% over 7 days. Higher magnification studies revealed

malformation of brain, eye, and heart in some, and ciliary dysfunction in pronephros, neuromasts, and olfactory pit. Intracellular transport of melanosomes is disrupted in crispants of one isotype. The results show that loss of gene function for 10 of the 11 tubulins tested caused at least a degree of dysmorphology and death, indicating that each has retained critical developmental functions.

B093/P0089
Tubulin subtype expression patterns developmental origins and cellular morphology of the developing cornea
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Background: Corneal development is highly dynamic involving diverse changes to the cytoskeleton. A common progenitor for the cornea, periocular neural crest cells (NCCs), undergo migration and/or mesenchymal-to-epithelial transition (MET) to produce both mesenchymal (stromal keratocytes) and epithelial (corneal endothelium) derivatives, respectively. These processes are dependent on microtubules, composed of alpha-beta tubulin heterodimers. Tubulins in the cornea have been studied in the context of pathology but largely in a subtype and isotype non-specific manner. However, growing evidence in NCCs suggests that tubulin isotypes show differences in spatiotemporal expression and function during development. The goal of the current study is to investigate the role of tubulin proteins, specifically tubulin alpha-4a (TUBA4A), which has also been implicated in cancer cell state changes. Here, we utilize integrated single cell RNA/ATAC-sequencing, immunohistochemistry (IHC) and in ovo perturbations in developing chicken (Gallus gallus) embryos to investigate tubulin isotypes in corneal formation and patterning. Results: Single cell RNA-sequencing integration of human, chick and mouse developing corneas revealed heterogeneous expression of tubulin subtypes. Subtypes were expressed either ubiquitously (TUBA1A, TUBA1B, TUBB2A) or focally in certain cell types (TUBA4A, TUBB3). Specifically, we noted an epithelial bias for TUBA4A across multiple species which aligned with IHC protein expression in a stage specific manner in the chick embryo. TUBA4A ATAC peaks were found to be correlated with epithelial associated gene peaks (CDH1, EPCAM1, CLDN1, etc.) and opening of transcription factor sites associated MET and NCC differentiation (PITX2, FOXC1, etc.) TUBA4A was highly expressed in the developing surface ectoderm-derived corneal epithelium. Additionally, significant TUBA4A expression was found in leading edge of the migratory periocular NCCs and maintained in the corneal endothelium (epithelial derivate) but lost in the stromal keratocytes (mesenchymal derivate) upon differentiation. Transient morpholino knockdown of TUBA4A reduced migration and differentiation of periocular NCCs. Conclusion: Cytoskeletal elements, particularly tubulins, define lineage trajectories of the developing corneal progenitors. Tubulin isotype expression is heterogenous. TUBA4A is localized to epithelial cells of the cornea independent of their developmental origins.

Cytoskeleton and Motility - Molecular Motors

B114/P0108
A Conserved Kinesin-2 Motif for Neuronal Cargo Targeting
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Intracellular transport relies on motor proteins like kinesins to deliver cargo along microtubules, yet how they recognize cargo remains unclear. Here, we present high-resolution cryo-electron microscopy structures of the heterotrimeric kinesin-2 complex (KIF3A/KIF3B/KAP3) bound to the cargo protein APC. Our findings reveal a previously uncharacterized KIF3 tail hook-like motif, termed the "HAC" domain, which mediates binding to both KAP3 adaptor and APC cargo.Within this domain, the KIF3A helical regions ensure cargo specificity, while a β-hairpin and KIF3B provide structural support. Biochemical and neuronal experiments confirm its functional importance. Notably, the HAC/KAP3 structure resembles hook-like architectures seen in kinesin-1 and dynein, suggesting a shared cargo recognition framework. These findings also shed light on kinesin-2 cargo specificity and offer a structural framework for understanding related neuronal transport mechanisms

B127/P0121

A metaphase, septin-associated myosin filament cage ensures lumen integrity in intestinal organoids by promoting metaphase cell rounding and planar cell division

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Here we investigated the relationships between myosin contractility, cell division plane and lumen integrity using mouse intestinal organoids, which normally maintain a single lumen as they grow. Attenuating myosin function inhibits interkinetic nuclear migration and metaphase cell rounding, causing the division plane to shift from planar to orthogonal and resulting in organoids possessing multiple lumens. Following the apical domain marker ZO-1 showed that new lumens originate at the interface between stacked daughter cells created by orthogonal divisions, and that subsequent lumen maturation involves passage through a transitional epithelial state. Super-resolution imaging of rounded metaphase cells revealed a never-before-seen myosin filament cage that we show resists surrounding tissue pressure and contributes to contractile ring formation. Finally, staining showed that septins 7 and 9 co-localize with the myosin filament cage, suggesting that this metaphase contractile structure may be templated by septin filaments. We conclude that a septin-associated myosin filament cage maintains lumen integrity in intestinal organoids by supporting metaphase cell rounding, a prerequisite for planar cell division.

B140/P0134

Cells stably expressing shRNA against MYO10 display altered cell motility and also serve as the foundation for a modern cell biology CURE

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Myosin-X (MYO10) is an actin-based motor protein that contributes to cytoskeletal dynamics, membrane interactions, and integrin-mediated adhesion. It is thought to play a key role in filopodia formation and function. To investigate MYO10's cellular roles, we generated MYO10 knockdown (MYO10KD) HeLa and COS7 cell lines using lentiviral transduction to establish cell lines stably expressing shRNA against MYO10. We then performed functional assays to determine the consequence of MYO10 knockdown on filopodia- and migration-related cellular functions. Using quantitative fluorescence approaches, we assayed for properties of filopodia,

wound-healing, and cell attachment & spreading. Compared to wild-type cells, MYO10KD lines exhibited reduced proliferation, impaired migration, and decreased filopodia density. The impact on filopodial density was particularly apparent in HeLa cells. MYO10KD cells also showed increased spreading on laminin-coated substrates, suggesting altered integrin activation and cytoskeletal linkage. To further explore MYO10's mechanistic contributions, we have attempted rescue experiments via transient expression of GFP-MYO10 and GFP-MYO10HMM, and then comparing the their effects on filopodia density and cell area in the MYO10KD cells. Together, these findings demonstrate that MYO10 plays a central role in regulating cell proliferation, adhesion, and migration. These MYO10KD and rescue models provide a valuable platform for dissecting MYO10's functions in filopodia dynamics and cell behavior. Additionally, these experiments were completed as part of a course-based undergraduate research experience (CURE) as part of an upper-level biology course. The students in this course contributed to the experimental design, image acquisition, image segmentation & analysis, and data interpretation. In addition to presenting their work to a cytoskeletal experts in a lab meeting via video conference, the students in the class are also contributing to authoring and submitting journal articles describing the work.

B123/P0117

Critical Role of the Non-Helical Tailpiece of Myosin-II in Regulating Filament Architecture and Function

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Non-muscle myosin-2s (NM2s) play essential roles in cytokinesis, cell migration, and cell adhesion during tissue morphogenesis. These functions depend on the dynamic assembly and disassembly of NM2 filaments. Mammalian non-muscle myosin-2 isoforms (NM2A, NM2B, and NM2C) each contain a non-helical tailpiece (NHT) at their C-terminus. Stop-codon mutations in the NHT of NM2A are linked to diseases such as macrothrombocytopenia. However, the role of the NHT in NM2 filament assembly, function, and disease mechanisms remains unclear. To address this, we purified full-length NM2 isoforms, their NHT chimeras, and NM2A variants with distinct NHT lengths or amino acids compositions from insect cells, and compared their filament structures by negative staining EM. Our results show that the NHT length significantly influences NM2 filament size. NHT truncations, including those associated with MYH9-RD, lead to the formation of larger bipolar filaments with reduced bare zones. We further examined NM2A-NHT variants in human osteosarcoma-derived U2OS cells using immuno-gold labeling PREM, super-resolution iSIM, and FRAP. Variants lacking the NHT or with disease-associated truncations form larger bipolar filaments, thicker stress fibers with lower turnover rates, and tend to aggregate during filament disassembly. Correlative immunogold labeling PREM (CL-PREM) revealed that these aggregates contained filamentous myosins arranged not only in parallel arrays, as in stress fibers, but also in mixed orientations. This finding provides insight into inclusion bodies observed in neutrophils of patients with NM2A NHT-linked thrombocytopenia. Importantly, the NHT-less NM2A impairs cell migration. Together, these findings reveal the critical role of the NHT in regulating NM2 filament assembly, turnover, and cell migration and provide a mechanistic explanation for disease phenotypes caused by NHT truncations in MYH9-related disorders.

B130/P0124

Decreased MYO19 expression leads to altered differentiation in C2C12 cultured myocytes

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Myosin XIX (MYO19) is an actin-based motor protein that binds to mitochondria. MYO19 is hypothesized to be associated with mitochondrial dynamics throughout the lifetime of the cell. Very little is currently known about the specific roles of MYO19 in normal cell physiology. What is known is that MYO19 protein is broadly expressed in many tissues and cell types. To investigate MYO19's role within muscle cell differentiation, we generated MYO19 knockdown (MYO19KD) C2C12 cell lines. We generated semi-clonal lines stably expressing shRNA against MYO19 using a lentiviral transduction system. Wild type C2C12 cells were infected with lentiviral particles containing shRNA against MYO19. When grown in puromycin-containing media, cells where the shRNA-coding sequence integrated into the genome were able to survive and replicate in the presence of the antibiotic. We verified MYO19 knockdown by quantitative PCR of MYO19 mRNA and western blot analysis of MYO19 protein. Under the appropriate growth conditions, C2C12 cells can be differentiated. They take on a myocyte-like phenotype where the cells fuse, become multinucleate, and reorganize their ultrastructure. MYO19KD cells displayed delayed differentiation compared to wildtype cells when assayed using Jenner-Giemsa staining. Although multinucleate cells were still apparent, there were fewer of them in the population. Phenotypic changes during differentiation likely correspond to gene expression changes, and we are analyzing the expression of muscle differentiation markers to determine the extent of differential expression between wild type cells and MYO19KD cells. Taken together, these results indicate that MYO19 may play a role in muscle cell differentiation.

B137/P0131

Direct optogenetic manipulation of myosin 2

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The dynamic activity of the actomyosin cytoskeleton determines the dynamic architecture of the cell. To function, actin and myosin form cortical networks that interact with adhesion and other complexes at the plasma membrane. To investigate and control actomyosin activity in these cortical domains, optogenetic tools have been developed with an anchor at the plasma membrane that can recruit molecules to activate signaling pathways like RhoA. While these tools have proven useful and provided great insight, they have multiple limitations. First, plasma membrane recruitment may not efficiently initiate the desired downstream events, and more precise recruitment to the cortex or proteins of interest may prove more beneficial. Second, RhoA activation lacks precision, inducing an array of downstream signaling events. For general activation of contractility, RhoA-driven actomyosin assembly may be desired. However, other experiments may benefit from more precise independent activation of actin or myosin. To circumvent these limitations, we generated an iLID-based optogenetic system to directly recruit modulators to myosin 2. We tagged the N-terminus of the cardiac (MYL2) and non-muscle (MYL12) regulatory light chains (RLC) with the blue-light sensitive iLID complex (LOV2-ssrA). These RLC-iLIDs efficiently integrate into their respective myosin 2 structures and reversibly recruit cytoplasmic SspB-RFP upon blue light stimulation in both neonatal rat ventricular myocytes and fibroblasts. To generate a direct myosin 2 activation system, we co-expressed RLC-iLID in fibroblasts with a recruitable catalytic domain of MLCK (SspB-MLCKcat), which should phosphorylate the RLC at Thr18/Ser19 and activate myosin when recruited. Upon blue light stimulation, we observe robust recruitment of SspB-MLCKcat to RLC-iLID and a concomitant increase in cell contractility measured with traction force microscopy. We are currently expanding the recruitable toolkit to spatiotemporally activate and inhibit myosin 2 with both iLID and Magnet optogenetic systems.

B135/P0129

Dysfunction of Smooth Muscle Myosin in Aortic Disease

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Aortic aneurysms and dissections are major vascular diseases and a leading cause of death world-wide. In this study, we focused on the 20% of cases that are due to inherited mutations in essential genes. One such gene, MYH11, encodes smooth muscle myosin (SMII), a class II myosin motor protein vital to the contraction of vascular smooth muscle cells (vSMCs), which maintain the integrity of the aortic wall. We investigated a pathological mutation, R712Q, located in the converter subdomain of SMII, which is necessary for transmitting force to the lever arm during smooth muscle contraction. We believe that the R712Q mutation may disrupt communication between the motor domain and lever arm, potentially resulting in reduced contractile force and vSMC dysfunction, as well as weakening of the aortic wall. To study the mutation and its effects, we made a SMII construct with the R712Q mutation and utilized the baculovirus insect cell expression system to express and purify the mutant and WT protein. We then analyzed the mutation's impact on SMII function by examining how it alters ATP hydrolysis, force generation, and formation of the autoinhibited interacting heads motif (IHM). We found that the R712Q mutation increased IHM formation ~2-fold at physiologic ionic strength and slowed in vitro actin sliding velocity in the motility assay ~2-fold. In parallel, we identified a novel variant of unknown significance (VUS), A733V, in a similar region of SMII. SMII-A733V was tagged with EGFP and transfected into primary human vSMCs. Single-cell myosin assembly assays and FRAP were performed to characterize SMII exchange dynamics, revealing a decrease in myosin bulk assembly and an increase in mobile fraction, indicating a destabilized SMII filament. Traction force microscopy was performed on the transfected vSMCs to measure the strain energy per area, revealing a two-fold decrease in contractility. Complementary biochemical assays will follow. Collectively, these data suggest that SMII mutations likely reduce force generation in smooth muscle and may increase the risk of aortic aneurysm and aortic dissection. Overall, our research may help guide therapeutic development aimed at enhancing vSMC function and preventing aortic aneurysm /dissection.

B120/P0114

Enhanced axonal transport in large vertebrates: KIF5A adaptations in giraffes and pythons

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Efficient axonal transport is essential for neuronal function, particularly in species with exceptionally long axons. The Kinesin-1 motor protein KIF5A plays a key role in this process, but whether and how it adapts to the transport demands of large vertebrates remains unclear. Here, we show that KIF5A from giraffes (GcKIF5A) and pythons (PbKIF5A), moves 25% faster than its mouse counterpart (MmKIF5A) on neuronal microtubules in vitro and within axons of cultured mouse hippocampal neurons. This enhanced velocity, driven by three unique amino acid substitutions (R114Q, S155A and Y309F), facilitates long-distance transport in these species and represents a case of convergent evolution. Our structural analysis reveals that accelerated ADP release underlies the increased speed of GcKIF5A. Despite exhibiting a reduced force generation, GcKIF5A maintains efficient cargo transport under load. Furthermore, GcKIF5A exerts less drag in mixed-motor environments, an

adaptation particularly beneficial for multiple motor-driven long-distance axonal transport. These findings reveal that KIF5A has evolved specific adaptations to facilitate efficient axonal transport in large vertebrates, highlighting the evolutionary plasticity of kinesin motors.

B138/P0132

Exploring phosphoregulation of MYO3A using quantitative fluorescence image analysis in COS7 cells

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Cellular protrusions are an important component of the auditory and vestibular systems. Myosin IIIA (MYO3A) is an unconventional myosin involved in the formation and maintenance of hair-cell stereocilia of the sensory epithelia in the inner ear. Disruptions in MYO3A expression have been linked to nonsyndromic deafness, and thus understanding its regulatory mechanisms is of importance to human health. As a class III myosin, MYO3A protein contains an N-terminal kinase domain followed by a myosin motor domain, a lever-arm region, and a cargo-binding tail domain. The kinase domain has been implicated in phosphoregulation of MYO3A activity through intermolecular autophosphorylation, as demonstrated previously by in vitro assays where kinase-domain containing MYO3A constructs were shown to phosphorylate MYO3A constructs lacking the kinase domain (MYO3AΔK). Cell-based assays demonstrated that the ability of MYO3A to stimulate filopodia formation was decreased by MYO3A autophosphorylation. To gain a better understanding of the phosphoregulation of MYO3A, we transfected COS7 cells with mchr-MYO3AΔK in combination with GFP-tagged full-length MYO3A (GFP-MYO3AFL), or GFP attached to just the kinase domain of MYO3A (GFP-MYO3Akin). Coexpression of mchr-MYO3AΔK with either construct resulted in decreased mchr-MYO3AK levels at the tips of filopodia and fewer filopodia at the edge of the cell, compared to cells expressing mchr-MYO3AΔK alone. These results suggest that MYO3A phosphoregulation may be influencing filopodia initiation at the earliest stages of filopodia formation, in addition to regulation that may occur in established filopodial structures. Previous studies using mass spectrometry identified two potential phosphorylation sites in the motor domain. Our informatic analyses and structural predictions suggest that these sites might influence actin-MYO3A interactions. To investigate the regulatory roles of these sites, we generated aspartic acid point mutations in mchr-MYO3AΔK to mimic phosphorylation. We assayed the constructs for their ability to tip-localize and influence filopodial density via transfection into COS7 cells. These analyses will enhance our understanding of regulation of MYO3A function by phosphorylation of the motor domain, and its influence on actin-based protrusions.

B113/P0107

Gear change to go through: Cooperation of two different kinesins enables fast and efficient axonal transport

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Neurons are highly polarized cells with multiple dendrites and a single long axon. Selective vesicle transports from cell body to axonal compartment play fundamental roles in neural survival and synaptic functions. Kinesin-1 and kinesin-3 are key players in long-range anterograde deliveries of various axonal cargos. Despite extensive body of prior studies which revealed specific cargo-kinesin interactions, how various cargos selectively utilize different types of kinesin motors according to their individual transport characteristics has remained unclear. A representative example is amyloid precursor protein (APP). Although APP is known to be

transported by kinesin-1, it is delivered at faster velocities than single-molecule velocity of kinesin-1 in vitro and molecular mechanism achieving this acceleration is largely unknown. In this study, we revealed that not only “slow” kinesin-1 but also “fast” kinesin-3 contributes to APP transport. Combination of single-molecule imaging, knockdown experiments and reconstitution assays in neurons or in vitro showed that kinesin-1 and kinein-3 play distinct roles during the delivery of APP. “Slow” kinesin-1 was required for APP to go through axon initial segment (AIS). “Fast” kinesin-3 was important to achieve fast transport velocity in distal axon. This cooperation of two kinesins was observed in other various presynaptic cargos, indicating the importance of this mechanism in synaptic functions. Further quantitative in vitro evaluations suggest that switching from kinesin-1 to kinesin-3 can be mechanically regulated based on their different physical properties and surrounding cytoplasmic environments. By increasing the numerical ratio of kinesin-3 to kinesin-1, fast transport by kinesin-3 was achieved; however, in the crowding solution which mimics the AIS cytoplasm, the velocity was predominantly governed by kinesin-1. This indicates that a single cargo can automatically switch its motors depending on the degree of crowding. Together, these results demonstrate a general mechanism of fast and efficient axonal transports and update the view of multimotor cooperation.

B133/P0127

Intermicrovillar adhesion complex motor MYO7B exhibits tail domain oligomerization

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Intestinal epithelial cells promote nutrient absorption and host defense via the brush border; an apical organelle composed of thousands of actin-supported finger-like protrusions known as microvilli. The intermicrovillar adhesion complex (IMAC) drives the tight packing of these protrusions to maximize their numbers on the apical surface. IMACs localize to the distal tips of microvilli and consist of extracellular adhesion links formed by interactions between the protocadherins CDHR2 and CDHR5, cytoplasmic adapters ANKS4B and USH1C, and the actin-based motor MYO7B, which is proposed to link the entire complex to the cytoskeleton. MYO7B was shown to interact with other IMAC proteins through its tail domain and promote their enrichment at the distal tips of microvilli. However, MYO7B lacks a clear dimerizing motif such as a coiled coil, found in most processive motors, and the mechanisms that promote tipward transport remain unclear. We found that when overexpressed in non-polarizing HeLa cells, which lack a majority of IMAC proteins, the MYO7B tail domain forms discrete cytoplasmic puncta. Immunofluorescent staining of USH1C, the only IMAC component endogenously expressed in these cells, only weakly correlated with MYO7B tail puncta. This led us to wonder if the MYO7B tail forms complexes with itself, either directly or indirectly through binding partners. If so, this would provide a mechanism for oligomerizing MYO7B motors, to enable IMAC transport. To test this hypothesis, we employed an in-cell nanoscale pulldown technique; we co-expressed a tip-directed chimeric motor ‘bait’ consisting of the MYO10 motor domain and its native coiled-coil fused to the MYO7B tail (M10mdcc-M7Btail), along with MYO7B tail as ‘prey’. These assays revealed robust MYO7B tail enrichment at filopodial tips, but only when the chimeric motor was co-expressed. This data suggested that MYO7B tails can oligomerize and this interaction is sustained during active transport. Our findings also revealed that endogenous USH1C did not accumulate at the tips of protrusions with M10mdcc-M7Btail and MYO7B tail, further suggesting that oligomerization of MYO7B tails is independent of known IMAC proteins. We next sought to determine if MYO7B tail oligomerization could enable processive motor transport in the absence of other self-association motifs. Indeed, the addition of MYO7B tail to the MYO10 motor domain lacking its coiled-coil was sufficient to promote distal tip targeting. These results demonstrate that the MYO7B tail oligomerizes and forms complexes stable enough to facilitate active transport to the distal tip compartment. Further studies will focus on dissecting the molecular requirements that enable MYO7B tail oligomerization.

B124/P0118

Investigating localization and expression patterns for Nonmuscle Myosin IIA interacting proteins in murine and human kidneys using super resolution microscopy.

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Our lab has previously demonstrated critical roles for Non-muscle Myosin II isoforms, MYH9 (NMIIA) & MYH10 (NMIIB) in trafficking of unique proteins uromodulin (UMOD) and sodium, potassium & chloride co-transporter 2 (NKCC2) in adult murine kidney epithelium, specifically in the thick ascending limb (TAL) cells. We performed a proximity-dependent biotinylation assay (BioID) using NMIIA or Myo18A heavy chain fusion proteins to identify protein partners that may interact with NMII isoforms to facilitate cargo transport within TAL cells. We utilized a novel SV-40 immortalized mouse TAL (imTAL) cells generated in our lab for the BioID experiment. This approach identified several cytoskeleton-associated proteins, including Myo18A, Ladinin-1 (Lad-1), Septin-9 and unconventional Myosin 1c (Myo1c) as potential Myh9 interacting proteins. We assessed the expression and cellular localization of these BioID candidate proteins in vibratome sections of mouse and human kidney using immunofluorescence staining followed by super resolution microscopy. Our results show expression of these candidate proteins in cellular compartments that express NMII isoforms and/or also associated with the actin cytoskeleton. Our images are in nanometer scale; they represent 3D localization and expression within intact kidney tissues. Expression of Myo18A was observed in the brush border of the proximal convoluted tubule (PCT), basolateral infoldings and the apical membrane of the TAL, and apical and basolateral membrane of the collecting duct (CD). Lad-1 was found in the glomerular capsule, basolateral membrane and brush border of the PCT, basolateral infoldings and the apical membrane of the TAL, and apical and basolateral membrane of the collecting duct (CD). Interestingly, we observed high expression of Septin 9 in glomerulus and CD of the human kidney. In murine kidney, Septin 9 was highly expressed in the brush border of some segments of the PCT and low expression along the basolateral side of the TAL. Myo1c was observed mainly along the basolateral infoldings and the apical membrane of the TAL and DCT. These localization and expression patterns of BioID candidate proteins in both murine and human kidneys suggest they may interact in tandem with NMII isoforms to regulate actin dynamics maintaining cellular and organelle architecture in various renal epithelial cells in adult kidneys.

B117/P0111

KIF1A-Mediated Trafficking is Required for Autophagosome Biogenesis and Maturation in Human Neurons

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KIF1A Associated Neurological Disorder (KAND) is a group of neurodevelopmental and neurodegenerative diseases caused by mutations in the KIF1A gene. To develop effective treatments for KAND patients, we need to better understand the molecular and cellular basis for the disease and how aberrant KIF1A-dependent trafficking leads to disease manifestation. Autophagy is a highly conserved pathway required for neuronal homeostasis that is frequently disrupted in neurodegenerative disease. There is evidence implicating KIF1A in neuronal autophagy, but whether KIF1A is required for autophagy in human neurons is unknown. To address this question, we investigated the role of KIF1A in autophagy biogenesis and maturation using human IPSC-derived neurons gene-edited to express a pathogenic C92* mutation in KIF1A. First, we compared wildtype (WT) neurons to C92* homozygous null mutant neurons, which lack KIF1A expression as shown by qPCR and

immunoblot. We examined the effects of KIF1A loss on the trafficking of ATG9, a transmembrane scramblase necessary for autophagosome biogenesis. Using immunohistochemistry, we found that ATG9 vesicles accumulated at the trans-Golgi network and were depleted from the axon in C92* null neurons. Consistent with this observation, confocal live-cell imaging revealed a decreased rate of autophagosome biogenesis events in the distal axon, which led to a significant deficit in LC3-positive autophagosomes distributed along KIF1A-null axons compared to WT. Loss of KIF1A in the C92* mutant also drastically reduced lysosomal density along the axon. This depletion of axonal lysosomes induced a deficit in autophagosome maturation, as we observed significantly less acidified autophagosomes in KIF1A-null axons. Finally, to extend these findings to a more clinically relevant context, we are examining the dynamics of autophagy in neurons gene-edited to heterozygously express the pathogenic variant p.C92*, a de novo mutation linked to a Rett-like syndrome in KAND patients. Neurons heterozygous for the C92* variant also displayed significantly reduced lysosomal density along the axon, which we hypothesize will lead to deficits in autophagy maturation, a possibility we are currently exploring. Together, these results suggest that KIF1A-mediated transport is critical to maintain neuronal autophagy and that targeting deficits in autophagy could be a promising therapeutic strategy for alleviating symptoms of KAND.

B116/P0110

KIF3B/B/KAP3 tail domain facilitates the transport of TRIM46, specifically to axon initial segment

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Intracellular transport is essential for neuronal function, yet how motor proteins achieve cargo specificity remains unclear.Kinesin superfamily proteins (KIFs) are vital components of the microtubule-based intracellular trafficking system. Among them, the heterotrimeric KIF3/KAP3 complex, consisting of KIF3 motors and the cargo-binding adaptor KAP3, plays crucial roles in neuronal intracellular transport. This study investigates the diversity of KIF3/KAP3 hetero-oligomerization states and their specific functions. We newly identified KIF3B/B/KAP3 in addition to KIF3A/B/KAP3 in neurons, each with distinct roles. Notably, KIF3B/B/KAP3 forms a stable complex that specifically binds TRIM46, a specifically localized protein at the axon initial segment, unlike other reported KIF3/KAP3 cargoes. Structural analyses revealed distinct cargo-binding mechanisms for KIF3A/B/KAP3 and KIF3B/B/KAP3, which determine their cargo specificity and functional differences. These findings provide new insights into the structural and functional diversity of kinesin-2 complexes, highlighting their multifaceted roles in neuronal intracellular transport.

B118/P0112

Loss of KIF5A Autoinhibition Disrupts Mitochondrial Localization in Motor Neurons

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Amyotrophic lateral sclerosis (ALS) is an incurable, fatal neurodegenerative disorder characterized by muscle weakness and paralysis, resulting from the degeneration of motor neurons (MNs). More than 30 distinct genetic mutations are linked to ALS, many of which affect mitochondrial health, RNA processing, and/or axonal transport driven by the molecular motor proteins kinesin and dynein. One such mutation is in the C-terminus of KIF5A, a neuronally-expressed kinesin-1 motor critical for anterograde axonal transport. However, we do not

yet understand the specific role of KIF5A in MNs, nor the consequence of ALS-associated mutations on axonal transport. The C-terminus of kinesin functions to autoinhibit the protein, preventing the motor from binding to microtubules. ALS-linked mutations in Kif5a cause exon 27 skipping during mRNA processing, resulting in a frameshift that alters the sequence of the distal C-terminal tail to include 39 novel amino acid residues in a variant termed KIF5AΔ27. The KIF5AΔ27 neopeptide disarms autoinhibition such that the motor is hyperactive, resulting in an accumulation of KIF5AΔ27 motors at the distal ends of neurites. Thus, we asked if canonical kinesin-1 cargos, such as mitochondria, are mislocalized in KIF5AΔ27 MNs. To answer this question, we utilized microfluidic devices to image human iPSC-derived MNs, comparing wild-type (WT) to heterozygous KIF5AΔ27 mutant lines. We find mitochondria accumulate at the distal ends of KIF5AΔ27 MN axons, but measurements of mitochondrial size indicate no changes between genotypes, suggesting mitochondrial fragmentation is not impacted. We turned to single-molecule motility assays to probe how KIF5AΔ27 results in distal mitochondrial accumulation, asking if the mutation affects regulation by the mitochondrial adaptor proteins TRAK1 or TRAK2. Our initial data suggest neither TRAK1 nor TRAK2 further activates KIF5AΔ27 anterograde run frequency. These data indicate the loss of KIF5AΔ27 autoinhibition is sufficient to dysregulate mitochondrial localization in MNs. Overall, this work highlights a critical role for KIF5A in the maintenance of intraneuritic mitochondrial organization and points to a possible mechanism for the distal axonal degeneration observed in ALS.

B112/P0106

Mutations in the kinesin KIF12 promote MASH in humans and mice by disrupting lipogenic enzyme turnover

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As a common cause of liver cirrhosis, metabolic dysfunction-associated steatohepatitis (MASH) is regarded as a target of therapeutic intervention. However, a successful therapy has not yet been found, partly because the molecular pathogenesis is largely elusive. Here we show that KIF12 kinesin suppresses MASH development by accelerating the breakdown of two lipid biosynthesis enzymes, acetyl-CoA carboxylase 1 (ACC1) and pyruvate carboxylase (PC), in hepatocytes. We report three familial early-onset liver cirrhosis pedigrees with homozygous KIF12 mutations, accompanying MASH-like steatosis and cholestasis. The mouse genetic model carrying the corresponding Kif12 nonsense mutation faithfully reproduced the phenotypes as early as between 8 and 10 weeks of age. Furthermore, KIF12-deficient HepG2 cells exhibited significant steatosis, which was ameliorated by overexpressing a proline-rich domain (PRD) of KIF12. We found that KIF12-PRD promotes the degradation of ACC1 and PC, and this effect is likely to be through its direct interaction with these enzymes. Interestingly, KIF12 enhanced the ubiquitination of ACC1 by the E3 ligase COP1 and colocalized with these proteins as seen by super-resolution microscopy imaging. These data propose a role for KIF12 in suppressing MASH by accelerating turnover of lipogenic enzymes

B139/P0133

MYO19 knockdown alters differentiation in the neuron-like CAD cell line

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CAD cells are derived from the catecholaminergic cell line Cath.a, originating from the mouse central nervous system. Upon serum starvation, these cells undergo morphological changes, extending processes resembling neurites. Myosin-XIX (MYO19), an unconventional myosin motor protein, plays a role in mitochondrial

movement and distribution along actin networks, such as those found in neurites. Proper mitochondrial positioning is essential for energy-intensive cellular functions, in regions such as growth cones, which require high ATP input. CAD cells are easily cultured, and the clear separation of neurites from the soma makes their processes a useful model for observing MYO19 and mitochondrial movement along actin filaments. In this study, we characterize the CAD cell transcriptome to evaluate their suitability as a neuronal model and to investigate the effects of MYO19 knockdown on neurite morphology. To assess the CAD cell transcriptome, we performed RNAseq analysis on both undifferentiated and differentiated cells. Using KEGG pathway gene sets, Pathview analysis identified 13 nervous system-associated pathways that were upregulated upon differentiation. This supports the reliability of CAD cells as a model for studying neuronal features, including the distribution and morphology of mitochondria and MYO19 in neuron-like cells. To explore the functional role of MYO19 within CAD cells, we generated stable MYO19 knockdown (MYO19KD) cell lines using shRNA constructs and evaluated differentiation and neurite morphology within the cells. Compared to wild-type (WT) cells, MYO19KD cells exhibited delayed differentiation, developing shorter, fewer neurites than WT cells at the same stage of differentiation. These findings suggest that MYO19 knockdown impairs neurite outgrowth, potentially by disrupting mitochondrial distribution, thus limiting ATP availability. We also carried out transcriptomic analysis comparing mitochondrial transport genes in differentiated WT and MYO19KD cells and did not observe significant differences in expression. Given the transcriptomic similarities between CAD cells and neurons, our results may also indicate that reduced MYO19 expression could contribute to nervous system pathologies.

B131/P0125

Myosin 18A regulates myosin 2 filament assembly and stability

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Myosin 18A (M18A) is an essential, myosin 2 (M2)-like myosin that lacks motor activity and does not assemble into filaments on its own but does co-assemble with M2 to make mixed bipolar filaments (Billington and Beach et al CB 2015). Because alternatively spliced versions of M18A possess N- and C-terminal extensions harboring both recognizable and uncharacterized protein: protein interaction domains, it is thought to drive the functional diversity of M2 filaments by recruiting proteins to them and/or attaching them to cellular structures (Alexander et al FASEB 2021). Using TIRF-SIM imaging of M2 filament assembly in GFP-M2A knockin (KI) MEFS, Beach and colleagues (NCB 2017) described a pathway termed sequential amplification in which individual M2A filaments appear to replicate via a splitting process that is promoted by actin dynamics (see also Fenix et al MBoC 2016). Subsequent work by Quintanilla and colleagues (JCB 2024) showed that sequential amplification actually involves the separation of adjacent filaments that are not resolvable by SIM, and that new filament assembly is promoted by preexisting filaments. Here we used dynamic imaging to explore the contributions made by M18Aα, which harbors a nucleotide-insensitive actin binding site in its N-terminal extension, and M18Aβ, which lacks this N-terminal extension, to the sequential amplification of M2A filaments in GFP-M2A KI MEFS. While both isoforms were found in mixed filaments as expected, they differed in that transient interactions with M2A filaments appeared to trigger filament splitting in the case of M18Aα but not M18Aβ. Current efforts are directed at determining if this difference is due to the nucleotide-insensitive actin binding site in M18Aα's N-terminal extension, as it could be facilitating actin dynamic-dependent filament splitting. To look for possible effects of M18A on the stability of M2 molecules within filaments, control and M18A knockout Rat 2 fibroblasts were transfected with GFP-M2A or GFP-M2B and subjected to

photobleaching. The kinetics of fluorescence recovery indicated that the lifetime of both M2 isoforms within filaments is reduced when M18A is absent. Together, our results are consistent with M18A playing significant roles in M2 filament assembly and stability, which could have significant impact on multiple cellular mechanisms driven by M2-dependent force.

B128/P0122

Opposing kinesins regulate the spacing of nuclei in the multinucleated myofiber through diverse microtubule dependent mechanisms

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One of the first genes identified to regulate the spacing of nuclei in the multinucleated myofiber was Kinesin-1. However, the mechanism of Kinesin-1-dependent nuclear spacing is not known. One popular model that has never been tested is that Kinesin-1 slides antiparallel microtubules (MTs) that emanate from neighboring nuclei (MT-sliding). To determine whether the MT organization could support MT-sliding we identified MTs that originate from each nucleus and found that there were significant regions of antiparallel MT overlap between adjacent nuclei. We then found that myonuclear spacing in a null Kinesin-1 mutant was rescued by a CRISPR-Cas9 knock-in of WT Kinesin-1 but not by a sliding-deficient Kinesin-1, suggesting that Kinesin-1 spaces nuclei by sliding antiparallel MTs, similar to the mechanism by which it elongates a mitotic spindle. We therefore tested whether other kinesins that regulate MT-sliding in the mitotic spindle such as the bipolar Kinesin-5 and the (-) end directed kinesin ncd, are also necessary for myonuclear spacing. We found that Kinesin-5 and ncd had opposing effects on nuclear movement during Drosophila embryogenesis; knockdown of Kinesin-5 reduced myonuclear movement speed and knockdown of ncd increased myonuclear movement speed. This supports a model where Kinesin-5 and ncd work against one another, with the former driving antiparallel MTs apart and the latter sliding them together during muscle development. While this model describes active myonuclear movement in the embryo, we were curious if these kinesins also affected spacing in the differentiated larval myofiber. Interestingly, we found that only depletion of ncd in larva disrupted MT organization and caused a dramatic clustering of myonuclei. These data suggest that beyond its role in embryonic myonuclear movement, ncd dynamically crosslinks MTs to anchor myonuclei during robust muscle growth. Together, our data supports a model where myonuclear spacing is regulated by a counteracting force generated by different kinesins during embryonic development. Furthermore, one kinesin, ncd, is repurposed in the differentiated myofiber to dynamically crosslink MTs, a function necessary to anchor nuclei in place. Thus, kinesin motors regulate myonuclear spacing across developmental time by leveraging opposing forces across several diverse mechanisms.

B126/P0120

Proximity Biotinylation Identifies Dynein-Dynactin Interactors in Pathogen-Infected Host Cells

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Dynein is the major retrograde microtubule (MT) motor that transports diverse cellular cargos along the MT network. The megadalton complex is composed of multiple dynein subunits that associate with dynactin and versatile sets of adaptors proteins that link dynein to its cargo. Several bacteria and viruses hijack vesicular transport pathways to promote their propagation within host cells. Following entry, intracellular bacterial

pathogens such as Salmonella and Chlamydia survive and replicate within a membrane-bound compartment. The bacteria compartments utilize dynein-mediated trafficking pathway to facilitate their trafficking on microtubule tracks to the cell center. To investigate how dynein activity affects Chlamydia intracellular movement, we treated infected HeLa cells with dynarrestin, a small-molecule dynein inhibitor. Cells were infected and fixed at 5, 6, and 7 h post-infection and co-stained for γ-tubulin (microtubule-organizing center, MTOC) and Chlamydia major outer membrane protein (MOMP). Quantification of MTOC-Chlamydia distances showed that Chlamydia was further from the MTOC in dynarrestin-treated cells than in control cells. To identify dynein interactors engaged during infection, we used proximity-dependent biotinylation (BioID/TurboID) coupled with mass spectrometry to profile proteins proximal to dynein in live infected cells. We generated an inducible miniTurbo-3×FLAG–DIC2 fusion in a Flp-In HeLa cell line. Expression was induced with 1 µg/mL tetracycline overnight, followed by 6 h biotinylation. Fusion-protein expression and biotinylation were verified by immunoblotting with anti-DIC2, anti-FLAG, and streptavidin. Moreover, cells were methanol-fixed (−20 °C, 10 min), blocked (3% BSA/1% FBS), and stained with anti-FLAG/anti-DIC2 and streptavidin to detect the fusion and biotinylated targets. Both FLAG and DIC2 exhibited strong colocalization with our biotinylated construct at the MTOC. We performed BioID assays on Chlamydia- and Salmonella-infected cells and analyzed the samples by mass spectrometry (in collaboration with Dr. Raught's lab, SickKids) using a SAINT cutoff of BFDR < 0.05 (FDR < 5%). High-confidence interactors were visualized in Cytoscape (v3.6.1). Proximity ligation assays further confirmed close spatial proximity of DIC2 with two prospective interactors: HAUS1 and NDEL1. We are now using siRNA to deplete HAUS1 and NDEL1 during Chlamydia and Salmonella infection to compare bacterial phenotypes between knockdown and control groups to examine their roles in dynein-dependent MTOC movement.

B122/P0116

Reactivation delay of catch-bonded dynein allows bidirectional transport

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Intracellular transport is crucial for a multitude of cellular functions, aided by motor proteins like kinesin and dynein, which walk on the microtubule network inside cells. Unlike kinesin, dynein can form a catch bond with microtubules, allowing it to endure high loads without detaching. Dynein experiences a catch bond during the "tug of war" of equally active opposing motor proteins in bidirectional transport. However, the effect of such a bond on bidirectional transport remains elusive. To investigate the precise role, we artificially subjected dyneins under high load to induce catch bond using an optical trap on early phagosomes (EPs) inside J774 cells. This condition was termed as "Superstall", which resulted in a 25% increase in motion reversals from dynein to kinesin (DK transition). This suggests that when dynein detaches under high load, it promotes kinesin binding on the microtubule. We reproduced similar results by superstalling isolated latex bead EPs from Dictyostelium using an in-vitro motility assay. Our findings led to the hypothesis that dynein shows transient inactivity post-catch bond. This speculation was validated in a robustly dynein-driven cargo - Late Phagosomes (LPs), where a larger population of dyneins was superstalled and observed a 3.4-fold increase in the re-attachment time of the motor. We discovered that dynein re-attachment to the microtubule follows a two-step sequential process, indicating an intermediate/inactive dynein (triggered only after the catch bond). We coined a novel term, "reactivation delay," to describe the time required to transition from the inactive (D*) to the active (D) state of dynein. The reactivation delay for dynein was experimentally calculated to be 1.31 ± 0.026s. Following this, the introduction of an inactive dynein state recovered bidirectional tracks in the Monte Carlo simulation with 2 kinesins versus the 6 dyneins regime, which was not reported earlier. Overall, we present a new model for bidirectional transport, which is regulated by the reactivation delay of the post-catch bonded dynein. In a

broader aspect, this concept of reactivation delay can be extended to other proteins known to form catch bonds, which helps preserve specific activities.

B125/P0119
Smooth muscle myosin activity drives vascular smooth muscle cell differentiation
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Vascular smooth muscle cells (VSMCs) are highly dynamic and contractile, playing a key role in blood flow regulation. VSMCs exist on a functional spectrum between the differentiated “contractile” phenotype and the dedifferentiated “synthetic” phenotype. While their contractile nature is important for regulating blood pressure, their ability to downregulate contractility and promote migration is necessary for tissue repair in response to injury. Misregulation of these transitions can be maladaptive, contributing to diseases such as atherosclerosis. The mechanisms linking VSMC contractility and phenotype are currently not well understood. Smooth muscle myosin II (SMII) is the essential motor driving VSMC contractility. SMII expression inversely correlates with VSMC dedifferentiation, and the field has suggested that SMII downregulation is an effect of this phenotype switch. However, contractility has been established throughout cell biology as causative in cell fate and differentiation. We hypothesize that SMII lies upstream of the differentiation switch and therefore helps drive VSMC phenotype. We engineered an endogenous EGFP-labeled SMII murine model to directly quantify changes in expression, localization, and dynamics of VSMC contractile machinery throughout phenotypic transitions. We cultured the EGFP-SMII aortic tissue ex-vivo and observed VSMCs migrating onto the culture dish within days. In this transition, we observed a notable difference in EGFP-labeled SMII filament exchange dynamics using FRAP: the native tissue ex-vivo (differentiated) has stabilized SMII bundles that show little exchange of monomers, while the individual VSMCs isolated from the tissue (dedifferentiated) exhibit significant SMII monomer exchange. Additionally, we observed that the transduction of human primary VSMCs expressing pathology-associated SMII motor domain variants increases SMII monomer exchnage and decreases whole-cell contractility. These results suggest that SMII activity plays a causative role in driving VSMCs from one phenotype to another. To further test this hypothesis, we designed constitutively active and inactive SMII regulatory light chain constructs by modulating the phosphorylation site at Thr18/Ser19. We observed that inhibiting SMII activity via the light chain increases SMII turnover and decreases the fraction of SMII monomers assembled into filaments, while activating SMII stabilizes the filaments, further supporting our hypothesis. Additional studies to determine the downstream phenotypic impact of manipulating SMII activity are ongoing. Our work reveals molecular insights into the regulation of VSMC differentiation and suggests that SMII expression itself plays a significant role in determining VSMC phenotype.

B134/P0128
Spatial Regulation of Dynein in a Model Large-Cell Interphase Aster
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In interphase cells of the early embryo, dynamic microtubules (MTs) assemble into radial arrays called asters, with their minus ends facing in. These networks, which vary in size from the micron to millimeter scale, serve as tracks for motor proteins, including dynein. As it moves toward the aster interior, dynein exerts force on

both its cargo and the microtubules that it traverses, thereby rearranging the intracellular space. Prior work from our lab in Xenopus laevis egg extract showed that dynein-mediated transport is not constant in the large interphase aster. Instead, organelles exhibited a burst of inward transport at the aster periphery before slowing down in the interior. We speculate that spatial restriction of dynein activity to the periphery may prevent concentration of organelles at centrosomes, which would interfere with their spatial segregation in cleavage divisions. To test if this spatial regulation is a property of dynein or of specific organelle cargoes, we tracked the inward transport of beads decorated with the dynein adaptor HOOK2 in egg extract asters. This system enables us to modulate cargo properties and to quantitatively measure their effects on transport dynamics. We find that, like endogenous organelles, our artificial cargoes exhibit fast inward transport at the aster periphery, which slows dramatically as they move inwards. This effect is general to a range of dynein cargoes with diverse properties, including size and dynein occupancy, as well as those functionalized with an alternative activating adaptor (BicD2). To test if internal transport is slowed by crowding, we tracked inert probe diffusion dynamics and found that the aster's interior is not substantially more crowded than its periphery, suggesting the internal slow-down is due to spatial regulation rather than steric occlusion. We next used proteomics to identify proteins in the dynein-dynactin system that are present at low concentration in egg extract and might therefore participate in rate limiting steps. NDEL1, which acts catalytically to facilitate active dynein-dynactin complex assembly, was present at ~30nM, while most other components were present at 300-600nM. Addition of excess NDEL1 stimulated dynein-mediated cargo transport, showing that it is indeed rate-limiting. We are currently investigating the effect of changing NDEL1 concentration on spatial regulation.

B129/P0123

Steady-state epithelial apical flatness depends on MLCK morphodynamics and asynchronous Ca2+ oscillations, but not underlying ECM geometry

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The canonical simple epithelium is a flat sheet-like tissue of horizontally packed cells. While the basal surface is delineated by the basement membrane of extracellular matrix (ECM), little is known about how a flat apical surface is maintained, or if apical/basal dynamics are coordinated. The current study tests the role of the apical domain, to define mechanisms involved in maintaining a flat apical geometry in an epithelium. Pharmacological and transgenic disruption of non-muscle myosin ATPase, and MLCK activity results in an uneven apical structure, and overall loss of the flat geometry typical of a confluent epithelium. MDCK cells evoke Ca2+ transients autonomously and Ca2+ imaging reveals an asynchronous calcium flux across a confluent epithelium suggesting a myosin II-mediated mechanism for maintaining a flat apical architecture. When internal Ca2+ stores are depleted, apical flatness is lost in the epithelium. Finally, when the basal geometry is modulated, MDCK cells adjust their morphology to maintain overall apical flatness of the confluent layer. Our results highlight that apical/basal cellular surfaces may not be tightly coordinated, but rather independently regulated. This study provides a new paradigm for how apical flatness is regulated at steady-state for epithelial resilience during human development and disease.

B132/P0126

The Interplay Between the Tubulin- and MAP-Codes and Their Effects on Kinesin Motility

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Microtubules (MTs) play a critical role in many cellular processes, most significantly in cellular trafficking. It has been proposed that signals that regulate motor-driven intracellular transport are deposited onto MT tracks, either by post-translational modifications (PTMs) and isotypes of tubulin (tubulin-code) or MT-associated proteins (MAP-code). In vitro studies showed that MT motors are tightly regulated by MAPs, but not by tubulin PTMs. Therefore, it remains unclear whether tubulin modifications directly regulate MAPs and motors on the MT. Using recombinant tubulin, we investigated the interplay between the tubulin- and MAP-codes and how they impact motor activity in vitro. We found that different tubulin modifications have a minor impact on kinesin motility when MTs are stabilized by taxol, because taxol expands the MT lattice and outweighs the effects of tubulin PTMs. However, these changes can significantly affect MT-MAP decoration, which in turn strongly affects motility. In dynamically growing MT experiments, we observed different tubulin modifications selectively tune the recruitment of MAPs and motors to the MT. We showed that tubulin PTMs affect the MT affinity of tau by altering its affinity to bind free tubulin. Our results are consistent with a two-layered code, in which the tubulin code selectively recruits MAPs onto MTs, and the MAP code dictates which motors can transport cargo along those tracks.

B121/P0115

The Kinesin-14 Tail Interacts with the Tubulin C-terminal Tails and the Lattice for Microtubule Interaction

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Proper spindle function requires the activity of molecular motor proteins that contribute to spindle morphogenesis. Kinesin-14 motors are minus-end directed motors that cross-link both parallel and anti-parallel microtubules (MTs) and contribute to spindle pole organization, spindle length control, and centrosome clustering. Our previous work identified two MT-binding domains (MBD1 and MBD2) in the Kinesin-14 tail domain wherein MBD1 facilitates faster anti-parallel MT sliding, and MBD2 promotes static MT cross-linking. How a small, 13 kDa domain can mediate distinct types of MT binding and cross-linking is unclear. Using full-length tail constructs with mutations that disrupted MT binding of either MBD1 or MBD2, we found that subtilisin-mediated removal of both α- and β-tubulin C-terminal tails (CTTs) reduced MT binding by ~50%, indicating that tail MT binding is partially dependent on the disordered tubulin CTTs. In contrast, the binding of the motor domain was unaffected by subtilisin treatment, consistent with the kinesin motor domain predominantly interacting with the MT lattice. Competition assays revealed that functional MBD2 can compete with the motor domain for MT binding, suggesting that MT binding of the tail may overlap with the kinesin binding domain. Structural modeling studies predict that the tail is highly disordered, suggesting that it likely becomes structured upon binding to MTs. To further elucidate how Kinesin-14s interact with MTs, we solved the cryoEM structure of the Kinesin-14 motor domain (no nucleotide state) bound to MTs and found its binding site is predominantly on beta tubulin, similar to that of other kinesin motor domains. Current efforts are focused on elucidating the structure of the Kinesin-14 tail bound to MTs. Together our work will provide insight into how MT cross-linking motors mediate their multiple MT contacts through their motor and tail domains to mediate MT sliding in the spindle.

B136/P0130
The role of a Filopodial Myosin-Talin interaction in Dictyostelium filopodia formation
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Filopodia are thin dynamic membrane protrusions, characterized by parallel bundles of actin filaments with a protein complex at the tip that cells use to explore and interact with their environment. Filopodia formation requires a MyTH-FERM myosin, such as DdMyo7 in amoeba, yet the mechanism of myosin-based filopodia formation is not fully understood. The major DdMyo7 binding partner is TalinA (TalA), a conserved mechanosensor required for cell adhesion with a predicted structure similar to HsTLN1 – an N-terminal FERM domain followed by a long C-terminal rod consisting of 13 helical bundles (R1-R13). The functional significance of their interaction and the role of TalA in filopodia formation are not known. Live cell imaging reveals that TalA is found at both the initiation site of filopodia and at their tips, similar to DdMyo7. Interestingly, TalA-null cells exhibit a decrease in filopodia number that corresponds to reduced cortical targeting of DdMyo7, suggesting that binding to TalA is important for DdMyo7 cortical recruitment. The functional significance of the TalA:DdMyo7 interaction was investigated by identifying the binding sites on DdMyo7 and TalA using a combination of deletion mapping and AlphaFold (AF) multimer prediction. Deletion of the DdMyo7 candidate dimerization domain (cDD, AA 884-952) resulted in disruption of the TalA:DdMyo7 interaction. The DdMyo7-cDD was then predicted to bind TalA-R11 by AF multimer, and indeed, deletion of R11 abolished TalA interaction with DdMyo7, as seen by loss of co-immunoprecipitation. Interestingly, TalA-ΔR11 was no longer found at filopodia initiation sites or at tips, and was instead strongly mis-localized to the rear of the cell. Similarly, TalA was also localized to the rear of DdMyo7-null cells. The results reveal that DdMyo7 and TalA mutually regulate each other's localization and function for optimal filopodia formation. Supported by NIH, NIGMS R01GM122917 to MAT

B119/P0113
Uncoordinated-76, essential for neurite outgrowth, is a regulator of microtubule-microtubule sliding by kinesin-1
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Kinesin-1, a major plus-end-directed microtubule motor, is essential for transporting many cellular cargoes along microtubules. Past findings from our lab have shown that, in addition to cargo transport, sliding of cytoplasmic microtubules against each other by kinesin-1 is crucial for neuronal development. However, the kinesin-1 interactions that regulate its multiple activities remain incompletely understood. Uncoordinated-76 (UNC-76) is a conserved cytosolic protein important for axonal outgrowth and transport across species. It was initially proposed that UNC-76 functions as a kinesin-1 cargo adaptor. However, our new data demonstrate that UNC-76 regulates microtubule–microtubule sliding by kinesin-1, rather than organelle transport, in neurons and other cell types. Depletion of UNC-76 leads to a dramatic increase in microtubule sliding. Remarkably, axonal outgrowth is stunted, and the defects can be rescued by the human ortholog of UNC-76, fasciculation and elongation protein zeta-1 (FEZ1), demonstrating its conserved role in regulating neuronal development.

B115/P0109
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 mutations 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. Using a bioinformatics approach, we identified nodes of direct interaction between SPGs, several of which included proteins with non-overlapping gene ontology groupings. The largest of these nodes contains the neuronally enriched kinesin KIF5A (SPG10), one of its known light chains (KLC2/SPG68), and 11 additional SPGs. While only three SPGs directly interact with KIF5A, expanding our bioinformatic analysis to include indirect interactions identified 13 additional SPGs with the potential to interact with KIF5A. To determine whether these protein complexes form in neurons, we have performed a series of co-localization/co-migration studies, as well as co-immunoprecipitation studies in neuronal cell culture. Using time-lapse fluorescence microscopy, we have observed puncta of Kif5A co-localizing and co-migrating with Klc2, Smn1, Tsg101, and Itsn1 in neurons. In co-immunoprecipitation studies using epitope-tagged Kif5A as bait, we have pulled down Klc2 and Smn1 from neuronal lysates. While these experiments are ongoing, our early studies suggest that these complexes form in neuronal cells and may contribute to the cellular pathway that is affected in the context of HSP caused not only by mutations in KIF5A, but also its interacting partners. Here we will present our ongoing analysis of interactions in the SPG network to broaden our understanding of the HSP disease mechanism.

Developmental and Stem Cell Biology - Cellular Asymmetry and Polarization

B010/P1142
Actin cytoskeleton regulators for cardiac cell chirality
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The heart is the first asymmetric organ in the development of the human body. Cell chirality has been heavily implicated in the early asymmetric looping of the cardiac tube. However, the biophysical mechanisms that govern cardiac cell chirality, leading to asymmetric morphogenesis, are poorly understood. Prior works assessing the chirality of fibroblasts have indicated that alterations to the actin cytoskeleton can regulate cell chirality. The OBJECTIVE of this study is to examine the function of the putative regulators, including Actinin 1, CapZβ, and Profilin 1, in modulating cardiomyocyte chirality. Genetic manipulation, using siRNA-mediated knockdown or plasmid transfection for overexpression, was used to examine the role of these three proteins in chirality. The transfected cells were seeded onto ring-shaped micropatterns to assess their changes in chiral alignment. We found that loss of α-actinin 1 resulted in reversal of cardiomyocyte chirality from clockwise

(CW) to counterclockwise (CCW) biases. While knockdown of CapZβ and Profilin 1 increased the CW bias, overexpression of these two proteins was not sufficient for chirality reversal. However, loss of CapZβ or Profilin 1 in α-actinin 1 knockout cells recovered a CW bias, indicating that α-actinin 1 safeguards a CW bias. In addition, it also suggests the existence of a CW-driven chiral mechanism, which doesn't require the presence of any of these three proteins. Our findings provide a deeper understanding of how chirality is regulated in different cell types and add new insights into how cell chirality is regulated through actin crosslinking and polymerization-associated proteins.

B021/P1156

Cluster assembly dynamics drive fidelity of planar cell polarity polarization

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Planar cell polarity (PCP) signaling orients epithelial cells along an in-plane tissue axis and is a required feature of embryonic development in bilaterians. In PCP signaling, distinct molecular subcomplexes segregate to opposite sides of each cell, where they interact across intercellular junctions to form highly polarized clusters. How signal amplification and error correction occur to achieve the observed level of asymmetry is not known. Here, we used quantitative photobleaching to quantify the number of monomers of core PCP proteins within individual clusters in living Drosophila pupal wings. These data, combined with mathematical modeling, allowed us to determine how the growth of PCP signaling complexes is coupled to robust and accurate polarization. We find that clusters become increasingly polarized and correctly oriented as they become larger. Further, mutations that inhibit cluster growth inhibit cell- and tissue-level polarization, confirming that a population of large clusters is required for successful polarization. Our results reveal how cluster assembly provides the signal amplification and error correction necessary for robust polarization at the molecular, and hence cellular and tissue, levels, a mechanism that may apply to the many signal transduction pathways that feature the assembly of large, nonstoichiometric signalosomes.

B019/P1826

Cytoskeletal Control of Rosette Remodeling and Lumen Formation in the Zebrafish Left-Right Organizer

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Left-right asymmetry in vertebrate embryos is initiated by the Left-Right Organizer (LRO), with the zebrafish Kupffer's Vesicle (KV) serving as a model to dissect the cellular mechanisms of symmetry breaking. During early KV formation, dorsal forerunner cells (DFCs) interface with the enveloping layer (EVL) via transient tight junctions and organize into small rosettes. These DFC-EVL contacts are lost as DFCs transition to forming a contiguous cyst of cells, coinciding with the onset of lumenogenesis. Prior to this transition, we observe that microtubule (MT) bundles that arise from non-mitotic origins at sites preceding nascent ZO-1 puncta at DFC-EVL interfaces. Live imaging and targeted ablation reveal that LRO cells divide and orient their cytokinetic bridges, with associated MT bundles, toward ZO-1–positive sites that promote actin recruitment and rosette expansion. Disruption of cell division or spindle orientation impairs rosette coalescence and lumen formation. These findings highlight a temporally restricted role for MT architecture in spatially patterning ZO-1 and actin-

enriched rosettes, which ultimately fuse to form the KV lumen. Our work reveals how cytoskeletal organization and division history govern epithelial morphogenesis at the earliest stages of LRO development.

B014/P1146
Cytoskeletal remodeling during cell-cell fusion in the human placenta
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The human placenta is essential for fetal development, and its proper function is required for pregnancy success. Central to nearly all critical placental roles—including nutrient and gas exchange, endocrine function, and immune protection—is the syncytiotrophoblast (STB). The STB is a multinucleated, polarized, terminally differentiated epithelial cell that covers the branching villous trees of the placenta. This expansive surface grows throughout gestation to meet the increasing metabolic needs of the developing fetus, reaching 12–14 m^2 and containing billions of nuclei at term. Growth of this unique cell depends on continuous cell-cell fusion of mononucleate cytotrophoblasts (CTBs). Proper STB formation is required for function, as placental pathologies including preeclampsia and intrauterine growth restriction display abnormal STB morphology and subcellular organization. Increasing evidence points to the cytoskeleton as a key regulator of growth, polarization, and fusion, yet its specific contributions to STB development and function remain understudied. Here, we investigate cytoskeletal remodeling during trophoblast fusion and differentiation using primary trophoblast 2D and 3D organoid models. We characterize cytoskeletal organization in mononucleated CTBs, fusing intermediates, and syncytial STBs. Our analysis reveals distinct network changes that suggest the cytoskeleton plays a role in mediating fusion and establishing polarization. We observe decreased microtubule density and polarity between CTBs and STB, alongside an increase in post-translational modifications which is consistent with a transition in network stability and function. Actin filaments also decrease in density and size, with increased disorder in filament orientation in STBs. At fusing membranes, F-actin reorganizes from long bundles to branching networks with dynamic cell protrusions, corroborating its essential role in cell-cell fusion. Septin family members 2, 7, and 9 display distinct localization patterns, associating with subsets of actin or microtubules and enriching at dynamic membranes, depending on the Septin. These findings suggest Septins act as scaffolds that integrate cytoskeletal networks with membrane dynamics and polarization in trophoblasts. Together, these results support a model in which cytoskeleton filaments dynamically remodel to coordinate cell-cell fusion, regulate STB growth, and maintain polarity across a meters-scale syncytium. By defining cellular mechanisms that underpin trophoblast morphogenesis, this work provides new insight into fundamental placental biology, and highlights potential pathways disrupted in pregnancy complications.

B009/P1141
Distinct roles of E-cadherin and N-cadherin in hepatocyte polarization and apical lumen formation
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Simple epithelial cells, such as intestinal enterocytes and kidney tubular cells, exhibit apical-basal polarity, with their apical surface facing the external environment and their basolateral membrane adhering to neighboring cells and the extracellular matrix. In contrast, hepatocytes, the parenchymal cells of the liver, display a unique polarity by forming a narrow elongated apical lumen known as a bile canaliculus (BC) between adjacent cells at the center of their contacts. However, the mechanisms underlying hepatic polarity and BC biogenesis remain largely unknown. Using the rat hepatocyte cell line Can 10, we previously showed that hepatocyte polarization

and BC formation are closely linked to cytokinesis, and that BC elongation involves oriented cell division. Unlike most epithelial cells that primarily express E-cadherin, hepatocytes express both E- and N-cadherin, though their respective roles have not been defined. We found that while both cadherins are jointly required for establishing hepatic polarity, they play distinct roles in BC elongation and polarity maintenance. Furthermore, a proximity-labeling biotinylation assay identified candidate effectors for E- and N-cadherin. Analyses of these candidates in Can 10 cells and ex vivo mouse fetal liver cultures indicate that the two cadherins regulate hepatic polarity and BC biogenesis through distinct yet coordinated mechanisms.

B013/P1145

Ebp50 regulates hepatocyte junction assembly and transport during apical lumen formation

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Hepatocytes are specialized epithelial cells that form narrow, elongated apical lumens called bile canaliculi (BC) between adjacent cells, structures essential for liver architecture and function. The mechanisms underlying BC formation, particularly elongation during liver development and regeneration, remain poorly understood. Using the rat hepatocyte cell line Can 10, which recapitulates features of developing and regenerating hepatocytes, we found that the Ezrin Binding Protein 50 (Ebp50), an apical scaffold protein, plays critical roles in both apical transport and BC formation. Strikingly, loss of Ebp50 not only delocalized selective apical transporters, but also prevented tubular BC formation. Knockdown studies and drug treatment revealed that Ebp50 selectively stabilizes apical transporters by preventing their endocytosis. Furthermore, functional, biochemical, and dynamic studies showed that Ebp50 regulates multiple proteins at the adherence junction (AJ) in an Ezrin/Radixin-independent manner. We propose that Ebp50 is essential for BC elongation by coordinating AJ assembly and apical transport.

B011/P1143

Elucidating role of membrane voltage in epithelia apicobasal polarization and fluid transport

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Ion transporters and membrane proteins are asymmetrically distributed along the apicobasal axis of epithelial monolayers. In the renal tubule, water flows from the apical side toward the basal side, enabling water reabsorption in the kidney. In contrast, basal-to-apical water transport drives fluid accumulation into lumens during organoid and embryonic development, providing the hydrostatic pressure essential for lumen expansion, tissue morphogenesis, and organ formation. Classically, the direction of water transport is attributed to the asymmetric localization of ion transporters along the apicobasal axis, which generates trans-epithelial osmotic pressure gradients driving water flux. Interestingly, while membrane voltage regulates many ion channels and membrane proteins, its role in epithelial polarization and fluid transport remains poorly understood. In this work, we investigated the relationship between membrane voltage and fluid transport using MDCK II cells expressing a novel ratiometric membrane voltage indicator, JEDI-2P-CyOFP1. While MDCK II monolayers are well-known to establish apicobasal polarity and generate transepithelial potential across the epithelium, our measurements further revealed that individual cells exhibit a distinct apicobasal gradient in membrane voltage. The apical membrane is more hyperpolarized than the basal side. During active fluid transport on 2D surfaces, where water flows apical-to-basal to inflate fluid-filled dome structures, basal membrane transiently becomes more hyperpolarized, surpassing the apical voltage. These hyperpolarization

events evolve dynamically within tens of minutes and return to baseline when domes deflate and fluid effluxes. We further demonstrate that anionic lipids and ion transporter activity contribute to these voltage changes. Together, our study provides novel insights into the relationship between cell membrane voltage and epithelial fluid flow. These results have strong implications for understanding the regulation of fluid transport during lumen formation, organoid morphogenesis, and embryo development.

B020/P1154
miR-190 is a Key Regulator in Establishing Cell Polarity and Specification in the Drosophila Nervous System
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Asymmetric cell division generates cellular diversity in developing tissues, particularly in the CNS. In Drosophila neuroblasts, this process relies on polarity complexes and fate determinants, yet its molecular regulation remains unclear. Here, we identify miRNA-190 as a key regulator of neuroblast polarity and differentiation. Single-cell RNA sequencing and transcriptome analysis reveal that miR-190 deficiency disrupts CNS cell populations, reducing neurons while increasing neural progenitors and glia. Mechanistically, miR-190 is required for proper localization of the Par complex and basal determinants during mitosis. In miR-190 mutants, these factors mislocalize, leading to defective polarity and fate specification in embryonic neuroblast. qPCR analysis shows that miR-190 targets RhoGAP, which modulates Cdc42 activation and Par-6, crucial factors in neuroblast polarity. We propose a model in which miR-190 ensures proper Cdc42 activation and polarity establishment by targeting transcripts for degradation. miR-190 has been implicated in various cancers, and our findings provide a mechanistic framework for understanding miR-190's roles in tumorigenesis and its broader involvement in metabolic diseases.

B016/P1148
Multivalent assembly of PAR-3/aPKC complexes establishes cell polarity in C. elegans zygotes
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Cell polarity is a fundamental organizing principle in metazoans. The conserved scaffold protein PAR-3, together with the kinase aPKC and its co-factor PAR-6, establishes polarity across diverse contexts and organisms. PAR-3 oligomerization is essential for recruiting aPKC and PAR-6, yet how oligomerization promotes PAR complex assembly remains unclear. Here, we show that PAR-3 and aPKC oligomerize cooperatively, due to multivalent interactions, in C. elegans zygotes. Using single-cell, single-molecule pull-down, we found that monomeric PAR-3 rarely binds aPKC or PAR-6, whereas oligomerized PAR-3 strongly associates with them, indicating that oligomerization enhances binding affinity. Conversely, PAR-3 binding to aPKC further strengthens PAR-3 oligomerization, revealing a reciprocal cooperativity. This cooperativity requires PDZ–PDZ ligand interactions and PAR-6 involvement. Prior to zygotic cytokinesis, the cell cycle regulator PLK-1 downregulates PAR-3/aPKC cooperativity, demonstrating cell-cycle–dependent control of polarity. Together, our results show that PAR-3 recruits aPKC and PAR-6 through cooperative assembly to establish polarity, and that this process can be dynamically switched off by cell-cycle regulation.

B012/P1144
Pathways restraining multipolarization in plant stem cells
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Asymmetric cell divisions (ACDs) in stem cells often depend on the formation of a single polar domain that controls division orientation and daughter cell fate. Despite their importance, how polar site number is constrained within stem cells during multicellular development remains mysterious. Here, we identify a pathway that restricts polarization in an essential progenitor population in plants, which similarly depend on ACDs to pattern tissues during morphogenesis. Stomatal precursors in the developing Arabidopsis leaf epidermis form a single polar domain before each ACD that depends on the plant-specific proteins, BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL) and BREVIS-RADIX family (BRXf) proteins. We found that pharmacological disruption of microtubule dynamics unexpectedly triggered multipolarization in precursors, creating the first model to study ACD-associated multipolarization in plants. Quantitative time-lapse imaging revealed that microtubule perturbation decoupled normal kinetics of polarization and depolarization from the cell cycle, allowing a new BASL/BRXf domain to form in cells with a pre-existing polarity axis. On observing that the rate of multipolarization increased over time and that many multipolarized cells were also multinucleated, we tested whether cytokinesis failure and an associated increase in ploidy could be driving multipolarization. We generated stable tetraploid plants and found that increasing ploidy was sufficient to trigger multipolarization, even without division defects. Because recent work found that protein concentration constrains multipolarization in yeast and an increase in ploidy is known to increase protein concentration in plants, we titrated the expression of BASL in tobacco leaves and found a positive association between the expression of polarity proteins and the number of polarity domains. Taken together, our data points to limited polarity protein synthesis as a key parameter preventing multipolarization during plant development. Furthermore, our model shares striking similarities to those that explain polarization in yeast, revealing shared polarity programs across evolutionary time.

B017/P1149
Regulation of convergent extension and anterior-posterior patterning by Aquaporin-3b during Xenopus gastrulation
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Convergent extension (CE) is a key morphogenetic process during gastrulation that drives anterior-posterior axis elongation through coordinated cell behaviors and signaling pathways, including noncanonical Wnt signaling. Our studies have revealed that the aquaglyceroporin Aqp3b (Aqp3L) plays a role in Xenopus gastrulation. Using morpholino oligonucleotides to inhibit aqp3b translation, we observed CE defects in Keller explants, which were rescued by Disheveled, PKC and RhoA, but not Rac1 or Cdc42, implicating Aqp3b in noncanonical Wnt signaling. Rac1 and Cdc42 have been shown to promote cell migration in dorsal mesoderm cells. Their failure to rescue the absence of Aqp3b suggested that Aqp3b may not promote active cell migration, but instead promoted CE via PKC and RhoA. Inhibition of aqp3b also impaired PKC localization to the cell membrane in animal caps. Other studies have shown that PKC can activate Cdc42 downstream of Dsh-independent Wnt/Ca2+ signaling, which promotes CE. Since PKC, but not Cdc42 rescued aqp3b morphants, Aqp3b may instead act upstream of Dsh-dependent PKC activation. In activin-treated animal caps, normal elongation was enhanced by Aqp3b misexpression and reduced by its inhibition. RT-PCR of these animal caps

revealed that Aqp3b downregulated anterior markers (goosecoid, otx2, noggin) and upregulated posterior markers (Xbra, HoxB9). Anterior dorsal mesoderm migrates, but does not undergo CE, while posterior dorsal mesoderm undergoes CE. Thus, these data are consistent with the rescue experiments and suggest that Aqp3b may inhibit active migration of dorsal mesoderm cells. Finally, Aqp3b induced FGF2 and eFGF expression in activin-treated animal caps. Studies have shown activation of Xbra and other posterior genes by FGF signaling. Thus, Aqp3b may act upstream of FGF signaling in regulating Xbra and posterior genes. These findings suggest that Aqp3b may promote CE by antagonizing cell migration. This work was funded by the NSF.

B015/P1147

Understanding the functional interaction of PAR-3 and E-Cadherin in epithelial symmetry breaking

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Epithelial cells are essential for organismal development and homeostasis, and require precise coordination of polarity information to properly form and function. A wealth of studies determined the molecular players of cell polarity establishment largely in single cell systems, yet less is known about how these core protein complexes 'break symmetry' to initiate and define asymmetric domains within cell collectives. Using the C. elegans intestine, we previously defined the initial embryonic symmetry breaking event: the formation of large-scale molecular assemblies called local polarity complexes (LPCs). LPCs coalesce at cell contacts, comprise PAR complex proteins and junctional components E-cadherin and α-/β-catenin, and seed tissue level polarization by moving coordinately towards the midline of the intestinal primordium to establish the apical surface which become encircled by mature junctions. Our proximity-labeling proteomics, live-imaging of endogenously tagged proteins, and tissue-specific protein degradation approaches suggest that PAR-3 modulates E-cadherin-based adhesion, perhaps through a direct interaction between E-cadherin and PAR-3. Insights into this interaction come from a PAR-3 oligomerization mutant which displays precocious and increased cell-cell adhesion, significantly impacting embryonic morphogenesis. Ongoing work aims to better characterize this functional interaction and newly identified LPC regulators, including the GTPase activating protein srGAP/SRGP-1. Our data suggest PAR-3 modulates E-cadherin, supporting a molecularly antagonistic paradigm that would shape our thinking about adhesion and cell polarization. This work is supported by funding from the NIGMS.

B018/P1825

Vertex remodeling driven by PAK4 and Afadin maintains epithelial integrity and barrier function

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Cell-cell junctions play an important role in maintaining epithelial tissue integrity and barrier function. Cell adhesion molecules and the cytoskeleton are mechanically linked at cell-cell junctions and contribute to the formation of a robust and well-organized epithelium, but the mechanisms that regulate junctional remodeling are not yet fully understood. Here, we investigated the role of PAK4, a kinase that is localized to cell-cell junctions, in vertex remodeling. We found that PAK4 localizes to apical cell-cell junctions and preferentially accumulates at multicellular junctions in both MCDKII cells and gastrula-stage Xenopus laevis embryos. Small molecule inhibition of PAK4 kinase activity in MDCK II cells led to an increased number of multicellular vertices. Similarly, PAK4-knockout (KO) MDCK II cells exhibited discontinuities at cell-cell junctions and an increased

number of multicellular vertices. We turned to the Xenopus embryonic epithelium to enable dynamic imaging of PAK4 and junction remodeling. We found that PAK4 dynamically accumulated at vertices undergoing remodeling. PAK4 inhibition hindered resolution of multicellular vertices following cytokinesis as well as resolution of multicellular rosettes. Furthermore, disrupting PAK4 function by expression of a dominant negative (DN) disrupted vertex remodeling and caused cytokinetic failure in the Xenopus epithelium. Recruitment of PAK4 to cell-cell junctions in MDCK II cells was dependent on the multidomain scaffolding protein Afadin. Afdn-KO MDCK II cells exhibited severe disruption of junction continuity. Remarkably, when PAK4 was artificially localized to junctions in Afdn-KO cells, the junction discontinuity phenotype was largely rescued. Global barrier function was significantly reduced in both PAK4-KO and Afdn-KO MDCK II cells. A live imaging barrier assay in Xenopus embryos showed barrier function defects – specifically leaks at multicellular vertices – upon either PAK4 inhibition or PAK4 DN expression. Taken together, these results suggest that PAK4 contributes to the maintenance of epithelial integrity and barrier function by promoting vertex remodeling.

Developmental and Stem Cell Biology - Embryogenesis and Organogenesis

B106/P1232
A Keratin-Notch Mechanobiological Axis coordinates Cell Fate and Mechanics during Epidermal Morphogenesis
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Epidermal morphogenesis relies on keratin intermediate filaments, yet how keratins coordinate keratinocyte mechanics with differentiation remains elusive. Here, we identify FAM83H as a potent, previously unrecognized regulator that links keratin organization to epidermal cell fate. Using mouse models and a human 3D microphysiological system, we show that loss of FAM83H disrupts epidermal integrity by impairing basal cell-cell adhesion, organization, and differentiation. Mechanistically, FAM83H localizes at cell–cell contacts, where it scaffolds keratin filaments into a circumferential network essential for desmosome assembly and cortical actomyosin organization. Strikingly, this structural role also couples keratins to Notch1 activation: FAM83H enables keratin–Notch1 interactions that are required for Notch1 proteolytic signaling and basal keratinocyte differentiation. These findings uncover an unexpected mechanism by which keratin filaments integrate tissue mechanics with developmental signaling, redefining their role in epidermal morphogenesis.

B079/P1205
A light-induced neurohormone circuit for metamorphosis in the protovertebrate Ciona
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Thyroid hormones (THs) initiate metamorphosis in vertebrates, although the evolutionary origins of this process persists as a central mystery in developmental biology. Here, we show that most TH processing genes are present in the protovertebrate model Ciona and play an instructive role in initiating metamorphosis, whereby swimming tadpoles with a chordate body plan are transformed into sessile filter feeders. Exogenous thyroxine (T4) accelerates metamorphosis, whereas TH inhibitors delay its onset. Most notably, we present evidence that TH activates Opsin2 (Opsin1/2b) in a subset of photoreceptor cells in the simple brain of swimming tadpoles and acts via a transient GnRH circuit to initiate attachment. Precocious activation of Opsin2 is sufficient to prematurely trigger this first step in metamorphosis. Our findings suggest a deep evolutionary

origin of TH-driven visual plasticity in vertebrates such as smoltification, whereby young salmonid fishes switch from UV to blue opsins for their transition from fresh to saltwater.

B108/P1234

A mitochondrial redox switch licenses the onset of morphogenesis in animals

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Embryos undergo rapid proliferative cleavage cycles before transitioning to morphogenesis. Mechanisms underlying this transition have traditionally centered on genetic cues from nuclei, as repressing zygotic genome activation halts development. Despite precedents that oxygen depletion can similarly suspend development in early embryos, hinting at a pivotal role for oxygen metabolism in this transition, whether there is a bona fide chemical switch that licenses animal morphogenesis remains unknown. Here, we discover that a mitochondrial redox switch licenses animal morphogenesis. Concomitant with mitochondrial activation during fly blastoderm formation, an electron leakage from the ubiquinol-oxidizing site of Complex III leads to a burst of mitochondrial superoxide (O2-) production. Suppressing this leak, or mitochondrial O2- production altogether, reversibly suspends development and does so only at the onset of morphogenesis, but not after. Our mechanistic analyses demonstrate that hydrogen peroxide (H2O2), and not peroxynitrite (ONOO-), nitric oxide (NO), or hydroxyl radicals (HO•-), single-handedly drives this response. Our LC/MS metabolomics experiments further reveal dynamic changes in carbon and nitrogen metabolism during suspended animation, as relevant to a potential metabolism-chromatin-transcription crosstalk regulating stages of early embryogenesis. Notably, we find that depleting mitochondrial O2- similarly prevents morphogenesis in vertebrate embryos and ichthyosporea, close relatives of animals. We postulate that such redox-based metabolic licensing of morphogenesis is an ancient trait of holozoans that couples the availability of oxygen to development, conserved from early diverging animal relatives to vertebrates.

B095/P1221

A new mesenchymal source and mode for expanding blood vessels of the embryonic lung

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Stromal tissues are essential for the development and function of organs. However, the developmental origin and patterning of diverse stromal cell types during organogenesis, including fibroblast, smooth muscle, and endothelial cells, remain understudied. Here, using single-cell RNA-seq and multiplexed in vivo imaging, we uncovered diverse mesenchymal progenitor populations and their fate decisions in the early embryonic mouse lung (E10-E12.5). Most unexpectedly, a Wnt2+Flk1+ mesenchymal progenitor population is spatially restricted to the posterior lung buds and supplies Etv2+ angioblasts, progenitors of endothelial cells, within the organ. This de novo endothelial-producing phenomenon is in stark contrast to the prevailing model of major pulmonary blood vessel formation, which takes place anteriorly at the onset of lung organogenesis. The discovery of spatiotemporally heterogeneous sources of endothelial cells within the lung raises the question of how different parts of the vascular networks are connected. Through live imaging and tracking individual cells, we discovered that these posteriorly-born angioblasts migrate anteriorly towards and integrate into pre-

existing vascular networks, as they differentiate into endothelial cells. In the absence of differentiated endothelial cells, angioblast patterning is disorganized, a phenotype that is rescued by the re-introduction of an endothelial network. This process is distinct from the existing models of blood vessel formation, and provides a new solution for rapidly expanding vascular networks in growing tissues.

B090/P1216

A novel regulatory paradigm: microRNA mediated translation induced mRNA decay in early development

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Following fertilization, metazoan embryos undergo a series of rapid and coordinated mitoses during which post-transcriptional control of translation is one of the predominant regulatory modes of gene expression. We discovered microRNA-31 (miR-31) and many of its target mRNAs (Fascin, Rab35, Gelsolin and β-Actin) to be enriched on the mitotic spindle of the sea urchin cleavage embryo and mammalian cell lines. Proteomic data reveals that inhibition of miR-31 leads to an upregulation in a large number of cytoskeletal and chromatin remodeling genes, with several of them containing miR-31 binding sites. Inhibition of miR-31 and removal of miR-31's binding to one of miR-31 target, Fascin mRNA, lead to a significant decrease of Fascin transcript and increased protein in the sea urchin embryo. Similarly, sea urchin embryos injected with a Renilla luciferase construct fused with Fascin 3'UTR mRNA containing mutated miR-31 binding site was significantly depleted compared to construct with WT miR-31 binding site, indicating that miR-31's binding has a protective effect on the stability of its target transcript. Further, prevention of de novo translation with puromycin significantly restores the level of Fascin transcript in miR-31 inhibited embryos. These results lead to our hypothesis that miR-31 post-transcriptionally inhibits the translation of its targets and concurrently protects the transcript from undergoing translation-dependent RNA decay. Interestingly, multiple spindle-enriched mRNAs in both the sea urchin embryo and HCT-116 (colon cancer) cells upon inhibition of translation with puromycin also display an accumulation of their mRNAs, suggesting that translation-dependent mRNA decay may be a general and evolutionarily conserved regulatory mechanism during mitosis. To further test our hypothesis, we use single molecule resolution live imaging (the SunTag system) to measure active translation and transcript stability. We will reveal the novel regulatory mechanism of miR-31 in dynamically modulating transcript stability and translation rates that impacts mitosis and early development.

B085/P1211

A quantitative model of phenotypic variability

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Mutations in approximately 5000 genes are associated with Mendelian disorders, or disorders that are caused by mutations in single genes and follow predictable patterns of inheritance. Incomplete penetrance and variable expressivity are hallmarks of these diseases. Given that the presence of a pathogenic allele alone is not sufficient to predict an associated phenotype, it is important to understand the contributions to phenotypic variability in order to determine the best course of treatment for patients. The underlying causes of phenotypic variability are thought to be a combination of modifier genes, environment, and stochasticity, or randomness, but disentangling these contributions remains challenging. Here, we establish the Drosophila larval tracheal system as a model for studying phenotypic variability. We focus on the specification of terminal

cells (TCs), which are extensively branched single cells that supply oxygen to surrounding tissues during larval stages. We study the dorsal TCs, which are present in 8 pairs along the anteroposterior axis of the larva; these repeated segments are referred to as tracheal metameres. Gain- and loss-of-function mutations in FGF-ERK signaling, which is required for proper specification of TCs, results in gain and loss of TCs, respectively. In flies, the FGF ligand Branchless (Bnl) is haploinsufficient; removing one copy of Bnl results in a severe reduction in the number of dorsal TCs per larva. We show that this effect can be partially rescued by introducing an additional gain-of-function mutation in MEK, the penultimate kinase in the Ras-ERK cascade, highlighting the contribution of modifier genes to phenotypic variability. Additionally, we find that in both the half-dose Bnl and rescue conditions, the tracheal metameres respond differently to the same perturbation, with some metameres being more strongly affected than others. Thus, we identify cryptic variation across metameres that is hidden in wild type conditions but is revealed upon introducing perturbations to the system. To determine the origins of this variation, we are using an FGFR pYtag, a receptor tyrosine kinase biosensor, to identify any differences in receptor activation across metameres that may exist prior to TC specification. We are also performing quantitative live imaging of TC specification in both wild type and Bnl half dose embryos to determine when this structural defect arises. We find that in wild type embryos, TC specification follows a stereotypic trajectory. In mutant embryos, developing tracheal cells either fail to induce expression of DSRF, a transcription factor that marks the TC identity, or express a low level of DSRF that is not sufficient to induce the characteristic morphological change associated with the TC.

B105/P1231

Actin crosslinking is required for force sensing at tricellular junctions

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The organization of the actin cytoskeleton into force-generating networks is essential for the dynamic behaviors that build multicellular tissues, but how cells modulate their behavior in response to mechanical forces in their environment is not well understood. In epithelial sheets, tricellular junctions where three cells meet are dynamically remodeled by actomyosin forces that drive cell rearrangement and epithelial morphogenesis. These forces also create the risk of fractures or tears that could compromise tissue integrity and function, raising the question of how cells withstand the forces required for epithelial remodeling. In particular, how forces induce the molecular changes required to stabilize cell adhesion at tricellular junctions is not known. Here we identify a mechanotransduction pathway involving the conserved actin crosslinking protein Fimbrin and show that Fimbrin acts as a central regulator of multiple cellular force responses that promote junctional and cytoskeletal remodeling at tricellular junctions under tension. We show that Fimbrin rapidly localizes to tricellular junctions in response to mechanical forces in the Drosophila embryo and is required to recruit several force-sensitive junctional proteins that reinforce cell adhesion during cell rearrangement. In the absence of Fimbrin, the actin cytoskeleton fails to remodel and ruptures occur between cells, indicating a failure to stabilize adhesion under tension. Conversely, increased Fimbrin crosslinking activity promotes the organization of actin networks at tricellular junctions into a high-tension state and enhances the recruitment of force-sensitive proteins, aberrantly stabilizing adhesion. Consequently, cells rearrange faster when Fimbrin is absent and slow down or stall when Fimbrin activity is increased, indicating that Fimbrin tunes the rate of cell rearrangements required for tissue morphogenesis. We further demonstrate that Fimbrin promotes force responses through a mechanical feedback pathway that locally enhances myosin-II cortical localization and amplifies myosin-II contractility at tricellular junctions under tension. These results identify Fimbrin-dependent actin crosslinking as an essential component of the cellular response to force at tricellular

junctions, revealing a mechanism that dynamically modulates cell adhesion and cytoskeletal organization during epithelial remodeling.

B098/P1224
Actin-driven cytoplasmic flows and microtubule transport organize metabolically distinct mitochondria during mouse oocyte maturation
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Mitochondrial metabolism is essential for cellular function and is patterned across space and time by variations in activity and intracellular localization. While disruption of this spatiotemporal organization is associated with neurodegeneration and infertility, the mechanisms underlying its formation and dysregulation in disease remain poorly understood. During meiotic maturation, mammalian oocytes arrest at metaphase II with an asymmetrically positioned spindle, creating a robust gradient of mitochondrial activity. To uncover the basis of this pattern formation, we used quantitative live and fixed fluorescence microscopy combined with biochemical inhibition and micromanipulation. We found that mitochondrial metabolism in mouse oocytes is not set by local biochemical environment but by intrinsic proteomic differences. The positioning of these proteomically distinct mitochondria is regulated by actin-driven cytoplasmic flows and microtubule-based transport. Live imaging and perturbation experiments indicate that subsets of mitochondria display differential adherence to microtubules, driving their selective enrichment near the spindle. To interpret these findings, we developed a hydrodynamic theory that couples mitochondria to cytoplasmic flows while incorporating metabolism-dependent adherence to microtubules. This model quantitatively reproduces the observed spatial patterning and recapitulates the effects of pharmacological perturbations and mechanical spindle displacement. Together, these results establish a mechanistic framework for the spatial organization of functionally distinct mitochondria and provide a predictive description of mitochondrial self-organization during oocyte maturation. Our findings offer insight into how cytoskeletal mechanics regulate subcellular metabolism in early mammalian development and suggest its contribution to age-related infertility.

B110/P1236
An insulin receptor-independent role for FoxO/DAF-16 in early embryos
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Forkhead box O (FoxO/DAF-16 in C. elegans) transcription factors play a conserved role in aging, metabolism, cell cycle regulation, and development in multiple systems. In the context of aging, loss of insulin receptor in C. elegans (InR/DAF-2 in C. elegans) significantly extends lifespan in a FoxO dependent manner. Canonically, FoxO's nuclear localization is inhibited when the Insulin/insulin like growth factor signaling (IIS) is turned on, however FoxO is also regulated by IIS-independent mechanisms. Interestingly, InR/daf-2 and FoxO/daf-16 double deletion mutants are 100% embryonic lethal despite little to no embryonic lethality in either single mutant alone. The cause of this synthetic embryonic lethality is unknown but suggests FoxO/DAF-16 might function in parallel with InR/DAF-2 at least during embryo development. Despite this severe embryonic phenotype, the exact role of FoxO/DAF-16 during embryogenesis remains unclear. To investigate a possible IIS-independent role for FoxO/DAF-16 in embryogenesis we utilized quantitative live imaging with different

combinations of RNAi depletions and loss of function alleles for FoxO/daf-16 as well as other members of the IIS pathway. We first imaged an endogenously tagged FoxO/DAF-16::GFP strain during the early stages of development and found a unique expression and localization pattern. In 2- to 4-cell embryos, FoxO/DAF-16 localized uniformly at low levels to all cell nuclei, but starting in the 8- to-16 cell embryo, it became enriched in 1 or 2 posterior cell nuclei, continuing through the 32- to 64- cell stage embryo. Cell lineage-tracing experiments revealed the identity of these cells as the germ lineage cells and this enrichment was regulated by the CCCH Zn-finger germ fate determinants (PIE-1, POS-1). Additionally, this enrichment was dependent on the IIS lipid phosphatase PTEN/DAF-18, the IIS lipid kinase PI3K/AGE-1, but was InR/DAF-2 independent. Functional analysis uncovered that loss of FoxO/DAF-16 significantly shortened the length of the cell cycle during early embryonic divisions, suggesting FoxO/DAF-16 functions as a cell cycle pacer. Furthermore, high resolution time-lapse imaging of InR/daf-2; FoxO/daf-16 double mutant embryos showed striking defects in genome stability and cell cycle regulation, as early as the 1-cell stage. Together, our results suggest an IIS-independent role for and patterning of FoxO/DAF-16 that functions synergistically with IIS to ensure proper embryogenesis.

B080/P1206

Asymmetric divisions and resulting cell cycles promote dynamic heterogeneity in the Drosophila germ cell primordium

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During early embryonic development of many species, germ cell progenitors – called primordial germ cells, or PGCs – are set aside from their somatic counterparts. In addition to the PGC-soma distinction, significant intercellular differences often emerge within the population of PGCs and can play an important role in their subsequent development. Because previous studies have largely relied on fixed embryos, the dynamic emergence of inter-PGC variability has remained poorly understood. We quantify these origins in Drosophila embryos expressing endogenously tagged Cyclin E-sfCherry and the pole cell marker Vasa-GFP. By live imaging, segmenting, and tracking PGCs from their formation to their cell cycle arrest, we constructed a forest of lineage trees for each embryo, with each tree corresponding to the lineage of a single nascent PGC. We identified several mechanisms that promote both inter-PGC variability and PGC-soma differences. First, the germ plasm – a specialized plasm inherited during PGC formation – is unequally partitioned across rounds of divisions in a spatially biased way. Labelling progeny as somatic or PGC in the resulting PGC lineage trees, we find that these unequal divisions are associated with asymmetries in the tree with respect to fate and cell cycles. Based on this evidence, we propose a mathematical model in which each cell is a non-linear phase oscillator that divides unequally after completion of a cycle, but whose phase progression is inhibited by translation of inherited mRNAs. This model provides insights into variability in final cell number across embryos and inter-PGC variability within an embryo. Since mitoses and the emergence of intercellular differences are often linked during development, we expect that similar mechanisms likely operate in other primordia.

B100/P1226

Branching Morphogenesis Coordinates Mesenchymal Differentiation in the Developing Kidney

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Background: Nephrons, the functional unit of the kidney, emerge from nephrogenic niches throughout kidney development and are scaled to meet a lifetimes demand through elaboration of the ureteric bud (UB) epithelium. Each nephrogenic niche consists of a UB tip enveloped by a pool of mesenchymal nephron progenitor cells (NP) known as the cap mesenchyme (CM) and separated from neighboring CM by mesenchymal stromal progenitors (SP). Balancing NP renewal and differentiation within CM maximizes nephron production over kidney development, yet how these fates are both successfully maintained in one niche remains unclear. The rate of nephrogenesis matches UB tip branching over a large window of mouse kidney development suggestive of a tighter coupling between these processes. This study aims to identify a connection between UB branching dynamics and differentiation within the nephrogenic niche. Methods: Embryonic day (E)13 mouse kidney explants were grown in a 1:1 (v/v) 1 mg/mL collagen I:Matrigel hydrogel for 3D explant culture. We performed spatial sequencing using the Xenium 5k Prime platform (10x Genomics) on kidneys from mice at E15, E17, and E18 and from human at gestational week (wk)17 or wk20. Nephrogenic niches were pseudo-aged along the UB tip branching 'lifecycle' according to geometric features of the UB tip and CM. Niches were classified manually into discrete stages (pre-, mid-, or post-branching) or computationally along a continuous scale between 0-100. Results: Time-lapse microscopy of 3D mouse kidney explants revealed an enrichment for nephrogenesis at the pre-branching stage. Further, spatial-sequencing revealed CM fluctuate in NP identity over the UB branching 'lifecycle'. Pre-branching CM were enriched for NPs from subclusters primed for differentiation while mid-/post-branching CM were enriched for NPs from naïve subclusters. Pathway overrepresentation analyses from pseudo-bulked CM along a sliding window over the continuous scale also revealed evidence of offset transcriptional activity corresponding to NP renewal and differentiation (ex: enriched YAP vs WNT activity, respectively). Intriguingly, our spatial-sequencing analysis also revealed 'lifecycle' dependent changes to the surrounding SPs. Pre-branching niches are more abundant for SPs from differentiating stromal subclusters than mid-/post-branching niches. In addition, we found a significant increase in average stromal nuclear aspect ratio and phospho-myosin light chain intensity in pre-branching niches suggestive of a SP mechanical transition over the branching 'lifecycle'. In conclusion, we identified UB tip branching as an apparent mechanical pacemaker guiding nephrogenesis possibly indirectly through concurrent, mechanically tuned SP differentiation.

B114/P1240

Centrosome-assisted assembly of the germ plasm aggregate.

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The Balbiani body (Bb), which was discovered about 170 years ago, is a membraneless organelle in the oocyte in most species. In organisms like Xenopus and Zebrafish, Bb accumulates mitochondria, endoplasmic reticulum (ER), and germline determinants and regulates the proper localization of germline determinants. The Bb forms around the centrosome in the oocyte during early oogenesis. The mechanism behind its assembly has gained attention only very recently. We found that overexpression of the germ plasm matrix protein Xvelo leads to the formation of a 'Bb-like' structure in somatic cells. The 'Bb-like' structure assembles around the centrosome and selectively recruits mitochondria, ER, and germline determinants. Taking advantage of this system, we investigated the roles of centrosome components in the assembly of Xvelo. Our results reveal that multiple components of the centrosome, including Sas6, Cenexin, and DZIP1, interact with Xvelo and promote its assembly, with Sas6 exhibiting the most prominent activity. Importantly, knocking down Sas6, Cenexin, and DZIP1 individually or in combination resulted in reduced Xvelo aggregates. Taken together, our work suggests

that the centrosome may function as a nucleation center to promote the initiation of Xvelo assembly, resulting in the formation of the Bb around the centrosome.

B104/P1230
Comparison of reproductive biology of the invasive zebra and quagga mussels from warm and cold environments.
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The dreissenid zebra mussel (Dreissena polymorpha) and quagga mussel (Dreissena rostriformis bugensis) are invasive freshwater bivalves that cause significant ecological and economic damage to waterways of the eastern, central, and southern United States. Originally found in cold-water regions, these mussels have spread southward into warmer regions including Texas and Louisiana (zebra mussels) and Nevada and southern California (quagga mussels). A key component to their invasive success is their reproductive strategy of broadcast spawning which allows their gametes and subsequent larval development and dispersal. To better understand the potential for southern range expansion, we addressed several aspects of their reproductive and general biology. We took a comparative approach looking for similarities and differences between populations of mussels from colder climates (Wisconsin, Michigan) and those adapted to warmer waters (Texas). Factors examined included gamete longevity including both egg and sperm viability. Overall survival of mussels at high temperatures was evaluated for both zebra and quagga mussels. Additionally, we looked at gamete compatibility and potential for hybridization between zebra and quagga mussels. Dreissenid mussel gametes showed significant longevity following spawning (several hours). Both species showed high mortality at elevated temperatures. Interspecific gamete interactions including chemotaxis and sperm binding were observed between the two species.

B082/P1208
Development of Chromosomally Stable Embryonic Stem Cell Lines for Studying Oocyte Mitochondrial Function
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Objective: To develop mouse embryonic (mES) cell lines for investigating the effects of multi-drug resistant transporter-1 (MDR1) mutations on mitochondrial function in oocytes. Methods: MDR1-mutant and wild-type mice (CF-1 background) were hormone primed and either mated for collection of 2-cell embryos or used to collect M2 oocytes subjected to SrCl2 activation in the presence of 1 ug/ml cytochalasin D for production of fertilized or diploid parthenogenic eggs. Eggs were cultured in fully defined, protein-free medium until blastocyst stage, then transferred to feeder-free stem cell medium containing 2i (MEK and GSK3 inhibitors), ACTH, and LIF. DNA was isolated from stem cell lines at first or second passage and subjected to array comparative genomic hybridization (aCGH) analysis to assess chromosomal stability through detection of copy number variations (CNVs). This protocol yielded stable early-stage mES cell cultures from 64% of fertilized blastocysts and 16.7% (2/12) of parthenogenic blastocysts. Cell proliferation rates were estimated by measuring time to first or second passage. Results: Sixteen (64%) mES cell lines were derived from MDR1-mutant embryos, of which 14 (5 male, 9 female) underwent aCGH analysis. Male lines remained euploid for all autosomes and sex chromosomes, as were male wild type lines. All female lines, both mutant and wild type, lost one copy of chromosome X. The only somatic chromosome aberration among female mES lines were 4 of

the 9 mutant lines showed partial gain of chromosome 8. In contrast, both parthenote stem cell lines were at least 50% triploid 8 by passage two. The gain of chromosome 8 was not associated with decreased cell cycle times in all cell lines. Conclusions: The overall chromosomal stability in these early passage mouse ES cell lines supports their potential value in studies of oocyte mitochrondria. None of the seven male lines exhibited aneuploidy. All female lines exhibited loss of one X chromosome, four of the nine mutant female lines showed partial gain of chromosome 8, and both parthenote lines gain chromosome 8. This characterization provides valuable insight into genetic stability patterns in stem cell models. These early lines serve as a resource for investigating mitochondrial metabolism in oocytes, though careful monitoring of karyotypic stability will be important for future studies.

B124/P1250

Developmental Stage-Specific Centrosome Remodeling by Cenexin-Pericentrin in Vertebrate Embryogenesis

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The centrosome, the primary microtubule-organizing center of the cell, comprises centrioles, mother centriolar appendages, and pericentriolar material (PCM). Evidence shows that organization and function of these structures, and the proteins that compose them, shift across development. We hypothesize that during early embryogenesis, when cell proliferation is rapid, the PCM plays a predominant role in spindle organization, whereas later, as proliferation slows, centrioles and their appendages increasingly support tissue-specific processes such as ciliogenesis. To test this, we examined the role of Cenexin (Cnxn), a mother centriole subdistal appendage protein, during this developmental transition. Post fertilization (pre-1k-cell stage), Cnxn did not localize to appendages but instead to the PCM in a Pericentrin (Pcnt)-dependent manner, mediated by a conserved phosphorylated C-terminal motif (S796 in humans; S829 in zebrafish). After gastrulation, Cnxn relocalized to centrioles independently of Pcnt. The Cnxn–Pcnt interface was essential for Cnxn mobility and exchange at the PCM early, but dispensable for its later stable centriolar localization. Functionally, Cnxn loss increased mitotic defects and multinucleation during early divisions. Together, these findings demonstrate that the dependence of Cnxn on Pcnt is stage-specific, safeguarding centrosome function during rapid early cell cycles while permitting centrosome specialization later in embryogenesis.

B073/P1828

Dishevelled regulates tube morphogenesis and regeneration

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Epithelial tubes are fundamental structures that make up organs. Understanding the basic, conserved mechanisms for building a solid tube with a stereotypical orientation and shape is fundamental for proper organogenesis and to prevent congenital disorders and tissue fibrosis. What are the cellular and molecular mechanisms that control tube orientation? We know that components of the Wnt pathways control patterning in different tube models, but we do not know the downstream mechanisms. In our lab we have developed a new system to investigate tube and organ morphogenesis, the sea star Patiria miniata larva. These larvae develop an optically clear structure called the hydro-vascular organ, which is formed by two long tubes that is used for buoyancy. Our recent data in the sea star larva show that the Wnt receptor Frizelled 1/2/7 is

localized in the elongating tubes and controls tube orientation. The gatekeeper of the Wnt pathway is Disheveled (Dvl), which upon binding with Frizzled receptors activates the Wnt pathway. Although the Wnt pathway is known to control early embryonic antero-posterior (A-P) axes, we lack a clear understanding of its role in organogenesis and regeneration. Here we used CRISPR/Cas9 to deplete Dvl protein in the larva without affecting earlier processes. Dvl knock out (KO) larvae have a shortened A-P axis and several tissues have compromised polarity. Lack of Dvl strongly influences morphogenesis of the hydro-vascular organ tubes that become spherical, grow towards the dorsal axis and have a bigger lumen, suggesting a novel role for Dvl in tube morphogenesis. Importantly, this phenotype differs from Fzd1/2/7 KO tubes, which also lost orientation but kept the correct shape. Because a role for Dvl in regeneration has not been clearly defined, we used dissection techniques to find that the anterior halves of Dvl KO bisected larvae fail to regenerate posterior structures, while the posterior half fully regenerates anterior structures. Taken together, our results show that Dvl plays important roles in tube orientation and shape and that this protein is key in regeneration.

B112/P1238

Dynamic Tissue-Specific Adaptations of Clathrin-Mediated Endocytosis During Zebrafish Development

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A long-held ambition of cell biologists has been to investigate subcellular processes in live, multicellular organisms. Such a process is clathrin-mediated endocytosis (CME), a major pathway by which cells regulate the composition of their plasma membrane and interactions with their environment. Despite its central role in cell communication, CME has been studied mainly in cell culture, where cells are confined to artificial conditions such as glass surfaces and culture media. To reveal how CME integrates diverse biochemical and mechanical cues to support cell-type-specific functions, it must be investigated in the intact, natural organismal context, under physiological conditions where these signals are naturally present. Studying CME in the context of living tissues has been technically challenging, as subcellular processes require both high spatiotemporal resolution and imaging depth. Here, we address this challenge by combining recent advances in genome engineering and light-sheet microscopy, applying them to zebrafish. By endogenously tagging components such as Adaptor Protein 2 sigma (Ap2s) with fluorescent proteins, we study CME at native expression levels for the first time in a vertebrate. Using oblique plane illumination microscopy, we directly visualize Ap2s-mNeonGreen (Ap2s-mNG) in tissues of living zebrafish embryos. Our imaging of the pectoral fin at 3 days post-fertilization revealed striking tissue-specific adaptations of Ap2s-mNG. We were able to resolve individual endocytic events and quantitatively distinguish between highly dynamic, motile processes in the periderm and long-lived, static Ap2s structures in the mesenchyme. Extending this approach to multicolor imaging, we quantified endocytic dynamics relative to cell shape and surface area, confirming cell type and tissue-specific adaptations of Ap2s.Next, we will extend this pipeline to investigate how CME adapts during tissue repair, focusing on the link between membrane trafficking, osmotic stress, and tissue reorganization. Ultimately, this work has created a framework for studying CME within its physiological context and sets the foundation for understanding how its regulation supports healthy development.

B092/P1218

Expanding the paradigm of tubulin autoregulation in vivo

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Tubulin autoregulation is the process by which cells maintain the appropriate concentration of soluble tubulin in the cell. The TETRATRICOPEPTIDE REPEAT DOMAIN CONTAINING PROTEIN 5 (TTC5) protein is crucial for mediating this process. Human genetics studies show that variants in TTC5 are associated with severe developmental brain malformations. We aim to demonstrate that dysfunction in tubulin autoregulation is responsible for cortical malformations and determine the underlying mechanism(s). We hypothesize that loss of tubulin autoregulation alters tubulin structures and causes defective corticogenesis. To test this, we generated a novel Ttc5 null mouse model. RT-qPCR of Ttc5-null mouse embryonic fibroblasts and mixed cortical cultures have shown that loss of Ttc5 notably increased total tubulin mRNA concentrations. Therefore, the Ttc5-null mouse demonstrates defective tubulin autoregulation. Furthermore, Ttc5-null mice faithfully recapitulate human brain malformation phenotypes including hypoplasia of the corpus callosum, ventriculomegaly, and microcephaly. Mechanistic studies into neurogenesis show changes in cell cycle dynamics with reduced proliferation at embryonic day (E)12.5, but increased proliferation at E14.5 in Ttc5-null mice. Examination of key tubulin structures revealed both a decrease in the length of the primary cilia and mitotic spindle defects resulting in severe chromosomal alignment and segregation errors. Both the mitotic spindle and primary cilia play essential roles in cell cycle progression and timing. Surprisingly, we found the alterations in the mitotic spindle are likely driven by increased gamma tubulin foci. We confirmed an increase of gamma tubulin mRNA via RT-qPCR. Gamma tubulin has not been previously associated with tubulin autoregulation. This finding thereby expands the paradigm of tubulin autoregulation and demonstrates the cellular mechanism responsible for tubulin autoregulation brain malformations.

B116/P1242

Heterogeneities of an epithelial-to-mesenchymal transition preceding developmental tissue invasion

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Epithelial-to-mesenchymal transition (EMT) occurs to varying degrees and migratory cell collectives have heterogeneous populations of cells, including leaders and followers. However, it is unclear how heterogeneity of EMT might relate to the initial invasion of a cell collective into the body from its epithelium of origin. In the Drosophila embryo, internalization of the anterior midgut (AMG) primordium involves a collective EMT at the embryo surface followed by invasion of the cells into the embryo interior. Before internalization, AMG cells lose adherens junctions, lose epithelial polarity, and gain protrusive activity. More recently, our unpublished data show that the surface AMG primordium gains expression of N-cadherin, but only at its posterior end. I seek to characterize the heterogeneous EMT at AMG, its transcriptional regulations, and its functions in AMG's collective invasion. I detected the expression of the endoderm marker Hindsight at AMG, limited to its anterior end. The Hindsight-positive anterior and N-cadherin-positive posterior sub-regions are apparent when the primordium has undergone EMT and is still present on the embryo surface. Between the sub-regions, we detected a cleft, suggestive of distinctive cell-cell interactions with each sub-group. During the internalization process, N-cadherin-expressing cells were consistently the last to remain on the embryo surface, suggestive of a leader-follower relationship during invasion. In parallel, we identified Drosophila orthologs of human EMT transcription factors with known expression in the early AMG primordium. A mini-screen of available mutants showed that the embryo surface EMT of the AMG primordium requires Snail and Twist, indicating that the process is orchestrated by these conserved master regulators of EMT. In the other transcriptional factor mutants, the EMT occurred. Of these, Serpent, a transcription factor known to promote Hindsight expression in other contexts, was required for the expression of Hindsight in the anterior of the AMG primordium and preliminary data suggests that Serpent is also required for the structural coordination of the two sub-groups of

the heterogeneous cell collective during its internalization. Overall, these data provide evidence of heterogeneous EMT of the AMG primordium at the embryo surface, and a regulator of this heterogeneity. Further studies will investigate the involvement of the EMT heterogeneity in AMG invasion into the embryo interior.

B113/P1239

How embryos keep time: mouse strain-specific developmental tempos and rates of branching morphogenesis

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Development occurs as a highly coordinated sequence of events, whose order and timing are critical for the generation of tissue form. Branching morphogenesis generates epithelial tissue form in several organs, including the lung, kidney, and salivary gland, and relies in part on the synchronized timing of proliferation and differentiation. This timing must also be regulated across the embryo to ensure functional maturity by the time of birth. However, what determines developmental tempo is unclear. To interrogate developmental tempos in mice, we took advantage of mouse strains with different gestational lengths. We evaluated the tempo of embryogenesis of each mouse strain using morphological measurements, including somite number, intranasal distance, digit webbing in forelimb and hindlimb, and length of long bones to assign a morphological age to each embryo. We found a strong inverse correlation between gestational length and developmental tempo, with shortest gestational length correlating with the fastest developmental tempo. In parallel, we evaluated the rates of branching morphogenesis in each strain by quantifying the number of epithelial branch tips. We found that rates of branching morphogenesis differed across mouse strains and similarly inversely correlated to gestational length. To distinguish between genome and environment, we performed mismatched pregnancy experiments, in which embryos were implanted into mothers of the same or different mouse strain. We found an additive effect, where both the strain of the mother and embryo influenced the morphological age and extent of branching. These data suggest that mouse developmental tempo and branching morphogenesis are regulated by genetic background and uterine environment, providing insights into how mouse embryos keep time and coordinate development. This work is supported through NIH Director's Pioneer Award (5DP1HD111539-02) and NIH F32 fellowship (1F32HD120006-01).

B093/P1219

Hydraulic pressure controlled by Wnt activity regulates semicircular canal morphogenesis

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Lumen pressure is essential for embryogenesis and the morphogenesis of luminal organs. However, how lumen pressure is regulated—and how it contributes to tissue morphogenesis—remains largely unknown. Here, we use the pressurized zebrafish otic vesicle (OV) and the semicircular canal (SCC) that form from it to show that Wnt activity regulates pressure within the OV lumen, and its expansion in turn shapes the morphology of epithelial pillars, which serve as the hubs around which the canals form. Previous studies show that between 18 and 40 hours post-fertilization (hpf), fluid transport into the lumen, driven by osmotic gradients, generates hydraulic pressure that feeds back to regulate further fluid influx and control OV expansion and size. SCC morphogenesis begins around 45 hpf, when epithelial buds extend from the OV, meet, and fuse to form transluminal pillars. We find that OV lumen continues to expand during this process. Using a convolutional

neural network–based segmentation pipeline, we found that ablation-mediated disruption of lumenal pressure results in smaller OV and shorter and wider pillars. While forskolin treatment that activates ion transporters inflates the OV and results in longer and thinner pillars. Beyond these transient perturbations, we show that genetic or pharmacological disruption of Wnt activity significantly reduces OV lumen volume and halts expansion by increasing epithelial permeability. These perturbations, likely through long-term depressurization, decrease pillar aspect ratio and disrupt tissue mechanics, as evidenced by reduced cell cortex myosin and tight junction protein levels. Together, these results reveal a functional role for Wnt activity in maintaining OV lumen pressure homeostasis and highlight the regulation of morphogenesis by lumen pressure.

B076/P1202

Identification of functional targets reveals novel GLD-2 functions in the C. elegans proximal germline

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RNA tailing is the post-transcriptional addition of untemplated nucleotides to the 3’ end of RNA molecules, central to RNA stability and translation. This process is mediated by tailing enzymes like GLD-2, a germline poly(A) polymerase in C. elegans, required for meiotic entry and early meiosis during gametogenesis. We set out to broaden the understanding of GLD-2 by uncovering novel targets and roles in fertility beyond the well-established target, gld-1, at the distal end. We used a Nanopore-based direct cDNA sequencing method to assess the effects of Auxin-inducible degron (AID) mediated GLD-2 loss on RNA tails and to identify the functional targets of GLD-2. GLD-2 depletion results in a significant decrease in length and proportion of A-tails for specific transcripts, identified as GLD-2 functional targets. The targets are enriched in transcripts involved in biological processes, including meiosis, chromosome condensation and segregation, cytokinesis, apoptosis, and eggshell formation. Consistent with the molecular evidence, we observed corresponding cellular and physiological defects in AID mediated GLD-2 depletion in adult worms: the loss of GLD-2 in the proximal germ line is also detrimental to terminal oogenesis and the oocyte-to-embryo transition. Interestingly, only the near complete depletion of GLD-2 (≥95%) in adult germlines (using RNAi + AID) phenocopies null mutants and upends meiotic entry and progression. In contrast, oocytes are continuously observed at the proximal end of gonads after AID-mediated GLD-2 depletion in adults (~90%). A consistent pattern of staggered and stacked oocytes does, however, point to defective terminal oogenesis in the terminal germline. DAPI-stained germlines exhibit normal meiotic progression to diakinesis and rule out meiotic defects. The resulting brood displays a fully penetrant embryonic lethality phenotype, characterized by eggshell and cytokinetic defects, and chromosome de-condensation. Crosses with wild-type males did not rescue oogenesis defects or embryonic lethality. These results highlight the critical role of GLD-2 in terminal oogenesis and embryogenesis. Future research will explore the dynamics of RNA tailing in the context of C. elegans aging and determine the effects of age-associated changes in GLD-2 expression and distribution on fertility decline.

B094/P1220

Investigating how differences in ALX1 coding and noncoding regions affect beak morphology within Darwin's finches

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Darwin's finches are a model for adaptive radiation and niche partitioning, showcasing species that recently diverged to occupy specific niches within the Galapagos archipelago. Whole genome sequencing of 120 individuals representing Darwin's finch species in Lamichaney et. al. (2015) identified fixed genomic regions between species possessing blunt vs pointed beaks. Among these, the ALX1 locus contained the greatest number of single-nucleotide polymorphisms (SNPs), including both coding and noncoding SNPs. Though ALX1 is implicated in craniofacial development, the extent to which differences in coding and noncoding genetic variants affect avian beak morphology has yet to be elucidated. I utilized Geospiza magnirostris and Geospiza difficilis as finch models given their beaks pertain to extremes on the morphological spectrum. I evaluated the effect of L110P and I209V mutations in the ALX1 coding region using chicken (Gallus gallus) in ovo RCAS-mediated manipulation. Next, I combined assay for transposase-accessible chromatin sequencing (ATAC-seq) from avian species and transcription factor binding analysis to identify candidate cis-regulatory elements (CREs) and tested their effects using luciferase reporter assays. Preliminary results show no overt phenotypic differences upon ALX1 overexpression in ovo. However, luciferase assays of candidate CREs revealed differential luciferase activity of regions 1 and 8. Further investigations will include deeper phenotypic analysis of ALX1 overexpression, as well as performing luciferase assays for the regions 1 and 8 for a spectrum of finch species including G. scandens, G. fortis, and G. fulginosa. Together, these experiments will contribute to our understanding of ALX1 regulation and function, providing insight on ALX1-associated human craniofacial conditions and informed conservation efforts.

B077/P1203

Investigating the roles of claudin proteins in notochord development using Ciona robusta

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In mammals, the 27 claudin family members display complex, tissue-specific expression and are essential components of tight junctions that regulate selective permeability between epithelial and endothelial cells. However, we recently discovered 13 claudin and claudin-like genes in Ciona robusta, a tunicate species and one of our closest invertebrate relatives in the chordate phylum. This expansive family of claudin proteins has dynamic spatiotemporal expression throughout Ciona embryogenesis suggesting they are performing tissue- and cell-specific functions in development. By utilizing tissue-specific CRISPR/Cas9-mediated mutagenesis, we investigated the regulation and functions of two claudins in Ciona, Claudin.h and Claudin.k in the development of the notochord, a transient structure that is present in all chordates. We show that these claudins are playing distinct, yet crucial roles in the developing notochord of Ciona. Specifically, we demonstrate that Claudin.h is vital for the early process of intercalation of notochord cells and Claudin.k plays a role in the later stage of tubulogenesis of the notochord. This work highlights the unique roles that claudin proteins play in cell behaviors during key stages of embryonic development in chordates.

B102/P1228

Is Ketogenic Diet Safe? Impact of Maternal Ketogenic Diet Consumption on Fetal Growth and Offspring Health

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Ketogenic diets (KGD) have risen in popularity as a nutritional strategy to lose weight. However, the use of KGD during pregnancy is being promoted without evidence of their effects on fetal growth and offspring health.

Using a mouse model, we are developing a periconceptional model of nutritional ketosis to investigate the effect of maternal consumption of KGD during the periconceptional period on fetal growth, postnatal health of offspring, including birthweight, cardiovascular profile and metabolic function. Three groups (C57/BL6) female mice at age of (3-4 weeks) were used in this study. Normal diet group (ND, mice were fed standard chow diet for 10 weeks), Ketogenic-diet group (KD, mice were fed standard chow diet for 6 weeks followed by 4 weeks of ketogenic-diet consisting of 67.4% fat, 0.6% carbohydrate, and 15.3% protein), and obesogenic-ketogenic-diet group (HF-KD, mice were fed 45% obesogenic high-fat-diet for 6 weeks followed by 4 weeks of ketogenic-diet). The control/ketogenic-diet group will help us to elucidate fetal growth and postnatal performance of offspring derived from non-obese mothers that switch to a ketogenic-diet during periconception, whereas the obesogenic/ketogenic group will be representative of obese mothers using ketogenic-diets to lose weight around the time of conception. After 10 weeks on respective diets, females were mated with C57/BL6 males. Vaginal plug detection defined embryonic day 0.5 (E0.5). Pregnant females were maintained on the ketogenic-diets until E3.5, when they were switched to standard feeding for the remaining days of gestation. During pregnancy, females underwent different analysis as shown below. Fetuses were collected and weighed at (E11.5; n=23) and (E14.5; n=23) corresponding to the mid- organogenesis and end-organogenesis. A total of 30 mothers (10 per group) were used to produce offspring. Ketogenic diets cause significant reduction in maternal weight during pregnancy as anticipated for such a diet. After two-weeks of ketogenic-diets consumption, ketone bodies showed elevated values in both KD and HF-KD groups compared with ND, indicating development of ketoacidosis. At E11.5 and E14.5, fetuses from KD and HF-KD exhibited a significant decrees in body weight (P = 0.000), independent of litter size compared with ND. Birthweight was significantly reduced in KD offspring compared with ND. KG group compared with HF-KD group revealed decrease fetal weight at E11.5, no differences were evident at E14.5 and birthweight. Cardiovascular profile, metabolic function of the offspring are under investigation. Maternal consumption of ketogenic-diets during the periconceptional period results in alteration in fetal growth trajectory and offspring birthweight.

B081/P1207

Left-right organizer cellular and molecular dynamics drive early cardiac asymmetries

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Abnormal left-right (LR) axis establishment during vertebrate embryogenesis disrupts the development of asymmetric visceral organs like the heart, presenting as a broad spectrum of severe congenital heart diseases (CHDs). LR asymmetric organogenesis is influenced by asymmetric mechanotransduction and Nodal signaling that initiate in a transient embryonic structure known as the Left-Right Organizer (LRO). We previously showed that LR asymmetry requires two populations of LRO cells – “pit” cells with flow-generating motile cilia and “crown” cells with mechanosensing/calcium signaling immotile cilia. Many gaps remain in our understanding of LRO formation and function, including how the crown and pit differentiate and how transcriptional asymmetries beget morphological asymmetries. LR asymmetry in vertebrate species utilizing this “two-cilia” mechanism also requires four evolutionarily conserved yet poorly understood genes, including MMP21, which encodes a matrix metalloproteinase implicated in epithelial-mesenchymal transition and metastasis in cancer. This study aims to identify the embryonic origins of LRO cell populations and elucidate the role of the MMP21 gene in LR development. To identify the transcriptional markers and differentiation trajectories of LRO cell subpopulations, we utilize a wild-type mouse single cell RNA-sequencing dataset spanning eight timepoints across gastrulation. Representation of LRO cells peaked transiently in embryos at the E7.75 timepoint. Focusing on the rare Shh and Noto positive “LRO” population (571 cells), we were able to identify six LRO subpopulations between E7.0-E9.0, including pit cells, crown cells, and putative precursor and descendant populations. Analysis of this data paired with imaging of mRNA transcript localization over time suggest

distinct transcriptomic and differentiation trajectories for pit and crown cells, with the pit cells potentially arising first. To expand upon the currently incomplete model of LR symmetry breaking, we also utilize hybridization chain reaction fluorescence in situ hybridization (HCR-FISH) and immunofluorescence (IF) experiments in mouse embryos. We observe asymmetric distribution of the MMP21 transcript to the right side of the LRO pit, suggesting pit cells are not a homogeneous population. MMP21 is the first LRO gene found to be asymmetrically localized in the pit cells. Furthermore, this asymmetry may be cilia-dependent, as mutant embryos lacking LRO cilia show a loss of asymmetry of MMP21 and other key LRO transcripts. We hypothesize the pit cells may require MMP21 to first remodel basement membrane to facilitate epithelial-mesenchymal transitions during LRO formation and to later propagate asymmetric signaling.

B109/P1235

LGL-1 and the RhoGAP protein PAC-1 redundantly control polarization of the C. elegans embryonic epidermal epithelium

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The formation of an apical–basal axis of polarity is essential for epithelial organization and function. Apical–basal polarity is established by cortical polarity proteins that define the apical, junctional, and basolateral domains. While these proteins are highly conserved and present in most epithelia, the mechanisms that drive polarity and the reliance on canonical polarity factors can vary between tissues and organisms. For example, the basolateral polarity protein lethal giant larvae (Lgl) is crucial for the polarization of most Drosophila epithelia, yet is dispensable for epithelial polarization and viability in C. elegans. To better understand the epithelial polarity program in C. elegans, we performed a whole-genome RNAi screen for synthetic lethality with an lgl-1 deletion mutant. We discovered that combined loss of LGL-1 and the RhoGAP protein PAC-1RhoGAP19D leads to embryonic lethality due to defects in elongation and rupturing of the epidermis. We found mislocalization of junctional proteins to the lateral domain of epidermal cells, presumably weakening tissue integrity. Furthermore, we observed expansion of the apical domain, sporadic aPKC mislocalization, and localization of the basolateral polarity protein LET-413Scribble in patches surrounded by DLG-1Discs large. These findings indicate that the combined loss of LGL-1 and PAC-1 leads to an overactivity of apical domain specifying factors. Consistent with this, partial inactivation of the apical polarity regulators aPKC or CDC-42 reduced the lethality of pac-1(RNAi); lgl-1(null) animals. Taken together, our work identifies pac-1 and lgl-1 as redundant inhibitors of apical polarity factors. While their relative contribution to the epithelial polarity program differ from fly epithelia, the canonical apical-basal polarity machinery is thus active in the embryonic epidermis of C. elegans – providing new insight into how conserved mechanisms are adapted across species.

B078/P1204

Long-range coordination of amniote gut tube morphogenesis through PCP-dependent elongation of the presumptive midgut endoderm

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The gut tube is an embryonic structure formed from endoderm that gives rise to the digestive and respiratory systems. It is subdivided along the antero-posterior axis into foregut, midgut, and hindgut, providing the basis

for organ development. Among amniotes, each of these three segments forms at distinct times and locations through seemingly disparate mechanisms, yet the result is the transformation of an epithelial sheet into a single continuous epithelial tube running the antero-posterior length of the embryo. In chick embryos, coordinated tissue elongation is required to properly shape these distinct regions from the primitive endoderm. While foregut and hindgut elongation have been studied to some extent, the contribution of midgut elongation—likely required to accommodate and support their extension—remains unclear. This project aims to map morphogenetic events driving midgut elongation at the tissue, cellular and sub-cellular scales, and to ultimately identify the biochemical and mechanical cues coordinating gut tube formation across a length scale of millimeters. Quantitative live imaging of electroporated chick embryos revealed that convergent extension is a key process in midgut elongation. At the cellular level, we observed T1 transitions and rosettes, hallmarks of tissue-scale convergent extension. Because convergent extension typically relies on planar cell polarity (PCP) to orient cell intercalations, we next examined PCP components during midgut elongation. Immunostaining confirmed that the PCP protein Vangl2 is expressed in the midgut during convergence extension. However, we noticed that Vangl2 abundance was not uniform; as midgut elongation progresses, long-range gradients in protein levels emerge alongside localized regions of polarization, indicating that both abundance and polarity are coordinated across the tissue. Current work focuses on identifying the biochemical and mechanical cues underlying this regulation during midgut morphogenesis. Finally, functional perturbations demonstrate that disrupting planar polarity in the midgut compromises hindgut extension, underscoring the role of midgut convergence extension in coordinating large-scale gut development. Together, these findings establish planar polarity–driven convergent extension as a central morphogenetic process of midgut elongation. The dynamic patterning of Vangl2 during midgut elongation further hints to biochemical and mechanical regulation of planar polarity. By linking midgut convergence extension to hindgut elongation, this work highlights the midgut as a key coordinator of large-scale gut morphogenesis and provides a framework to investigate how forces and signals are integrated to coordinate epithelial morphogenesis.

B083/P1209

Mapping Regulatory Networks Reshaping the Early Embryonic Proteome

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Early Drosophila embryos begin development as a pre-assembled machine, powered by maternal proteins deposited in the egg. Yet this maternal endowment must be replaced as zygotic control takes over, requiring both new protein synthesis and clearance of maternal factors. Transcriptomic studies have defined many features of this transition; yet, they alone cannot fully separate maternal from zygotic protein contributions, and regardless, mRNA levels often diverge from protein abundances. As a result, the proteome-level remodeling that underlies this handoff remains poorly defined at a systems scale. To disentangle maternal versus zygotic contributions, we used chromosomal loss mutants and quantitative proteomics. We profiled embryos lacking the 2R arm of chromosome 2 and identified 442 differentially expressed proteins (DEPs), representing 7% of the proteome, at the onset of ventral furrow formation. As expected, proteins encoded on 2R comprise over half of DEPs, providing a direct readout of the protein complement that must be restored by zygotic transcription. These proteins were enriched for signaling and transcription factors, including even skipped and scabrous, underscoring their developmental importance. Interestingly, 190 proteins encoded elsewhere in the genome also changed in abundance. These indirect effects reflect two classes of factors normally encoded on 2R: activators (30%), whose absence reduces expression of their targets, and repressors or degradative factors (70%), whose absence allows other proteins to accumulate. Our results establish a framework for dissecting maternal vs zygotic effect and reveal a global regulatory network shaping the early

embryonic proteome. Future work with finer chromosomal deletions will pinpoint the elements that coordinate this rapid developmental remodeling.

B123/P1249
Mechanical regulation of stromal progenitors in 3D embryonic kidney culture
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The kidney is an essential organ for filtration and homeostasis in the body. The mammalian kidney develops in utero with limited regenerative capacity after birth; thus, it is critical to study its development and the emergence of congenital anomalies to advance appropriate therapies. The kidney develops through branching morphogenesis of the ureteric bud (UB) epithelium, which exchanges signals with surrounding nephron progenitors (NPs) and stromal progenitors (SPs), leading to periodic nephron commitment. While the UB and NP lineages have been extensively studied, the stromal lineage remains poorly characterized, and mechanical interactions are largely unexplored. Here, we hypothesize that the mechanical conditions at the organ surface directly influence the contacting cortical SPs, which signal to modulate branching and nephrogenesis outcomes. Our approach is to embed mouse embryonic kidneys in 3D composite collagen I/Matrigel droplets to preserve in vivo-like 3D tissue architecture throughout prolonged culture. We demonstrate that explants without cortical SP-mediated adhesion exhibit poor development in culture. We also find that varying the collagen I concentration significantly affects the mechanical boundary condition, SP invasion into the surrounding matrix, and the number of committed nephrons per UB tip. These findings suggest that the kidney-3D matrix boundary conditions modulate cortical SP behaviors and branching/nephrogenesis coordination. To elucidate cell-level interactions driving the tissue-level phenotypes, ongoing work focuses on tracking fluorescently labeled cortical SPs using time-lapse confocal microscopy. We also use NP reporter (WT1-EGFP) mice to investigate the SP migration patterns in coordination with nephron commitment cycles and how changes in matrix mechanical properties offset the nephron per UB tip balance through stromal signaling. This study provides an in vivo-like platform for characterizing the tripartite SP-NP-UB interaction during kidney development with high spatiotemporal resolution. It offers new insights into the SP lineage and its role in translating mechanical boundary conditions into morphogenesis outcomes.

B122/P1248
Metabolic regulation of branching morphogenesis in the developing mammalian lung
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Metabolism plays a pivotal role in the early embryo, supporting its growth and energy production, establishing cell identity, and regulating signaling pathways. However, spatial and temporal patterns of metabolism during later stages of embryonic development remain largely unexplored. In the mammalian lung, the epithelium undergoes branching morphogenesis in response to molecular and mechanical signals from the surrounding mesenchyme and airway smooth muscle. We hypothesized that spatial patterns of glucose metabolism dictate the mechanics of branching in the lung. To map spatiotemporal patterns of glycolysis in the developing lung, we performed whole-mount immunofluorescence analysis for the canonical glucose transporter GLUT-1. We found that while both epithelial and mesenchymal cells expressed GLUT-1, the expression was most pronounced in vascular endothelial cells. To investigate the role of glycolysis in lung branching, we cultured embryonic day (E)12.5 mouse lung explants in the presence of the glycolysis inhibitor, 2-deoxyglucose (2-DG),

and found that this treatment significantly reduced epithelial branching. We also found that pyruvate oxidation in the TCA cycle was not necessary for branching morphogenesis. Instead, we found that pyruvate had to be converted to lactate via glycolysis to promote epithelial morphogenesis. Culturing 2-DG inhibited lungs in the presence of lactate rescued branching. To further validate our results, we cultured lung explants in medium deficient in glucose, and found that glucose was the primary substrate necessary for epithelial branching morphogenesis. Culturing lungs in glucose-deficient media in the presence of lactate rescued branching, confirming that lactate is essential in regulating branching morphogenesis. To understand the mechanisms by which lactate directs branching, we performed EdU-incorporation assay to assess spatial changes in proliferation. Deficiency of glucose significantly decreased EdU-positive cells, while lactate supplementation increased the number of EdU-positive cells in the epithelium. These results suggest that lactate-directed lung branching morphogenesis is spatially patterned. Our future work will investigate the relationship between lactate metabolism, mechanical forces, and biochemical signaling pathways, and how these are coordinated to ultimately sculpt lung epithelial branching.

B111/P1237

Microtubules Regulate Membrane Reservoir Dynamics during Embryo Morphogenesis

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Cell surface membrane reservoirs are plasma membrane protrusions or invaginations that store and release membrane to support cell surface growth or shrinkage during changes in cell shape. Membrane reservoirs are broadly utilized during cytokinesis, phagocytosis, cell motility and tissue morphogenesis. During cellularization of the syncytial Drosophila embryo, a membrane reservoir composed of microvilli unfolds to fuel the simultaneous ingression of cleavage furrows between ~6000 nuclei to build the primary epithelial sheet. In the cellularizing embryo, the kinetics of reservoir unfolding and furrow ingression are coupled and biphasic with a slow phase followed by a fast phase. How the switch from slow to fast phase is regulated is not known but cannot be explained by the mechanical properties of the cell cortex alone (Stark et al., bioRxiv, 2025). Recently, we found that microtubules abruptly transition from a centrosomal to acentrosomal organization at the same time as reservoir unfolding and furrow ingression switch from slow to fast phase. Thus, we hypothesized that the transition from centrosomal to acentrosomal microtubule organization may somehow control the biphasic kinetics of cellularization. To test this, we blocked the centrosomal to acentrosomal transition by knocking-down levels of atypical protein kinase-C (aPKCRNAi). Consistent with our hypothesis, aPKCRNAi embryos fail to switch from the slow to fast phase of reservoir unfolding. To then ask how microtubules interact with the reservoir, we used live dual-channel TIRF imaging. Unexpectedly, we found that during slow phase, centrosomally organized microtubules enter the microvilli of the reservoir, polymerizing perpendicular to the embryo surface, and alongside microvillar actin filaments. After the switch to fast phase, acentrosomally organized microtubules no longer enter microvilli, and instead polymerize parallel to the embryo surface, within the cortex at the base of disassembling microvilli. We suggest that centrosomal microtubules push out membrane of the reservoir into microvilli and resist unfolding to generate slow phase kinetics, while acentrosomal microtubules in the cortex are no longer oriented to resist unfolding and so allow fast phase kinetics. Additional changes due to microtubule reorganization may also contribute to the switch from slow to fast phases, including altered paths of membrane trafficking. However, our work provides a first hint that microtubules may regulate reservoir dynamics by deforming the cell surface membrane themselves.

B118/P1244

Modeling hypoblast function in early human embryonic development using a synthetic non-human primate embryo model

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Understanding the molecular and structural underpinnings of embryo implantation is critical for addressing infertility and early pregnancy loss. Implantation failure accounts for most unsuccessful pregnancies, yet the precise mechanisms governing stable uterine embedding remain elusive. Trophoblast cells, progenitors of the placenta, have long been central to implantation studies, as they establish the physical interface between the uterus and embryo. However, the roles of other early embryonic lineages remain poorly understood. Here, we uncover a previously underappreciated role of the hypoblast, a lineage derived from the inner cell mass, in orchestrating implantation in vitro. Using embryonic stem cells from the common marmoset, Callithrix jacchus, we reconstruct peri-implantation development by assembling epiblast, hypoblast, and trophoblast cells into a defined 3D embryo organoid system. Our laboratory introduces a novel approach for precise control over lineage composition: rather than differentiating all three lineages sequentially from one progenitor population, we separately differentiate each cell type and assemble them using a high-throughput microwell array. Each microwell accommodates a single organoid, allowing us to systematically vary cell ratios and investigate how specific compositions affect implantation potential. We find that hypoblasts play two essential roles in early embryo development. First, hypoblasts promote epiblast proliferation through an unidentified pathway, with sufficient hypoblast support proving critical for embryo growth. Second, hypoblast population and integrity strongly predict stable implantation in vitro. Using live imaging of organoids assembled with fluorescently labeled progenitor cells, we ruled out two intuitive explanations: hypoblasts neither influence trophoblast proliferation nor spatial organization. Instead, our data support a biophysical mechanism wherein hypoblasts are a structural scaffold between epiblast and trophoblast layers, physically stabilizing the peri-implantation embryo to facilitate attachment. Our findings reframe the hypoblast not as a passive secondary lineage but as an active contributor to implantation success. This dual structural-regulatory role challenges the trophoblast-centric narrative and opens new avenues for understanding embryonic contributions to uterine embedding. By integrating biological inquiry with a scalable, quantitative platform, our primate-based model bridges key gaps between murine research and human clinical relevance. Ultimately, our system provides a foundation for uncovering the cellular and molecular causes of implantation failure, paving the way for future therapeutic interventions in reproductive medicine.

B099/P1225

Morphological diversity reveals a role for Hippo signaling in buckling morphogenesis of the small intestine

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Buckling, the process by which a structure bends out-of-plane to relieve in-plane compressive forces, is a fundamental mechanism of vertebrate morphogenesis. While the physics of buckling is well-established, its biological regulation remains poorly understood. This study examines looping morphogenesis of the avian small intestine to investigate how conserved molecular pathways are modulated to generate species-specific intestinal morphology. The amniote small intestine is organized into compact, undulating loops that position the lengthy intestinal tube within the body cavity. Transformation from an initially straight tube, known as gut looping, is driven by elongation of the intestinal tube against the constraint of its attached mesentery, which

compresses the tube until it buckles into loops. Loop wavelength and curvature are conserved within a species, and can be predicted from growth rate, geometry, and stiffness of the tube and mesentery, each directly measurable in avian embryos. Comparative analyses revealed that while most birds display variations of the same basic looping pattern, the Long Island Duck (Anas platyrhynchos) is a remarkable exception. The duck intestine undergoes normal buckling initially, but its single central loop elongates without further buckling, flanked by segments that buckle normally. This suggests local disruption of buckling mechanics and their upstream biological controls in the central loop. We have exploited this morphological anomaly to elucidate regulators of buckling and intestinal development. Previous work in the lab comparing chromatin accessibility of central versus flanking mesentery revealed enrichment of TEAD-binding motifs in the elongated central loop. TEAD functions downstream of Hippo signaling and requires co-activators YAP/TAZ, known mechanotransducers, to drive gene expression. Here, we test the functional role of these candidate regulators through misexpression of a constitutively active YAP mutant (YAP5SA) using the replication competent avian retrovirus RCAS. Embryos expressing YAP5SA showed disrupted gut morphology, including looser and distended gut tubes. Immunostaining verified viral infection and confirmed that nuclear YAP and proliferative cells could be detected. Curiously, a subset of YAP5SA-expressing embryos displayed an inverse phenotype, with tightly packed, poorly organized loops. Work is ongoing to identify the cellular mechanism of the observed looping phenotypes, the mechanical effectors through which they act, and whether feedback mechanisms or temporally distinct roles of Hippo signaling explain the observed range. This work provides a framework for understanding how conserved signaling pathways contribute to shaping organ morphology across vertebrates.

B088/P1214

Myosin II is required for seamless tubulogenesis and unicellular branching morphogenesis in Drosophila tracheal terminal cells

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Transformation of epithelial cells into complex functional organs is a critical step in development. Organs such as the lungs and kidney are composed of multicellular tubes (two or more cells attached to one other by junctions), whereas a large percentage of capillaries in the vertebrate vascular system are composed of seamless tubes (single cells that lack junctions). Although the morphogenetic events that help generate a multicellular tube can vary, it is clear how cells joined together by epithelial junctions create a membrane-bounded lumen; what is not obvious is how a single cell hollows out to create an internal lumenal membrane de novo. Specialized tips cells in the Drosophila tracheal system called “terminal cells” go on to form branched seamless tubes. Using terminal cells as a model, we have uncovered a number of cytoskeletal and membrane trafficking regulators that help orchestrate this seamless cell-to-tube transformation in previous work. Here, we show a novel role for Myosin II during the process of seamless tubulogenesis and unicellular branching morphogenesis in the fly tracheal system. RNAi against the fly myosin II regulatory light chain (spaghetti-squash) and myosin II heavy chain (zipper) results in changes to terminal cell architecture and discoordination between branch and lumen formation. Myosin II depleted cells exhibit reduced spreading and lumen integrity defects compared to wild type control cells. Preliminary data of a zipper null allele also supports a cell autonomous role for Zipper during terminal cell morphogenesis. Efforts to determine the mechanism by which Myosin II regulates branching and tube formation in terminal cells are currently underway.

B120/P1246

Patterns of Mitochondrial ATP Predict Actomyosin contraction and Apical Constriction

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Contraction of the actomyosin network is used to construct tissue shapes during embryonic development and wound healing, generating mechanical forces that are fueled by chemical energy from ATP hydrolysis. Here we find that this chemical energy is spatially patterned prior to and during actomyosin contraction events. Specifically, mitochondria are locally enriched at the apical sides of epithelial cells during apical constriction, which is widely used across the animal kingdom to fold epithelial tissues. Timelapse imaging, spatial transcriptomics, and measurements of oxygen consumption rate reveal that mitochondrial density, potential, and ATP increase in epithelial cells prior to actomyosin contraction and tissue folding, which is prevented by inhibiting oxidative phosphorylation. Mitochondrial enrichment and apicobasal patterning are conserved during apical constriction of epithelial tissues in flies, chicks, and mice, and these subcellular patterns can be used to predict computationally patterns of epithelial folding. These findings highlight a spatial dimension of bioenergetics in the cell biological processes that drive embryonic development.

B097/P1223

Piezo1-driven Ca^{+} dynamics and Atp2b2 fine-tune cellular stretch responses in the tissue of the ear's endolymphatic sac

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Dysregulation of inner ear fluid pressure underlies common hearing and balance disorders such as Ménière's disease, affecting approximately 615,000 Americans annually. The endolymphatic sac (EDS), a specialized epithelial structure in the inner ear, functions as a pressure-relief valve that maintains homeostasis by periodically releasing excess fluid and pressure. However, the mechanotransduction pathways coordinating EDS function remain largely unknown due to the inaccessibility of the inner ear in most vertebrates as its encased in temporal bone. Zebrafish surpass this limitation with functional ears before bone formation, allowing optical accessibility of ear structures in their native environment. Mechanosensitive cells often use ionic calcium (Ca2+) as a secondary messenger linking mechanical cues to biochemical signals. Our live imaging of zebrafish embryos expressing genetically fluorescent Ca2+ sensors shows the presence of high-frequency Ca^{2+} transients in EDS cells during periods of elevated fluid pressure, alongside differential Ca^{2+} activity in distinct cell populations under low-pressure conditions. These findings suggest a novel mechanotransduction pathway where EDS cells utilize Ca2+ signaling to sense and respond to pressure fluctuations at both cellular and tissue levels and a functional heterogeneity within the EDS epithelium. Further, single-cell RNA sequencing data of dissected zebrafish inner ears revealed high EDS expression of Ca2+-permeable mechanosensitive ion channel Piezo1, mutations in which have been implicated in GWAS studies of Ménière's disease, and the deafness-associated gene atp2b2 (zebrafish PMCA2 ortholog). To precisely delineate the roles of Piezo1 and Atp2b2 in EDS Ca2+-dependent pressure regulation, our ongoing studies utilize ferromagnetic oil droplets to poke the ear via a magnetic actuator to control pressure induced strain while live-imaging in combination with Piezo1 and Atp2b2 perturbations. This study advances our understanding of in vivo cellular

mechanotransduction mechanisms for tissue homeostasis and establishes the EDS as a powerful model for understanding how epithelial tissues maintain integrity under fluctuating mechanical stress.

B089/P1215

PP1cb functions with myosin phosphatase-targeting subunits to prevent anterior rupture during embryo elongation

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Protein phosphatase 1 (PP1c) regulates multiple cellular processes by reversing serine/threonine phosphorylation. PP1c interacts with a diverse set of adapters to form a collection of active phosphatases that dephosphorylate specific targets. Here, we investigate the role of PP1c during embryonic development in C. elegans, where two catalytic isoforms, PP1cbGSP-1 and PP1caGSP-2, are active. These isoforms share ~90% sequence identity, are expressed at similar levels, and function largely redundantly during the early embryonic cell divisions. However, depletion of PP1cbGSP-1—but not PP1caGSP-2—causes embryonic lethality, suggesting a non-redundant, essential function for PP1cbGSP-1 during embryogenesis. Live imaging revealed that PP1cbGSP-1 is dispensable for germ layer specification but is required during embryo elongation. In the absence of PP1cbGSP-1, the embryos rupture from their anterior side as they attempt to elongate. Two-hybrid analysis revealed that the MYPT family proteins—MEL-11 (MYPT1) and GFI-2 (MYPT3) are PP1cbGSP-1-specific targeting subunits that likely target phosphatase activity to suppress actomyosin contractility. Depleting MEL-11 resulted in an anterior enclosure defect during elongation that phenocopied PP1cbGSP-1 depletion, while GFI-2 depletion resulted in a similar but less penetrant phenotype. To determine where and when the PP1cbGSP-1/MEL-11 complex functions during elongation, we developed a tissue-specific auxin-inducible degradation toolkit consisting of a set of heritable extrachromosomal arrays expressing the plant F-box protein TIR1, along with mCherry::Histone H2B. Elimination of maternally-provided PP1cbGSP-1 revealed strong zygotic expression of PP1cbGSP-1 in the skin, pharynx, intestine and neurons, and indicated that zygotically expressed PP1cbGSP-1 is sufficient for elongation. Tissue-specific degradation of PP1cbGSP-1 in pharyngeal cells led to a partial anterior rupture phenotype, while degradation of PP1cbGSP-1 in neuroblasts, which give rise to both neuronal and non-neuronal cells, fully recapitulated it. We are currently extending this analysis using neuron-specific promoters. Collectively, our results suggest that PP1cbGSP-1 is likely to function in both pharyngeal cells and neurons, where it partners with MYPT1 and/or MYPT3 to provide spatially-patterned myosin phosphatase activity. These results suggest that suppression of actomyosin contractility in tissues in the head is required to prevent anterior rupture as the embryo attempts to elongate. These findings have the potential to provide insight into Noonan-like syndrome, a rare human congenital disease linked to mutations in PP1cβ.

B121/P1247

Predicting Developmental Potential of Embryos from Polar Body Transcriptomes Using Machine Learning

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Over the past few decades, assisted reproductive technology (ART) has contributed to the birth of more than ten million babies worldwide. While ART has provided solutions for many infertile couples, its success rate remains suboptimal, in part due to the prevalence of poor-quality embryos that fail to develop to term. The biological determinants of embryo quality, including the specific genes and molecular pathways involved, are not fully elucidated. It is well established that unfertilized oocytes store maternal mRNAs within their cytoplasm, and the precise regulation of these transcripts is essential for supporting embryonic development after fertilization. However, the subset of maternal transcripts that are specifically associated with embryo quality remains largely unexplored, and a reliable method has not yet been established to identify maternal transcripts that can predict or reflect embryo quality. In this study, we tried to develop a method to simultaneously examine both the expression level of maternal transcripts and the subsequent embryonic development in a minimally invasive way for the purpose of identifying maternal transcripts whose expression is well correlated with the quality of fertilized embryos. We then focused on a second polar body (PB) released from a fertilized mouse embryo. Second PBs were removed from fertilized embryos of mice, and preimplantation development of the manipulated embryos was monitored. Subsequently, the second PBs collected from embryos that had developed to the blastocyst stage were classified as good PBs, whereas those that had been arrested were regarded as bad PBs. The cDNAs of good PBs and bad PBs were examined by RNA-seq and RT-qPCR. As a result, RNA-seq analyses revealed that most of the transcripts detected in PBs were expressed as maternal transcripts in sibling embryos and expression levels of transcripts in PBs were well correlated with those in the accompanying embryos, suggesting that PB transcriptomes can be used to deduce the expression level of maternal transcripts. We further identified several candidates for biomarker transcripts that can be used to predict the fate of embryonic development by comparing differentially expressed genes between good and bad PBs. Based on the biomarker expression, we established a predictive model using machine learning, which successfully predicted embryonic development with an accuracy exceeding 75% in prospective experiments. We propose that fertilized embryos with high developmental potential can be selected by assessing the expression level of biomarker maternal transcripts in PBs.

B119/P1245

Quantitative Proteomics of Cell Fate Specification in the Early Drosophila Embryo

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The transition from a single fertilized zygote into diverse tissues in a multicellular organism requires precise regulation of protein expression in space and time. However, current studies measuring comprehensive global protein dynamics during embryonic cell fate specification remain limited. Bulk proteomic studies in frog, zebrafish, and fly embryos have reported thousands of proteins during early development, but these approaches average heterogenous tissues. In vitro differentiation systems may not fully recapitulate developmental contexts, and single-cell proteomics, while promising, is still insufficiently reliable for global quantification of thousands of proteins that define cell fate. To overcome these challenges, we developed Drosophila morphogen-flattened (MF) embryos that have endogenous patterning cues (Bicoid, Hunchback, Capicua, and Dorsal) genetically fixed to uniform, flat levels across the embryo. This method generates multicellular embryos composed of a single cell type prior to gastrulation, which provide sufficient material for quantitative proteomic analysis of cell fate specific differentiation. Using MF embryos representing three distinct cell fates, neuroectoderm, hindgut, and dorsal ectoderm, we quantified approximately 6,500 proteins across four developmental time points. Although most of these proteins are suppled maternally and are

uniformly distributed in the unfertilized egg, already by the blastoderm stage when embryos are composed of one cell type, proteomes already diverged by 10% along the anterior-posterior axis and 25% along the dorsal-ventral axis. These differences heightened to 40% in both axes at the subsequent development time point two hours later, indicating that subtle changes in maternal positional information determine cell-type specific changes in global maternal proteome during differentiation in Drosophila embryos in ways not always predictable from transcript abundance. Our results demonstrate that early developmental patterning programs rapidly establish distinct, fate-specific proteomes, providing a framework for understanding how subtle differences in maternal positional inputs govern cell fate diversification in multicellular organisms.

B115/P1241

RTK dynamics in branching kidney organoids

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Branched tissues are commonly composed of a continuously self-renewing progenitor population surrounding an epithelium. This epithelium branches by switching between elongation and bifurcation regimes and often develops through crosstalk with the mesenchyme. In developing embryonic kidneys, the epithelial ureteric bud (UB) tree is surrounded at its tips by nephron progenitors and stroma. Their interaction is mediated primarily via activation of the RTK RET in UB tip cells driven by GDNF ligand secreted by the cap mesenchyme. Sustained and spatially homogeneous perturbations of RET and GDNF can drive several rounds of branching in ureteric bud organoids lacking nephron progenitors and stroma. However, UB organoids still fall short of the 10-15 generations of branching exhibited in vivo, possibly due to the absence of spatial and temporal GDNF/RET tuning ordinarily supplied by the neighboring mesenchyme. Properly tuning RET activity is likely critical for sustained renal branching morphogenesis, yet the dynamics of RET activation and its effects on collective cell behaviors are largely uncharacterized. Recently, our group has developed an optogenetically-controllable RET (optoRET) system that permits spatiotemporal control of RET activation. We first use immunofluorescence and confocal microscopy to examine RET activation in UB organoids. We then use optoRET to examine the effects of varying light intensity and spatiotemporal frequency on branching morphogenesis in UB organoids. We propose an extension of this system by integrating optoRET with a live reporter of downstream ERK/MAPK activity, creating an actuator-biosensor circuit that can be used to characterize RET-dependent ERK dynamics and cell homing. Finally, we design a strategy using mosaic organoids that permits live imaging of the homing behavior of ERK-activated cells and interrogation of effects of RET activation on neighboring cells. These approaches make important advances in our understanding of the dependence of branching morphogenesis on proper RET tuning and how a continuously replenishing progenitor niche can cue the neighboring epithelium to form a discrete branched structure.

B117/P1243

Self-organization of Early Embryo Shape Controls Developmental Potency

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Early embryogenesis requires proper spatial organization and patterning of cellular divisions; however, how the spatial organization of the embryonic cells regulates the development potential remains elusive. Here, we report that self-organization of early embryos shape is essential for developmental potency. To determine whether the shape of the fertilized egg regulates development, we established a system using the sphered

Xenopus early embryos. The removal of vitelline membrane at the 1-cell stage alters the shape of early embryos from a sphere to an oblate ellipsoid, leading to an altered pattern of embryonic cleavages starting from the 2-cell stage to early blastula, which causes developmental defects ranging from curvy body to embryonic death in the late tailbud. Interestingly, the oblate ellipsoid restores the embryonic shape into a sphere at the mid-blastula stage, exhibiting a robust self-organization of early embryogenesis. Importantly, the restoration of the embryo shape is essential for subsequent gastrulation and development as interference of the restoration process abolishes development, while artificial confinement of the embryonic shape rescues the development. Moreover, a time-course analysis of the de-vitelline shows a stage-dependent of developmental potency, and the de-vitelline of embryos after mid-blastula stage do not affect development. Further, transcriptome analyses of stage-specific de-vitelline demonstrate stage-dependent activation of zygotic genes and reveal the critical role of WNT signaling in mediating shape-induced gene expression. Finally, reversing the orientation of de-vitellined embryos before, but not after, the mid-blastula stage aggravates the developmental potency and induces early activation of zygotic genes, corroborating a critical role of early embryonic shape in controlling development. Together, our study provides a fundamental framework to reveal the importance of the self-organization of the embryo shape in regulating developmental potency in early embryogenesis.

B087/P1213

Single-cell Sequencing of the Left-Right Organizer in Zebrafish Reveals a Novel Class of Anteriorly Expressed Genes

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Vertebrates exhibit key asymmetries in both the positioning and patterning of their organs along the left-right (LR) body axis. LR patterning is essential during development, and failure to properly pattern the organs can cause heterotaxia and congenital heart defects (CHDs). CHDs are the most common structural birth defect in the United States; therefore, it is crucial to understand the mechanisms that establish LR patterning in the embryo to identify new causative genetic risk factors for CHD. In vertebrates, LR patterning is first initiated within transient embryonic structures known as LR organizers, which in zebrafish is Kupffer's vesicle (KV). Motile cilia within KV rotate and generate a leftward fluid flow. This flow culminates in the downregulation of the Nodal inhibitor dand5 on the left of KV, which restricts Nodal to the left side of the embryo. Although we know fluid flow is required for the establishment of dand5 asymmetry, we still lack an understanding of how flow is utilized by cells to regulate gene expression/asymmetry. To gain insight into KV functions during LR axis establishment, I performed single-cell RNA sequencing (scRNA-seq) of KV cells across developmental time. This analysis identified a class of genes asymmetrically expressed in the anterior cells of the KV, which do not express dand5. One identified gene, matrix metallopeptidase 21 (mmp21), has been reported to play a key role in LR patterning in Xenopus, mice, and humans. However, the mechanism of how mmp21 regulates LR patterning is not known. Knockdown of mmp21 in zebrafish embryos causes midline cardiac jogging, or the loss of the normal leftward displacement of the heart, a phenotype commonly associated with the loss of Nodal activity. These findings suggest that mmp21 functions upstream of Nodal signaling, which leads us to hypothesize that mmp21 could be activating Nodal expression in the lateral plate mesoderm. In addition to this finding, I have also identified key transcriptional changes throughout time that explain KV's function in LR patterning.

B107/P1233

Spatial Asynchrony of RNA Modifications Orchestrates Maternal-to-Zygotic Transition in Early Embryogenesis

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Early embryogenesis requires a maternal-to-zygotic transition (MZT), which includes the maternal factor clearance and the zygotic genome activation (ZGA) that are essential for early embryonic cell fate specification. How MZT is regulated in time and space is still not fully understood. We previously developed novel single-cell ZGA assays and revealed a spatially graded pattern of single-cell ZGA in Xenopus early embryos - ZGA onset occurs first in the cells at the animal pole (AP) and gradually propagate to the cells at the vegetal pole (VP). Nevertheless, how the spatial pattern of single-cell ZGA is regulated is still unknown. To test whether regionally enriched factors mediate the new pattern, we performed proteomics on dissected regions of the AP and the VP from embryos and discovered a significant enrichment of the N6-methyladenosine (m6A) RNA regulators, including its writer METTL16 and readers YTHDF1/2, in the AP. Consistently, Western blot assays confirmed significantly higher levels of these proteins in the AP than the VP across all early stages of embryogenesis before ZGA. Intriguingly, while the levels of these proteins in both regions are continually increased from the 1-cell stage to the mid-blastula stage when ZGA occurs, their differences between the regions are gradually diminished, indicating a potential link of the spatiotemporal asynchrony of m6A regulators to the spatially graded pattern of ZGA. To uncover the functional importance of the spatial pattern of m6A regulators in early embryogenesis, we assessed the phenotypes of embryos upon perturbation of the m6A regulators. Strikingly, blocking the functions of METLL16 with METTL16-IN-1 or YTHDF2 with YTHDF2-IN-1 delays early development, and overexpression of YTHDF2 by microinjection the YTHDF2 mRNA into the VP also impairs early development, suggesting a critical role of the m6A spatial asynchrony in orchestrating early development. Further, to characterize whether RNAs are differentially modified in space, we performed the direct RNA sequencing using Nanopore. Our preliminary analysis revealed significantly more m6A sites in thousands of transcripts in the AP than the VP, supporting a spatial asynchrony of the m6A RNA modification. Importantly, Gene Ontology (GO) analyses revealed the enriched pathways of RNA splicing and translation in the AP; the top AP-enriched m6A RNAs include serine/arginine-rich splicing factor 2 (srsf2), a key regulator during gametogenesis and ZGA. This finding is in line with our proteomic analysis which showed that the AP is enriched in the RNA splicing and processing, while the VP is enriched in fatty acid metabolic process. Collectively, our study uncovers a novel spatiotemporal role of the m6A epigenetic regulation of the MZT during early embryogenesis.

B074/P1200

Spatially ordered zygotic genome activation fulfills embryo quality control

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Early embryo development features autonomous, maternally driven cell divisions that self-organize the multicellular blastula or blastocyst tissue. Maternal control cedes to the zygote starting with the onset of widespread zygotic genome activation (ZGA), which is essential for subsequent cell fate determination and morphogenesis. Intriguingly, although ZGA onset is highly regulated at the level of the whole embryo, it can be non-homogenous and precisely patterned at the single-cell level. We previously demonstrated a stereotyped spatial and temporal ordering of ZGA in a model vertebrate embryo tied to a pattern of cell size along one

embryo axis. Unknown, however, was whether this precise ZGA patterning was required for development. To address this fundamental question, we devised a strategy to control cell division speed spatially across the embryo that perturbs blastula organization. We demonstrate the feasibility of spatially inverting the cell size pattern of embryos and find that these inverted embryos exhibit a flipped pattern of ZGA. Mispatterned ZGA along the animal-vegetal axis triggers embryo apoptosis, revealing that gastrula embryos have a built-in quality control system to sense inappropriate ZGA patterning, including regional defects in transcriptional onset. The quality control response is non-autonomous, dependent on an anti-apoptotic signal from which suppresses cell death outside the animal hemisphere. These results reveal the requirement of properly patterned ZGA for normal development and the existence of a surveillance system of embryo quality control exquisitely tuned to the spatial and temporal ordering of genome activation and zygotic gene expression. Our findings highlight the contribution of cell size patterning to the spatially choreography of events essential for the embryonic maternal-zygotic transition.

B103/P1229

Spatially restricted tissue failure through endolysosomal remodeling of junctional proteins promotes ear homeostasis

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Sensory and neural organs like the ear, eye, and brain rely on pressurized, fluid-filled cavities for structure, protection, and function. When luminal fluid pressure goes awry, it contributes to common disorders such as glaucoma, hydrocephalus after brain injury, and hearing loss in Pendred Syndrome or Meniere's Disease—yet we still know little about how these pressures are controlled. Using zebrafish, we previously discovered a tissue-scale pressure relief valve in the ear's endolymphatic sac that explains long-mysterious physiologies central to inner ear homeostasis. New characterization of this valve shows that relief comes through a single rupture in the endolymphatic sac epithelium, in line with material failure theory: heterogeneity in adhesion sets up points of weakness, and under strain, a viscoelastic sheet gives way where it is least reinforced. We set out to uncover the molecular basis of adhesion heterogeneity driving spatially restricted tissue failure. Using zebrafish embryos and larvae, where the inner ear can be imaged live instead of hidden in bone, we applied advanced imaging, genetics, and genome editing of endogenous proteins with fluorescent protein tags to tackle these processes head-on. Live-imaging and genetics show that mutants for wnt ligand secretion mediator (wls) develop distended sacs, much like lmx1bb mutants, and both mutants exhibit mispatterned endolymphatic sacs. In genome-edited fish, we see heterogeneity in nuclear β-catenin and junctional Claudin 7 as the endolymphatic sac valve begins to function in wild-type embryos. As the endolymphatic sac acquires its valve physiology, we find that a subset of cells in the endolymphatic sac target adhesion and junctional proteins to lysosomes. A small molecule agonist of casein kinase or an inhibitor of mTor accelerate the turnover of junctional proteins in the endolymphatic sac and this can break its ability to reform a barrier after pressure relief. We then found that Tgf-β-signaling, through activin whose expression is elevated in some patients with Meniere's disease, can weaken adhesion within the endolymphatic sac. Blocking activin signaling, either with follistatin or a small molecule inhibitor of activin receptors, slows the normal remodeling of adhesion proteins. Together, our work shows that Wnt-signaling is necessary for early sac patterning, and Tgf-β-signaling regulates endolysosomal remodeling of junctions to activate 'weakest point' release under strain—aligning with the conceptual framework of failure theory in material science.

B101/P1227
Study of sexual reproduction in softe coral Xenia sp.
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The Zheng Lab has been developing a fast-growing soft coral, Xenia sp., as a model to study coral–algal endosymbiosis, in which host corals benefit from dinoflagellates maintained within the coral gastrodermis cells. However, limited knowledge of Xenia sexual reproduction has hindered the establishment of Xenia as a genetically tractable model. Previous studies of coral anatomy reported putative female and male germ cells in gastrovascular cavity, yet their identity and developmental stages remained unconfirmed. To enable genetics in Xenia, this project focuses on characterizing Xenia gametogenesis, especially identifying mature eggs and sperm. To identify female germ cells in Xenia, we used a conserved germline marker ddx4 and detected ddx4^{+} cells in Xenia gastrovascular cavity. We performed Smart-seq to these ddx4^{+} putative oocytes and confirmed their germ cell identity through the expression of female germline markers such as piwil1. We observed that Xenia oogenesis begins with a cyst-like cell mass, and as oocytes enlarge, they become individualized. To further identify the mature eggs, we characterized the largest Xenia oocytes (800-900 µm in diameter). We found that the large oocytes are in primary meiotic arrest, indicated by their intact nuclear envelope and decondensed chromosomes localized to the nuclear periphery. Our next goal is to activate these arrested oocytes to obtain mature eggs. By genome mining in Xenia, we have identified a list of candidate neuropeptide precursors that share features with oocyte-activating neuropeptides in other cnidarians. We will test these neuropeptides to activate Xenia oocytes and induce maturation. Xenia spermatogenesis remains a large mystery. We identified a filamentous structure restricted to regions lacking female germline, containing densely packed nuclei and flagella. Our 10x scRNA-seq revealed a cluster with a classic male germline expression profile. We will further identify Xenia male germline by using markers identified from the scRNA-seq analyses.

B084/P1210
Temporal and mechanistic analysis of Bnl/Btl signaling during terminal cell branching morphogenesis in the drosophila tracheal system
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Improper branching due to abnormal cellular signaling is a driving factor for many congenital diseases involving angiogenesis in humans. To better understand these defects, developmental biologists have long used the Drosophila melanogaster tracheal system, the fruit fly respiratory system, as an effective model for branched tube formation. While previous research in this system has demonstrated a requirement for components of the Bnl/Btl (FGF/FGFR) signaling pathway during tracheal network formation, there is a limited understanding of the role, and essential time frames, of this pathway in promoting unicellular branching of specialized tracheal terminal cells over periods of larval growth. It also remains unclear whether the pathway promotes branching by interacting directly with the cytoskeleton or by inducing changes in gene expression. To investigate the requirement of this pathway during larval terminal branching, we will take advantage of tools that enable us to compromise signaling during larval stages while leaving the pathway unperturbed during embryonic differentiation events. Expression and activity of pathway components will also be monitored throughout larval stages to characterize Bnl/Btl signaling during periods of terminal cell growth. This research

will have wide-ranging implications for our understanding of the signaling requirements to form a branched single cell with subcellular lumens innervating each branch extension.

B075/P1201

The first line of life's defense: epithelial cells protect the early embryo from bacterial infection

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Early embryogenesis is a highly plastic stage in which cells proliferate, differentiate, and coordinate complex morphogenetic movements. In addition to intrinsic regulatory mechanisms, embryos develop within diverse biological environments. While external development exposes embryos to a wide range of microorganisms, internal development, as in humans, can also entail interactions with infectious agents that commonly colonize the uterus. How embryos respond to these microbial challenges before the formation of immune cells remains unknown. Here we identify a defensive biological role for surface epithelial cells of the embryo in protecting against bacterial infection. We previously showed that this epithelium eliminates defective embryonic cells through phagocytosis. Using advanced quantitative live imaging in zebrafish embryos, we now demonstrate that surface epithelial cells can efficiently detect, engulf, and destroy bacteria such as Escherichia coli and Staphylococcus aureus. Inhibiting this clearance impairs development, highlighting its crucial role in early embryogenesis in the context of infection. We observed actin-rich protrusions that capture bacteria via a zipper-like mechanism, enabling their engulfment and internalization, followed by trafficking to lysosomes for degradation. Single cell disruption of protrusion dynamics in live embryos revealed that bacterial phagocytosis depends on the GTPase Rac1 and the formin Fhod1, key components of the conserved molecular machinery employed by immune cells for pathogen internalization. Transcriptomic profiling of epithelial cells after bacterial exposure uncovered the activation of an immune program characterized by the regulation of proinflammatory cytokines, pattern-recognition receptors, antibacterial proteins, and macrophage-associated genes. These findings reveal an unexpected innate immune competence within early embryonic epithelial cells. Importantly, imaging of mouse embryos confirmed that this phagocytic program is conserved across vertebrates. Moreover, exposing human blastocysts to pathogenic bacteria associated with uterine disease demonstrated that the mammalian trophectoderm can internalize and destroy these infectious agents, underscoring an autonomous defense of the human embryo. This innate immune function challenges the traditional view of the early embryo as immunologically naive and has significant implications for understanding developmental robustness and embryonic failure linked to microbial exposure. Our interdisciplinary approach reveals how early embryos deploy epithelial cellular defenses to safeguard normal development under changing biological environments.

B096/P1222

Transcriptomic analysis of dental pulp development associated with sensory innervation

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Holomkova K, Svandova E, Kostolanska K, Tucker AS, Lesot H, Matalova E Laboratory of Odontogenesis and Osteogenesis, Institute of Animal Physiology and Genetics, CAS, v.v.i., Brno, Czech Republic Dental pulp innervation is a critical process in tooth development allowing for establishment of an organized system of fibres important for homeostasis maintenance of this specific soft tissue inside the mineralized tooth. This includes sensory as well as autonomic innervation. In the mouse first molar, the most common mammalian model of odontogenesis, sensory innervation happens around the postnatal day (PN) 4. The aim of this work was to provide a complex insight into transcriptomic profile of the dental pulp cells during sensory innervation network formation. To document and approved in situ situation, serial histological section of the mouse first molar at the PN2, PN3, PN4, PN5 and PN6 stages were labelled for unmyelinated sensory nerves by peripherin antibody using immunohistochemistry (IHC). The samples of the dental pulp were collected at the stage PN2, PN4 and PN6 and the isolated RNA was delivered for RNA sequencing (genseq.cz). Data analysis was performed in the Cytoscape plug-in ClueGo. According to IHC, the first appearance of the sensory nerve inside of the dental pulp happens at stage PN3. At PN4 are nerves seen more prominent in the middle of pulp and reach the cusps in the coronal part at PN6. These observations are consistent with reported findings in the literature. Transcriptomic analysis identified 420 differentially expressed genes (DEGs) between stage PN2 and PN4, and 588 DEGs in PN4 versus PN6. Among the enriched pathways, neuron projection development was the most significantly occurring pathway between stages PN2 and PN4. Genes associated in this pathway namely: Tnn and Kirell3, were in the top 20 most significantly upregulated genes. The RNASeq based analysis identified key neurogenetic pathways and specified genes with altered expression among the investigated stages. These findings were matched with the morphogenetic events in the tooth and expression of specific proteins associated with innervation. The comprehensive RNA dataset is also a background for further odontogenic investigations. Supported by the Grant Agency of the Czech Republic, project GACR 23-06660S.

B086/P1212

Using spatial transcriptomics to reveal the patterns of structural proteins during formation of the tooth and tooth-bone interface in human fetal development

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Collagens are a central component of the ECM in bones, teeth, and associated tissues. These collagens form a scaffold but also have roles in signalling between tissues. Defects in specific collagens leads to defects such as osteogenesis imperfecta (OI) and dentinogenesis imperfecta (DI), characterised by brittle bones and dentine. The collagen family comprises 28 members that contain at least one triple-helical domain. These can be divided into subfamilies, such as Fibril-forming collagens, fibril-associated collagens with interrupted triple helices, multiplexins and membrane collagens. Keratins are intracellular intermediate filaments that provide mechanical support to epithelial cells, and like collagens are formed of a large family divided into type I and type II. We have used spatial transcriptomics using the COSMX single-cell imaging system in human fetal samples to follow the pattern of different members of the collagen and keratin family as the tooth develops, focusing on expression in the tooth, forming alveolar bone, and between these tissues at the tooth-bone interface (TBI). We show that both collagens and keratins have a spatially restricted pattern from the late cap to the bell stage at 11 weeks post conception, with the TBI outlined by the expression of SPP1, MMP9 and ACP5. Some collagens, such as COL3A1, were associated with the dental follicle, while others, such as COL6A2, were expressed specifically in the dental papilla. ECM associated proteins, such as Decorin, also showed dynamic expression patterns in the mesenchyme around the tooth. In the dental epithelium, the different Keratins indicated previously unappreciated divisions of the epithelium, through nested patterns of KRT14,

KRT5, KRT15, KRT17 and KRT19. These nested patterns of keratins and collagens highlight the different compartments of the developing human tooth and can help shed light on dental defects associated with mutations in these components. Supported by the Grant Agency of the Czech Republic, project 23-06660S.

Developmental and Stem Cell Biology - Epigenetics and Reprogramming

B037/P1165
A baseline model of polymer-driven transcription for enhancer-driven gene activation
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Enhancers are key regulators of cellular decisions. They manage to convert transcription factor binding into target promoter activation with high fidelity in both space and time. Despite their prevalence and importance, the mechanism of enhancer-driven gene activation remains elusive. Bulk results demonstrate a decrease in transcription upon enhancer-promoter separation, suggesting a proximity-based activation mechanism. However, direct visualization of enhancer-promoter distance along with transcription does not always confirm this picture. Currently, it is unclear whether these results are compatible and whether they are consistent with a picture where enhancer-proximity drives promoter activation. To address these questions, we developed a simple model of enhancer-driven transcription. The model employs a Rouse model for polymer kinetics and considers a promoter that can activate only when the enhancer and promoter come within a threshold distance. We find that experimental data constrain the model to a regime where promoters display low sensitivity to enhancer contacts, requiring several contacts before successful gene activation. This implies a vanishing relation between enhancer-promoter distance and promoter state, providing a possible explanation for the sometimes-lacking correlation between enhancer-promoter distance and transcription observed in experiments. We then investigated how transcription varies with genomic separation and found average transcription and bursting kinetics to be simple hill functions of the contact probability. Interestingly, this allows for easy exploration of the effects of complex enhancer-promoter landscapes on gene activation. Together, these results provide a baseline model of enhancer-driven transcription that answers the question of what to expect when activation is governed by polymer-contacts alone.

B040/P1168
Emerin and Histone-Modifying Enzymes Interact to Regulate Genomic Reorganization During Differentiation
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Emerin is a resident inner nuclear membrane protein that is implicated in chromatin reorganization and transcriptional regulation through its interactions with chromatin modifying proteins, such as histone deacetylase 3 (HDAC3) and the histone methyltransferases (HMTs), G9a and EZH2. We previously showed that emerin directly interacts with HDAC3 and associates with it at the nuclear envelope in myogenic progenitor cells. This interaction was shown to be required for proper temporal positioning of myogenic loci during myogenic differentiation concomitant with their repression. Emerin also interacts with two HMTs, G9a and EZH2. Mutations in emerin that inhibit HDAC3 binding lead to increased binding of G9a and EZH2. Thus, we posit that emerin coordinates interactions between histone-modifying enzymes at the nuclear periphery to control the precise spatiotemporal localization of lineage-specific genes to regulate their expression. However, it is how emerin coordinates these interactions to facilitate differentiation that is not well understood. Here,

we show that G9a and EZH2 levels significantly decrease 48 hours after induction of myogenic differentiation, suggesting G9a and EZH2 activity are important early in differentiation. Previous data showed emerin was important for the transcriptional reprogramming that occurs during differentiation. Thus, we hypothesize that emerin associates with G9a and EZH2 early in differentiation to reorganize the genome to drive transcriptional reprogramming. Supporting this hypothesis, emerin-null myogenic progenitors have lower levels of H3K9me2/3 and H3K27me3, the targets of G9a and EZH2, compared to wildtype progenitors. We tested whether rescuing methylation of H3K9 and/or H3K27 improved differentiation of emerin-null myogenic progenitors, as suggested by our hypothesis. We treated emerin-null progenitors with demethylase inhibitors that targeted H3K9me2/3 (PFI-90), H3K27me3 (GSK-J4), or both (JIB-04) to confirm they increased the respective methyl mark. All three inhibitors increased methylation, as expected and led to little cell death. We are currently testing their ability to rescue emerin-null differentiation. Future studies would then examine how emerin and HMTs functionally interact during early differentiation to coordinate transcriptional reprogramming.

B041/P1169

Functional Characterization of Polycomb Repressive Complex 1 (PRC1) in Mammalian Germ Layer Specification

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The embryonic germ layer development into endoderm, mesoderm, and ectoderm is essential for the formation of a viable mammalian organism and is tightly regulated by chromatin modifiers. Polycomb Repressive Complex 1 (PRC1) plays a key role in transcriptional gene silencing and stable cell-fate decisions in development, yet the specific mechanisms it employs are not fully elucidated. Here, we use mouse embryonic stem cells (mESCs) to understand PRC1-mediated gene regulation and the functions of distinct PRC1 sub-complexes in early germ layer specification. We initially show that the mESC can overcome their instability during differentiation through a robust conversion to Epiblast Stem Cells (EpiSC), which then enabled us to reproducibly achieve germ layer derived cells. To characterize dynamic organization of canonical (cPRC1) and variant (vPRC1) complexes across germ layers, we performed affinity purification followed by mass spectrometry with the core PRC1 catalytic subunit, RING1B, and the vPRC1 subunit, RYBP, identifying their associated proteomes. Our data reveals a dynamic shift from CBX7-cPRC1 to CBX2-cPRC1 assemblies as germ layer specification proceeds. In contrast, PCGF paralogs show germ-layer specific stoichiometries, with PCGF2 and PCGF4 exhibiting endoderm and mesoderm-specific abundances, respectively. In addition, we discover unique CBX6-cPRC1 and CBX4-cPRC1 specific functional assemblies in the endoderm and mesoderm, respectively, highlighting the modularity of PRC1 complex formation. We then utilized a CRISPRi system to achieve stable knockdown of PRC1 subunits across distinct developmental stages to elucidate the temporal requirements of PRC1 during germ layer specification. Loss of RING1B and RYBP each leads to severely impaired endoderm formation, whereas ectoderm appears to be comparatively unaffected, regardless of the developmental stage at which the knockdown occurs. We propose that the endoderm is particularly sensitive to PRC1 disruption independent of temporal deviations. Subsequently, we have generated a roadmap of cell fate perturbations in each germ layer cell that occur due to the temporal deficiency of PRC1 subunits. These findings underscore that PRC1 is an essential transcriptional regulator, and its subunits are dynamically organized to specify correct germ layer patterns in early development. This ongoing research will shed light on the functional contributions of cRPC1 and vPRC1 and define the predominant PRC1 sub-complexes across development.

B038/P1166
Maternal Asymmetry of KLF4 Regulates Blastocyst Formation and ICM Differentiation in Porcine Preimplantation Embryos
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Efficient blastocyst formation is vital for assisted reproductive technologies but remains challenging in vitro. In early mammalian development, the first lineage segregation forms the inner cell mass (ICM) and trophectoderm, driven by zygotic and maternal factors. We identified Krüppel-like factor 4 (KLF4), a transcription factor, as asymmetrically expressed between blastomeres at the 2-cell stage, with greater enrichment in the ICM than the trophectoderm, based on transcriptomic data. Functional assays revealed high KLF4 expression at mRNA and protein levels during the morula stage. CUT&Tag analysis showed KLF4 enriched more genes at the morula stage than the 4-cell stage, particularly near gene promoters and termination regions. KLF4 knockdown at the MII stage impaired blastocyst formation, reduced OCT4-positive ICM cells, and decreased OCT4 protein expression. It also disrupted epigenetic regulation, reducing H3K4me3 and H3K9me3 while increasing H3K9me2. Lineage tracing confirmed that KLF4 depletion in single 2-cell blastomeres reduced ICM contribution. These findings indicate that maternal asymmetry of KLF4 contributes to unequal ICM differentiation between blastomeres and plays a crucial role in early lineage specification and blastocyst development.

B042/P1170
Mechanisms of Polycomb Domain Inheritance and Establishment Across the Maternal-to-Zygotic Transition
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During early Drosophila embryogenesis, Polycomb Group (PcG) proteins establish chromatin domains that heritably mark developmental genes for long-term epigenetic silencing. While some of these domains are thought to be inherited from the maternal genome of the oocyte, de novo acquisition of PcG domains must occur in the zygote following a period of intense cell cycle activity. Here, we integrate experimental and computational approaches to define the mechanisms governing PcG-driven H3K27 methylation in the context of the maternal-to-zygotic transition. Using ChIP-seq and live imaging of CRISPR-engineered PcG components, we show that Polycomb Repressive Complex 2 (PRC2)-dependent H3K27me3 emerges in nuclear cycle 14 at prospective Polycomb Response Elements (PREs), following a nucleation-and-spreading pattern. Surprisingly, while PRC2 components such as the histone methyltransferase E(z), localize to interphase nuclei throughout early cycles, PRE-binding factors Pho, Combgap, and GAGA-factor enter nuclei only after the tenth nuclear division. Zelda, a pioneer factor, is required for proper H3K27me3 and H2Aub deposition at a subset of PcG domains, in a context-dependent manner that distinguishes recruitment from licensing functions. To mechanistically interpret these dynamics, we developed and validated a mathematical model incorporating the nuclear concentration dynamics of E(z) and Pho, replication-driven histone dilution, and allosteric regulation of E(z) by existing methylation states. Stochastic simulations together with in vivo perturbations support a model where allostery preserves maternal H3K27 methylation in cis at lower-order states (H3K27me1/2), while de novo paternal methylation depends on nucleation in trans by Pho. At ZGA, broad H3K27me3 domains are re-established across both parental genomes. Together, our findings reveal that the early embryo initially relies solely on read-write allosteric feedback to copy one parental epigenetic state and that nucleating factor availability limits the onset of de novo zygotic establishment of the PcG chromatin

landscape. This two-step process for PcG domain formation during the maternal-to-zygotic transition provides a unified explanation for how Polycomb states are both inherited and re-established at the beginning of development.

B035/P1163
Pioneer factors induce global chromatin decompaction in early reprogramming
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Objective: Chromatin undergoes global physical changes during embryogenesis, with pluripotent cells exhibiting hyperdynamic chromatin compared to the more static state of differentiated cells, although the functional significance of this biophysical changes remains elusive. Our study aims to determine how somatic cells acquire hyperdynamic chromatin during reprogramming to pluripotency and how this influences transcription factor engagement with chromatin. Methods: We reprogrammed human fibroblasts into the pluripotent state by inducing OCT4, SOX2, KLF4, and c-Myc (OSKM). Combining live-cell imaging with single-molecule tracking (SMT), we quantified the motions of core histone H2B and the pioneer factor SOX2 in individual cells across both successful and unsuccessful reprogramming trajectories. Results: We found that within four days of inducing OCT4, SOX2, KLF4, and c-Myc (OSKM) in human fibroblasts, we observe a global increase in chromatin mobility and decompaction that occurs independent of reprogramming status. By day seven, however, only cells progressing toward pluripotency maintain this hyperdynamic chromatin, whereas non-reprogramming cells revert to a somatic-like compacted state. Mechanistically, we found that reprogramming factors OSKM drive changes in linker histone isoform dynamics and composition, marking the initial chromatin responses during fate induction. Importantly, global chromatin remodeling precedes enhanced SOX2 chromatin scanning, suggesting that early acquisition of hyperdynamic features creates a permissive landscape for pioneer factor search and binding at pluripotent genes. Conclusions: Our findings reveal a mechanistic link between large-scale chromatin dynamics and transcription factor scanning of the genome, providing a biophysical framework for understanding how pioneer factor-induced chromatin reorganization initiates cell fate transitions.

B036/P1164
Preserving Germline Integrity through the Transgenerational Silencing of SAEG-2/Dnttip1 and MiDAC-Dependent Chromatin Remodeling
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Transgenerational epigenetic inheritance (TEI) preserves germline integrity by ensuring that somatic gene programs remain silenced across generations. In Caenorhabditis elegans, the LOTR-1 protein, which contains LOTUS and Tudor domains and localizes to Z granules, helps facilitate this process (TEI). To define its targets, we analyzed three independent lotr-1 mutant alleles (ΔTudor, ΔLOTUS, and Δlotr-1) using mRNA-seq. Across all alleles, we identified robust upregulation of saeg-2, the worm homolog of human DNTTIP1. In other organisms, DNTTIP1 partners with histone deacetylases in the MiDAC complex, presumably to regulate chromatin accessibility; however, much remains unclear. We validated the saeg-2 upregulation in lotr-1 mutants by RT-qPCR and single-molecule FISH, which showed that the increased transcripts are primarily

restricted to the germline, with the most pronounced upregulation in the ΔLOTUS allele. However, polysome profiling and CRISPR tagging of endogenous SAEG-2 protein revealed that these germline transcripts are not efficiently translated in lotr-1 mutants, where SAEG-2 protein largely remains confined to somatic tissues. Previous studies have shown that SAEG-2/DNTTIP1 functions within the MiDAC/histone deacetylase complex to regulate neuronal gene expression and that its transgenerational silencing is essential for proper behaviors, such as chemotaxis and the basal slowing response. Because lotr-1 mutants increase saeg-2, we hypothesized that they would exhibit defects in neuronal regulation and behavior, which could then be suppressed by the loss of saeg-2. However, both lotr-1 mutants and saeg-2 deletion worms exhibited relatively minor or no defects in these behavioral assays, possibly through translational repression of ectopic saeg-2. We hypothesize that SAEG-2 and MiDAC-like activity promote somatic differentiation and remain silenced in the germline. LOTR-1 may help preserve germline integrity through this silencing, while ensuring that mechanisms for proper neuronal and behavioral responses can persist across generations.

B039/P1167

SIRT6 activation represses endochondral ossification to promote a stable articular cartilage phenotype in vivo

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Precise stem cell signaling cues are needed to induce chondrocyte differentiation and articular cartilage repair. Current stem cell therapies to form stable articular cartilage are suboptimal and can form bone through endochondral ossification. Our prior data demonstrates that activation of sirtuin 6 (SIRT6) can regulate chondrocyte and stem cell function, but its role in chondrogenic differentiation is unclear. In this study, we assessed the effect of MDL-800, a small molecule activator of SIRT6, to regulate chondrogenic differentiation and endochondral ossification processes in vitro and in vivo. Animal studies were approved by the Thomas Jefferson IACUC. We utilized our previously described 'Matrigel with BMP2 endochondral ossification model' which has been shown to promote ectopic bone formation in vivo. Twelve-week-old C57BL/6 mice were subcutaneously injected with 300 µl of Corning Matrigel Matrix Basement Membrane containing BMP2 (6µg/ml) or BMP2 plus MDL-800 (25 µM). After 14 days, mice were euthanized and Matrigel masses were analyzed by RT-PCR, microCT and Safranin-O/fast green staining. To define the effect of MDL-800 to transcriptionally regulate chondrogenesis in vitro, human bone marrow derived mesenchymal stem cells (hBM-MSCs) were cultured in 3D micromasses (n=9) and incubated in basal media or chondrogenic differentiation media (containing TGFß3) in the presence or absence of MDL-800 (10 µM) for 10 days. After experimental incubations, RNA-sequencing was performed, followed by gene set enrichment analysis (GSEA). Supplementation of MDL-800 into Matrigels containing BMP2 significantly decreased endochondral ossification processes and bone formation ($p=0.0154$), when compared to BMP2 controls. Matrigels displayed robust cartilage formation that was unchanged between groups. However, MDL-800 containing Matrigels displayed significantly increased COL2A1 and ACAN gene expression levels, compared to controls. In our chondrogenic differentiation model, RNA-sequencing and GSEA analysis demonstrated that administration of MDL-800 to chondrogenic media significantly decreased pathways associated with 'bone development' and 'activation of matrix metalloproteases' while enriching pathways associated with 'histone activity', 'FOXO signaling', and 'DNA repair'. This study demonstrates that SIRT6 activation represses BMP2-mediated endochondral ossification and maintains stable cartilage formation in vivo. Mechanistically, RNA sequencing revealed that SIRT6 activation promotes pro-anabolic signaling cascades and represses bone development processes. In conclusion, our findings demonstrate that SIRT6 may exert preferential effects on stem cell chondrogenesis to increase cartilage formation.

B043/P1171
The Role of SATB1 in the Transition from Presecretory Ameloblasts to Secretory Ameloblasts
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Enamel is the hard outer layer that provides the tooth with a protective barrier against damage and decay. Amelogenesis is the process of tooth enamel formation. Specific AT-rich sequence-binding protein 1 (SATB1) is a genome organizer which plays an essential role in amelogenesis. SATB1 expression is upregulated in presecretory ameloblasts (PABs) and gradually decreases as these cells transition into secretory ameloblasts (SABs). This study investigates how SATB1 drives the differentiation of PABs to SABs enhancing the expression of key enamel matrix proteins (EMPs). In mouse models with defective SATB1, enamel organ cells at the PAB and SAB stages of enamel formation were harvested for RNA-Seq and qPCR to identify changes in EMPs. Mouse hemimandibles were processed for immunohistochemistry and hematoxylin and eosin staining. ATAC-seq analyses were performed on wild-type and SATB1-deficient mouse SABs to identify SATB1's impact on chromatin accessibility. RNA-seq analyses showed that, compared to Satb1+/+ SABs, genes encoding amelogenin, ameloblastin, enamelin were downregulated, while the gene associated with epithelial cell proliferation (Ki67) was upregulated in Satb1-/- mouse SABs. QPCR analyses on presecretory and secretory stage of enamel organ cells from wild-type and Satb1fl/fl CK14-Cre (Satb1cKO) confirmed the RNA-seq findings. Immunostaining of sagittal sections of mouse incisors further demonstrated reduced expression of EMPs and increased expression of Ki67 in Satb1cKO ameloblasts from the presecretory to secretory stages of development. ATAC-seq analysis mapped genome-wide chromatin accessibility and revealed that the accessibility of regulatory regions for enamel matrix protein (EMP) genes in ameloblasts was SATB1-dependent. This study led to the further investigation of the molecular mechanism of SATB1's role in regulating EMP expression by driving the differentiation of PABs into SABs by suppressing cell cycle progression and enhancing chromatin accessibility of genes encoding major EMPs.

Developmental and Stem Cell Biology - Morphogen Gradients and Tissue Patterning

B029/P1157
A simple engineering method for spatially patterning morphogens on 2D stem cell monolayers
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Recreating the complex cellular organization of native tissues is a central challenge in tissue engineering. During embryonic development, this intricate architecture is orchestrated by precise spatiotemporal morphogen gradients. Recapitulating this dynamic signaling in vitro has been limited by methods that are often complex, costly, or lack the tunability of changing signaling cues over time. To address this, we have developed a custom-engineered transwell culture device that spatiotemporally patterns morphogen gradients in 2D within engineered stem cell colonies. The device leverages the strong tight junctions formed within stem cell colonies that prevent paracellular diffusion of morphogens and sharply separate the apical and basolateral sensing surfaces of cells. When this feature is combined with the natural apical-basal separation of key morphogen receptors (e.g. BMP4 is basolateral only), we can start to control where and when cells receive

a morphogen cue. We use precisely patterned PDMS stencils placed on the basal side of the transwell as masks to restrict morphogen access to only certain cells. The key advantage of this system is its flexibility: spatial control is easily customizable via the stencil shape while temporal control is achieved by simply swapping the media containing the morphogen of interest allowing even multiple dosing regimens to be carried out in parallel all while performing live imaging (transmitted light, epifluorescence, or confocal). To date we have been able to spatially pattern multiple primordial germ layers (endo/trophecto/mesoderm) by using appropriate morphogen dosing schemes. We first validated with Bone Morphogenetic Protein 4 (BMP4), demonstrating precise regions of differentiation towards trophectoderm. Similarly, dosing with Activin or Wnt3A triggered local differentiation into endoderm or mesoderm. Moreover, the interfacial collective cell dynamics within adjacent compartments (differentiated vs. pluripotent) result in surprising behaviors such as invasive migration, tissue migration, and even multi-layer formation. This simple yet robust tool provides an accessible method to probe the effects of complex signaling dynamics on cell fate and offers a powerful platform for engineering patterned, multi-lineage tissues for applications in disease modeling and regenerative medicine

B026/P1150

Cytoneme networks and pattern disruption analysis in the developing Drosophila peripheral nervous system

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Normal tissue function relies in part on the emergence of tissue-wide patterns from smaller scale cell interactions and fate decisions during development. During the patterning of the thoracic peripheral nervous system (PNS) in the fruit fly, bipotential cells extend actin-rich protrusions called cytonemes. Evidence suggests that cytonemes allow cells to contact distant neighbors and engage in signaling, leading to the selection of sensory organ precursors in a field of epithelial cells. However, the mechanisms by which cytonemes support signaling are unclear. Furthermore, methods for detecting pattern disruption have been limited. To address these issues, we have (1) investigated the mechanisms of cytoneme-mediated interactions, (2) performed targeted RNAi screens to identify factors that support PNS organization, and (3) developed a binary classification system for analyzing PNS patterns. Using live confocal microscopy, we find that cytonemes create dynamic meshworks during patterning. Individual cytonemes that interact with these structures have distinct behaviors and require E-cadherin to mediate interactions. Next, we have identified 21 genes that were not previously known to play a role in PNS patterning, as well as the MyTH4-FERM myosin XV, one of a class of myosin known to support the formation of cellular projections. Finally, we developed a binary classification system based on clustering algorithms to identify factors other than density that can differentiate between wild type and disrupted PNS patterns. We anticipate that our screen results will be broadly useful for the study of neurogenesis and cellular projections. Furthermore, our classification system may be applicable to the analysis of most biological spot patterns.

B031/P1159

Exosome Secretion is Required for Sonic Hedgehog Dispersal and Signal Gradient Formation in the Embryonic Limb Bud Mesenchyme

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Spatial gradients of Hedgehog (Hh) signaling coordinate cellular behaviors across diverse tissues, but how Hh is secreted from source cells and dispersed across target tissues to shape these gradients remains poorly understood. Studies in different cells and tissues have identified numerous mechanisms that contribute to Hh dispersal, including carrier-assisted diffusion, via molecular chaperones or extracellular vesicles, and directed transport along specialized filopodia known as cytonemes. However, the relative contributions of these mechanisms in shaping Hh gradients in specific tissues remains poorly defined. In particular, we know very little about the mechanisms that mediate Sonic hedgehog (Shh) dispersal in mesenchymal cells, even though mesenchymal tissues make up a large fraction of the vertebrate body plan. Here, we combine novel signaling assays with quantitative microscopy and molecular perturbations to establish a fundamental role for exosome-based secretion in forming Shh signaling gradients in the embryonic limb bud mesenchyme. We developed a novel synchronous release system that allows us to follow Shh, along with other proteins, through the secretory pathway in primary cell cultures and vertebrate embryonic tissues in vivo. Using this system, we show that the majority of Shh traffics through the exosome biogenesis pathway in both Primary Limb bud Mesenchymal Cells (PLMCs) and in the limb bud mesenchyme in vivo. This identifies exosome packaging as the predominant route for Shh secretion. Using a reconstituted mesenchymal tissue in which PLMCs secrete Shh into a 2D field of reporter cells, we demonstrate that exosome-based release is essential for establishing short range Shh gradients. Additionally, we show that a large fraction of cytoneme-localized Shh is associated with secreted exosomes that undergo directed transport along the external surface of these cytonemes. This suggests that the limb bud may also harness directed cytoneme-based transport of secreted exosomes to promote Shh signaling at longer length scales. Thus, we propose that exosome biogenesis provides a general and versatile platform for Shh secretion that can be coupled to different forms of extracellular transport (e.g. diffusive vs directed) to sculpt Shh signaling gradients across tissues with varied architectures and length scales in developing embryos.

B025/P1153

Fox Transcription Factors Regulate Follicle Stem Cell Differentiation and Ion Transport Gene Expression in the Drosophila Ovary

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Increasing evidence indicates that intracellular pH (pHi) dynamics contribute to cellular behaviors such as differentiation, proliferation, and epigenetic modifications. In the Drosophila ovary, a naturally occurring increase in pHi is required for differentiation in the follicle stem cell (FSC) lineage. However, the mechanisms by which pHi influences cell fate remain unclear. We used single-cell RNA sequencing (scRNAseq) to identify transcriptional changes caused by inhibition of the pHi gradient in the FSC lineage and found that Forkhead box (Fox) transcription factor (TF) activity is significantly altered. Therefore, we hypothesized that an increase in pHi regulates differentiation in FSC lineage by modulating the activity of Fox family transcription factors. To test this hypothesis, we first performed an RNAi and over-expression screen of the 17 Fox TFs in the fly genome. We found that RNAi knockdown of either FoxK or biniou (the Drosophila homolog of FoxF), caused significant follicle cell differentiation defects that resemble the phenotypes caused by disruption of the pHi gradient. ScRNAseq and in situ HCR revealed that biniou is expressed in escort and FSCs, and that FoxK is expressed broadly throughout the ovary. We also found that over-expression of biniou caused an accumulation of undifferentiated follicle cells but no change in the rate of proliferation. This, in combination with the expression pattern of biniou suggests that it may be an important FSC self-renewal factor. We performed bulk

RNA sequencing and gene ontology analysis of biniou and FoxK RNAi ovaries and discovered an enrichment of upregulated genes relating to ion transport. This suggests that these genes may act in a feedback loop to modulate pHi. Our current studies include the generation of an in vivo Fox TF reporter to assess the role of pHi dynamics in the patterning of Fox TF activity, clonal analysis, and epistasis analysis to investigate the genetic relationship between Fox TFs and the pHi gradient. Successful completion of these studies has the potential to uncover a direct molecular mechanism by which pHi dynamics regulate cell fate decisions within the adult stem cell lineage.

B033/P1161
How a fluid-like mesenchyme shapes the intestinal epithelium
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Tissue folding generates structural motifs critical to organ function. In the intestine, bending of a flat epithelium into a periodic array of folds gives rise to villi, the finger-like projections essential for nutrient absorption. Our lab investigates the cellular and mechanical mechanisms that initiate this transformation. By combining live imaging of intact mouse tissues with mechanochemical profiling and functional perturbations, we examine how epithelial-mesenchymal interactions break symmetry to drive coordinated tissue bending. We will highlight our recent work on villus initiation driven by mesenchymal dewetting, demonstrating that self-organizing collective mesenchymal mechanics can act as a symmetry-breaking force that generates emergent curvature in epithelia. Building on this framework, we will additionally show how molecular and mechanical feedbacks at tissue interfaces refine these active processes to stabilize and propagate curvature during morphogenesis.

B024/P1152
Investigating the roles of focal adhesion kinase and mitogen-activated protein kinase kinase in patterning smooth muscle during lung branching morphogenesis
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During lung development, airway branching is shaped by dynamic interactions between the epithelium and surrounding mesenchyme. Airway smooth muscle (ASM) forms in stereotyped patterns around emerging branches and is required for epithelial bifurcation, yet the regulatory mechanisms underlying the patterned distribution of ASM remain poorly understood. Specifically, it is unclear how mechanical and chemical signaling events cooperate to induce ASM differentiation at the sites of future epithelial clefts while sparing the adjacent epithelium. We hypothesized that focal adhesion kinase (FAK) may serve as a transducer of the chemical and mechanical cues that regulate this pattern of ASM differentiation. To test this hypothesis, we cultured embryonic day 12.5 (E12.5) mouse lungs in the presence of small-molecule inhibitors targeting FAK, the structurally and functionally similar proline-rich tyrosine kinase 2 (PYK2), and mitogen-activated protein kinase kinase (MEK), a key component of the Ras-Raf-MEK-ERK signaling pathway that is often suggested to act upstream of focal adhesion assembly. Immunofluorescence and immunoblotting were used to assess spatial distribution, expression level, and extent of activation of these proteins. While inhibiting FAK alone produced only mild defects in ASM patterning, inhibiting MEK induced robust mesenchymal-to-smooth muscle differentiation, resulting in epithelial branches fully surrounded by ASM after 24 hours in culture. To determine whether PYK2 compensates for loss of FAK activity, we cultured E12.5 mouse lungs in the presence of dual

FAK/PYK2 inhibitor but surprisingly observed no major differences compared to FAK inhibition after 24 hours, suggesting a dispensable and perhaps only indirect role of FAK in ASM patterning. We additionally performed immunoblotting to compare the levels and activity of focal adhesion components in MEK- versus FAK/PYK2-inhibited lungs. Together, our results suggest that while FAK-mediated mechanotransduction may be active during lung development, it does not appear to be required for inducing ASM differentiation. Instead, ERK signaling appears to play a more dominant role in establishing the extent and patterning of ASM around the branching epithelium. Future work will investigate how ERK signaling regulates the differentiation of smooth muscle-destined mesenchymal progenitors.

B023/P1151

Optogenetic Control of RET Signaling Directs Branching Morphogenesis in Human Kidney Organoids

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Kidney function depends on a branched collecting duct network that connects millions of nephrons to the ureter. This network forms during embryonic development through iterative branching of the ureteric bud (UB) epithelium, driven by signaling interactions between the REarranged during Transfection (RET) receptor and its ligand, glial cell-derived neurotrophic factor (GDNF). While organoids derived from human induced pluripotent stem cells (hiPSCs) have strong potential for congenital disease modeling, therapeutic development, and regeneration, they typically produce only two to three branching generations and lack consistent organization. Developing strategies that achieve spatiotemporal control over branching would enable engineering of extended epithelial networks that approach physiological complexity. We first show that pharmacological manipulation of GDNF-RET signaling alters epithelial branching in explanted mouse kidneys and in UB organoids derived from hiPSCs. However, these pharmacological methods fail to recapitulate the spatiotemporal pattern of RET activity localized to UB tips during development. To address this, we developed an optogenetic RET receptor (optoRET) responsive to blue light, enabling precise control of RET activity. We engineered iPSC lines to express optoRET, differentiated them into UB organoids, and stimulated them with blue light using a programmable LED platform and by confocal microscopy. Light stimulation of optoRET-expressing UB organoids significantly increased branching tip number compared to unstimulated controls. The increase reflected new bud initiation, suggesting that optoRET signaling controls the earliest steps of branching. Notably, this response occurred in the absence of exogenous GDNF, indicating that optoRET can functionally substitute for ligand stimulation. Organoids lacking optoRET were unresponsive to light, confirming photoactivation specificity. This work positions optogenetics as a powerful approach to dissect receptor tyrosine kinase-driven branching pathways in morphogenesis and opportunities to engineer organoid architectures with defined complexity using patterned light.

B032/P1160

Sequence-specific Control of Signaling Protein Activity during Animal Development using a Synthetic Protease Library

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Cell signaling is essential for coordinating morphogenesis during animal development. Embryonic cells deploy signaling proteins to guide the differentiation of nearby cells, ultimately forming various tissues such as muscle, bone, and blood. Secreted signaling proteins typically undergo proteolytic activation. To precisely control the activity of signaling proteins, we created the Synthetic Processing (SynPro) system, a library of proteases that were engineered to be functional in the secretory pathway of embryonic cells. Here we used proteases from the Potyviridae family, a class of enzymes that can cleave a unique seven-amino-acid sequence. More than 3,800 Potyviral proteases are known and are predicted to cleave peptides in a sequence-specific manner. To functionally validate protease-substrate specificity, we constructed the Protease Substrate Specificity Calculator (ProSSpeC), leveraging direct coupling analysis to predict protease performance that were then validated in animal cells in vitro and in vivo. ProSSpeC revealed co-evolutionary sequence features at single amino acid resolution, most importantly in its ability to predict experimental outcomes of any protease-substrate sequence pair. We demonstrate the ability of SynPro proteases to cleave key signaling proteins during early embryogenesis: Gdf1, Nodal, Lefty1/2, and BMP2/4/7, with concomitant phenotypes when these proteins are proteolytically processed. We further engineered the system for optogenetics to artificially induce developmental signaling pathways with light in a developing zebrafish embryo. To develop a light-inducible SynPro system, we fused each half of a protease with iLID and SspB proteins, which dimerize upon blue-light stimulation. We observed light-induced reconstitution of SynPro proteases and downstream activation of secreted proteins. Overall, our studies demonstrate that we can engineer sequence-specific proteases to control secreted proteins and their downstream signaling pathways at the cellular and whole-animal levels.

B030/P1158

The role of mechanical force in the patterning of progenitor domain gene regulatory network proteins Pax6 and Nkx6.1

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During early mammalian development, a sheet of epithelial tissue known as the neural plate undergoes neurulation - a highly complex folding event resulting in the tissue assuming a tube-like morphology. The resultant ‘neural tube’ will give rise to the spine and nervous system. Defects in neurulation can result in a variety of lethal and non-lethal diseases, such as exencephaly and spina bifida. The ventralizing morphogen, sonic hedgehog, is secreted at the midline of the neural plate and diffuses laterally, patterning cellular behavior by inducing discrete and stereotypical domains of protein expression known as progenitor domains. Progenitor domains are responsible for patterning the differentiation of cells in the neural plate into mature neuronal subtypes. Sonic hedgehog is also responsible for regulating mechanical behavior via cell shape change, acting as a negative regulator of apical constriction and cell height. Pax6 and Nkx6.1 are transcription factors downstream of sonic hedgehog involved in the patterning of progenitor domains, are induced early in development, and their expression is refined concomitantly with neural tube closure. Pax6 and Nkx6.1 are each necessary but insufficient for specifying multiple different progenitor domains, and are expressed in distinct portions of cells along the dorsal-ventral axis. In this study, we examine the role of mechanical force in patterning the gene network downstream of sonic hedgehog in the murine neural tube. Utilizing mice homozygous for mutant alleles of the calcium pump SPCA1, which are incapable of neural tube closure, we examined the spatial distribution of mRNA coding for Pax6 and Nkx6.1 at embryonic days 8 and 9, the period during which cranial neural tube closure normally occurs. Utilizing fluorescent in situ hybridization chain reaction probing for mRNA, we have examined the localization of Pax6 and Nkx6.1 mRNA during neural tube closure. We found that SPCA1 mutants that fail to fold have a similar pattern of Pax6 and Nkx6.1 expression to control embryos, indicating that mechanical force is not necessary to induce expression of these progenitor domain proteins. However, mechanical force may be necessary to refine their expression patterns, with shifts

in the exact positioning of these genes' expression domains. We are developing methods to quantify these shifts and will examine additional components of the progenitor domain gene regulatory network.

B034/P1162
Tissue fluidity: A double-edged sword for multicellular patterning
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Tissue fluidity, or the ability of cells to freely move, flow, and rearrange within a tissue, is a universal physical property of multicellular life that plays central roles in development, regeneration, and cancer. While it is clear that tissue fluidity is tightly regulated and varies drastically across biological contexts, we are only beginning to understand (1) how tissue fluidity is biologically controlled; and (2) how fluidity impacts critical tissue patterning mechanisms such as morphogen signaling and cell sorting. In recent work, we showed that tissue fluidity can easily be tuned to freeze, catalyze, or erase multicellular patterns, depending on the biological context (Garner et al., bioRxiv 2025) – suggesting that tissue fluidity is a key regulator of multicellular organization and must be tightly controlled to achieve robust patterning. Interrogating these questions in the context of zebrafish development, we have now established an in toto imaging pipeline to simultaneously track cell movements and morphogen signaling embryo-wide at single cell resolution over many hours. Using this approach, we have identified a fluidity transition in the early embryo in which initially stationary cells suddenly undergo rapid, random migration and scatter globally across the embryo. Similar early-embryonic movements have been reported in chick, mouse, and human. Performing high-resolution imaging of cell morphology and movement, as well as quantitative in toto cell track analysis, we establish that cell scattering is in fact driven by large-scale random migration. The cellular movement is rapid, with cells migrating an average of 1.8 µm/min (IDR 0.5-3.8 µm/min) – approaching the speed of immune cells. Mean squared displacement analysis revealed that cells move diffusively with an average effective diffusivity of 5.6 µm2/min (IDR 0.3-14.1 µm2/min), meaning they exhibit RMS displacements of 63 µm (IDR 14.2-100.8 µm) in just 2 hours, comparable to the size of the embryo (radius ~300 µm). Strikingly, the levels of random motility exhibit a gradient across the embryo, suggesting that the motility is developmentally patterned. Indeed, using an FGF/Erk reporter, we show that the onset of cell motility closely follows the initiation of a transient FGF activity gradient across the dorsal-ventral axis. Overall, our results demonstrate that a fluidity transition induces dramatic cellular mixing during early embryonic patterning. We are now actively investigating the consequences of the observed fluidity transition for early embryonic development. Using quantitative imaging, machine learning, and perturbations to control both motility and signaling, we are determining how major axis formation and fate specification can be robust in the face of such extreme random cell movements.

B027/P1155
Understanding BMP morphogen gradient interpretation
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Across the animal kingdom, morphogen gradients provide positional information to cells within developing embryos. Bone Morphogenetic Protein (BMP) acts as a morphogen to pattern the dorsoventral (DV) axis. In vertebrates, high levels of BMP signaling promote ventral fates, while low levels of BMP lead to the development of dorsal fates. Though we generally understand how the BMP signaling gradient is established,

we lack a mechanistic understanding of how different BMP signaling levels are interpreted by the cell to activate specific gene expression programs. BMP signaling begins when BMP ligand dimers bind to and assemble a complex of kinase receptors. Activated receptors within the complex then phosphorylate the intracellular effector protein, Smad. In zebrafish DV patterning, phosphorylated Smad5 then accumulates in the nucleus, where it binds and regulates BMP target genes. Recent work by our lab investigated models of BMP signaling gradient interpretation during DV patterning in zebrafish embryos. We identified 30 direct BMP target genes, many with known roles in DV patterning, and then found that they are expressed in three distinct DV expression domains. Our results indicate that the boundaries of these expression domains are regulated through Smad5 concentration thresholds, allowing us to now probe the molecular mechanisms underpinning the formation of these DV gene expression domains. To identify which cofactors facilitate Smad5 DNA binding and how Smad5 binding patterns differ among the DV gene expression domains, we validated two different internal Smad5 tags. Both triple V5 and single ALFA-tagged Smad5 constructs produced functional protein detectable through immunoprecipitation. ALFA-Smad5 also appears detectable by immunofluorescence and ALFA-Nanobody GFP. Using these tools, we plan to describe a mechanism of BMP signaling gradient interpretation. These findings will in turn uncover how different cell fates are specified during embryonic development. This work is supported by NIH grant R35GM131908 to MCM.

Developmental and Stem Cell Biology - Niche and Microenvironment

B125/P1251

Expansion Microscopy reveals changes in cellular and extracellular structures between healthy and de-tensioned tendons

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Tendons transmit mechanical forces between muscles and bones thanks to their uniform extracellular matrix (ECM), composed mostly of parallel collagen fibrils. When this ECM is damaged, resident tendon cells are responsible for regenerating this matrix to restore physiological function. However, in the case of tendinopathies, cells no longer respond to normal biomechanical cues and thus fail to regenerate the tendon tissue. Previous work in our lab, using isolated tendon cells for healthy and diseased patients, has shown aberrant nuclear chromatin remodeling in diseased state and loss of responsiveness of chromatin organization to mechanical cues. However, how chromatin is organized in the native tissue context and how that organization relates to the tissue ECM is poorly understood, since current methods are unable to visualize both chromatin and ECM structures at relevant length-scales within tendon tissue. To address this need, we have adapted Fluorescent Labeling of Abundant Reactive Entities (FLARE) along with Expansion Microscopy (ExM) to visualize ECM and chromatin structures throughout native tendon tissue. We demonstrate FLARE effectively resolves the dense network of collagen fibrils for multiple tendon types and species, while highlighting elastic fibers, glycan-rich intrafascicular regions and the architecture of the myotendinous junction. Using FLARE, we compared the chromatin organization of tendon cells with the uniformity and glycan composition of the surrounding cell ECM. Notably, we observed both a moderate thickening of ECM collagen fibrils and an increase in carbohydrate signal across the surface of resident tendon cells after tendon loss of tension. FLARE enables detailed visualization of the uniformity and composition of tendon tissue ECM and the distribution of tendon cell chromatin, across physiological and pathological conditions. We anticipate this approach will be valuable in studying the interplay between biomechanical signals, chromatin organization, and ECM structure composition and uniformity across various disease models, ultimately informing therapeutic strategies for tendon-related diseases.

B127/P1253
SEPT9 maintains colon homeostasis by controlling the microbiological environment via goblet cell maturation and mucin secretion
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Goblet cells (GCs) are essential for maintaining mucosal homeostasis and are closely linked to the pathogenesis of colorectal diseases, including inflammatory bowel disease (IBD) and colorectal cancer (CRC). Septin 9 (SEPT9), a member of the cytoskeletal septin family, plays a critical role in regulating colonic physiology. Our previous work demonstrated that intestinal epithelial cell (IEC)-specific SEPT9 knockout results in increased GC numbers, compromised barrier integrity, and enhanced susceptibility to inflammation. We hypothesize that loss of SEPT9 disrupts goblet cell regulation and mucin secretion, thereby impairing colon homeostasis and contributing to IBD and CRC pathogenesis and development. To test this hypothesis, we employed IEC-specific SEPT9 knockout (cKO) mice and littermate controls. Colon tissue sections, crypt-derived organoids, and isolated epithelial cell extracts were analyzed using super-resolution microscopy, qPCR, and western blotting. Fecal pellets were collected for microbiome profiling. We examined cell proliferation marker Ki-67, goblet cell markers (MUC2, SPDEF, TFF3), and intracellular trafficking/secretion markers (VAMP8, MARCKS). SEPT9 loss increased GC abundance and led to excessive intracellular accumulation of immature mucins in apical GCs. These alterations weakened the epithelial apical surface connections, compromising mucosal structure. Expression of the MUC2 and SPDEF genes was significantly reduced in cKO tissues, suggesting a higher proportion of immature GCs. Colonoids derived from SEPT9–mNeonGreen reporter mice revealed that SEPT9 localizes to vesicles containing WGA-labeled lectins, implicating SEPT9 in mucin intracellular trafficking. MARCKS expression, a key regulator of mucin secretion, was diminished in apical goblet cells of cKO mice but remained unchanged in crypt-resident goblet cells. Besides, Ki-67 was significantly elevated in mature cells of both cKO organoids and tissues, indicating a disrupted cell cycle regulation. Microbiota analysis revealed that cKO mice completely lacked Akkermansia muciniphila, a major microbial regulator of mucin turnover, and showed shifts in Lachnospiraceae spp., a family of commensals with established anti-inflammatory functions. Together, our findings identify SEPT9 as a critical regulator of colonic mucus production, goblet cell maturation, epithelial proliferation, and microbial homeostasis—independent of inflammation.

Developmental and Stem Cell Biology - Stem cell identity, Pluripotency, and Differentiation

B071/P1199
A Novel Doxorubicin-Adaptive hiPSC Model Links Early Stem Cell Stress to Impaired Cardiomyocyte Maturation
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Doxorubicin (Dox), a highly effective anthracycline chemotherapy, is limited by its cardiotoxicity, which is caused in part by the depletion of cardiac progenitor cells (CPCs). However, the mechanisms by which chemotherapy stress induces these maladaptive states in stem cells, thereby impairing cardiac regeneration, are not well-defined. We hypothesized that human induced pluripotent stem cells (hiPSCs) could serve as a renewable model to track how early stress persists into differentiated states. We developed a novel Dox-

adaptive hiPSC (D50-hiPSC) model by treating hiPSCs with 50 nM Dox for 2 hours, followed by five passages to stabilize adaptive responses. This system recapitulates acquired adaptation—defined as the ability to survive sub-cytotoxic stress while retaining pluripotency and differentiation potential—and enables direct tracking of how stem cell stress impacts lineage decisions. Morphometric analysis by deep-learning segmentation revealed a maladaptive 'Size-Structure Signature,' consistent with a failure to achieve a critical cell size (a 'Sizer' defect) required for normal maturation. Compared to controls, Dox-treated cells displayed significantly smaller area (1384 ± 477 vs. 1963 ± 657 μm^2, p = 0.0001) and higher nuclear-to-cytoplasmic ratio (0.66 ± 0.24 vs. 0.50 ± 0.17, p = 0.0001), indicating remodeling into smaller, rounder cells with a relatively enlarged nuclear compartment. Despite these changes, D50-hiPSCs retained pluripotency, with upregulation of stemness markers including SSEA4 (~2.8-fold), Nanog (~1.8-fold), and Oct4 (~1.6-fold). Functionally, cardiomyocytes derived from D50-hiPSCs at day 7 post-differentiation exhibited impaired contractile kinetics, with significantly prolonged time-to-peak contraction (1.06 ± 0.20 vs. 0.85 ± 0.05 s, p = 0.0001) and slowed relaxation, with delayed 30% decay (1.51 ± 0.13 vs. 1.17 ± 0.06 s, p = 0.0001) and 90% decay (2.03 ± 0.21 vs. 1.81 ± 0.27 s, p = 0.0001), while beat frequency remained unchanged. In conclusion, we discovered that Dox-adapted hiPSCs exhibit a distinct 'Size-Structure Signature' characterized by: (1) a reduction in cell size and altered nuclear morphology; (2) a concomitant reinforcement of the pluripotent state; which (3) functionally transmits to derived cardiomyocytes as impaired contractile maturation. Our novel Dox-adaptive hiPSC model provides a powerful platform to mechanistically dissect how chemotherapy stress reshapes stem cell fate and propagates functional deficits across lineages. This work advances our understanding of cardiotoxicity and provides a new paradigm for studying chemotherapy-induced tissue toxicities.

B047/P1175

Analysis of caspase-8 and caspase-9 activity during chondrogenic differentiation of equine adipose-derived stem cells with relevance to osteoarthritis

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Caspases-8 and -9 are key initiator cysteine proteases inapoptotic cascades, caspase-8 in the extrinsic one and caspase-9 in the intrinsic (mitochondrial) pathway. However, they also emerge in non-canonical roles such as regulation of differentiation, including mesenchymal stem cells (MSCs). MSCs, particularly bone marrow and adipose tissue (ADSC) derived, are a common source for regenerative approaches, e. g in case of cartilage repair and osteoarthritis (OA). The aim of this pilot investigation was to follow activation dynamics of caspase-8 and caspase-9 during chondrogenic differentiation of equine ADSC under normal and OA-like conditions. The cells were isolated from the subcutaneous gluteal adipose tissue and cultured in chondrogenic differentiation medium over a 21-day period. To mimic inflammatory (OA-like) conditions, cells were treated with pro-inflammatory cytokines interleukin-1β (IL-1β), tumornecrosis factor alpha (TNF-α), and a combination of both, respectively. Caspase activity was measured at 7-day-time points using a luminescent assay and quantified with theTecan Spark Microplate Reader. The results indicated changes in the activation pattern in the time course of differentiation within individual groups as well as among groups at the same time point. Notably, caspase-8 activity decreased between day 7 and 21 in all groups while caspase-9 activity dropped only in controls but was maintained in the OA-like environment. The most pronounceddifference among experimental groups during differentiation was observed in caspase-8 activity in TNF-α-treated cells. Overall, TNF-α-treated samples maintained significantly higher activities of both enzymes at the end of differentiationcompared with the other groups. This is in agreement with previous knowledge about TNF-αmediated caspase-8 expression which links this traditionally apoptotic caspase-8 with OA and possible anti-OA strategies. The different

caspase-8 and -9 activation patterns under control and OA-like conditions contribute to understanding of their emerging functions beyond cell death. Support of international cooperation by the Inter-COST scheme, project: LUC25127, www.msmt.cz

B064/P1192

Analysis of replication stress and DNA damage in induced pluripotent stem cells and induced mesenchymal stem cells

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Mesenchymal stem cells (MSCs) are non-hematopoietic adherent cells derived from bone marrow, adipose tissue, or placental tissue, with the capacity to differentiate into bone, cartilage, and adipose tissues. Owing to their regenerative potential, MSCs hold significant promise for clinical applications, particularly in tissue repair. Recently, the generation of functional MSCs from induced pluripotent stem cells (iPSCs) has emerged as a promising approach to obtain high-quality cells for regenerative medicine. In this study, we systematically investigated genomic alterations occurring from the initiation of iPSC generation to the differentiation of induced mesenchymal stem cells (iMSCs). Elevated phosphorylation of histone H2AX at serine 139 (γH2AX) in iPSCs was used as a marker of replication stress and DNA damage, both of which are critical to monitor for genomic stability. We quantified and compared γH2AX levels in human skin fibroblasts, SV-iPSCs, Epi-iPSCs, SV-iMSCs, and Epi-iMSCs. Both SV-iPSCs and Epi-iPSCs displayed higher basal γH2AX levels than fibroblasts and iMSCs, indicating increased replication stress and DNA damage. As anticipated, late-passage fibroblasts, SV-iPSCs, Epi-iPSCs, SV-iMSCs, and Epi-iMSCs exhibited more pronounced replication stress than their early-passage counterparts, correlating with greater genomic instability. Notably, γH2AX levels were not significantly influenced by reprogramming method (SV vs. Epi), suggesting that replication stress is comparable in iPSCs regardless of the approach used. By defining the genomic landscape during iMSC generation, differentiation, and passaging, our work provides valuable insight into the safety and efficacy of iMSC-based therapies. These findings highlight the necessity of comprehensive genomic assessments to ensure the clinical safety of iMSCs in regenerative medicine. Acknowledgements: This work was supported by the Korean Fund for Regenerative Medicine (23A0106L1), the KRIBB Research Initiative Program (KGM1232511), and the National Research Foundation of Korea (NRF) (RS-2023-00272798).

B048/P1176

Cell-cell mediated-actomyosin contractility is a potent suppressor of mesoderm induction by human pluripotent stem cells

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During morphogenesis, tissue organization is mediated by complex cell-cell and cell-extracellular matrix interactions, dependent on intracellular cell tension and tissue-scale force sensing. However, it remains unclear how, mechanistically, cells integrate the state of their environment to coordinate developmental responses. We hypothesize that mechanical forces, such as cell tension, regulate cell identity by de-sensitizing mesodermal genes to WNT activity. During mesoderm specification, we noticed that induced pluripotent stem cells (hiPSCs) experience high contractility, leading to stem cell colony retraction and increased myosin light chain phosphorylation. Surprisingly however, pharmacological or genetic inhibition of cell contractility

increased mesoderm gene expression and sped up epithelial-to-mesenchymal transition, a critical step for differentiation. Conversely, expression of a constitutively active Rho kinase (ROCK) to increase tension caused a total blockage of cardiac mesoderm conversion. Together, these data suggest that actomyosin contractility inhibits mesoderm identity in hiPSCs. Abolishing cell-cell force transmission using EGTA-mediated disruption of adherens junctions (AJ) or by creating CTNNA1 knock-out to uncouple AJ to actomyosin network, promoted mesoderm differentiation and mimicked ROCK inhibition, suggesting that force transmission across neighboring cells is important. Mechanistically, reduced contractility increased levels of active β-catenin in the cytoplasm and nucleus, and promoted WNT target gene expression, suggesting that lowering cell contractility licenses WNT pathway signaling. In addition, low contractility promoted β-catenin-TCF4 interaction and CUT&RUN experiment confirmed increased occupancy at TBXT promoter (encoding Brachyury), a key regulator of mesoderm lineage. Together, these data support a mechanism through which cell-cell-mediated actomyosin contractility modulates AJ stability to regulate free β-catenin, WNT signaling and mesoderm induction in hiPSCs.

B059/P1187

CLIPR-76 (CLIP4): A novel microtubule-binding protein and its role in striated muscle development

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CLIPR-76 (encoded by CLIP4) is a formally uncharacterized CAP-Gly domain-containing protein with splice isoforms capable of binding both microtubules (MTs) and membranes. Bioinformatics analysis of reported mRNA sequences indicate CLIP4 has multiple splice isoforms and exogenously expressed isoforms of CLIPR-76 have alternative subcellular localizations that appear to be isoform dependent. Isoforms 1, 2, and 3 localize to a subset of MTs whereas isoform 4 localizes to the endoplasmic reticulum (ER). We determined CLIP4 mRNA to be most highly expressed in striated muscle and found endogenous CLIPR-76 protein undergoes upregulation when murine C2C12 myoblast cells are induced to differentiate into skeletal muscle. These observations suggest a potential role for CLIPR-76 in the development of striated muscle, perhaps by helping direct the cytoskeletal and ER/sarcoplasmic reticulum rearrangements necessary for myogenesis. Here we utilize a combination of cell and molecular biological techniques as well as bioinformatic analysis to further study CLIPR-76 and its role in striated myogenesis, particularly during development of human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs). Updated bioinformatic analysis of reported CLIP4 mRNAs indicates remarkable conservation across vertebrate species at both the gene and protein levels, though different methods of alternative splicing appear to be utilized between human and mouse genomes. Immunofluorescence studies demonstrate CLIPR-76 expression and localization change throughout both skeletal and cardiac muscle development and alterations to CLIP4 using CRISPR editing is correlated to perturbation in the expected developmental timeline of iPSC-CMs. Taken together, these data suggest CLIPR-76 does play a role in early myogenesis, perhaps serving as a scaffold protein in the organization of MTs at critical areas of developing striated muscle.

B051/P1179

DIFFERENCES IN BASEMENT MEMBRANE INTEGRITY OF 3D VS 2D HUMAN INDUCED PLURIPOTENT STEM (HIPS) CELL CULTURES AFFECT MIGRATION ONSET DURING THE EPITHELIAL TO MESENCHYMAL TRANSITION

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The epithelial to mesenchymal transition (EMT) is a widely studied state change that occurs in both normal processes like development and wound healing as well as in pathological conditions like cancer and fibrosis. We induced an EMT in epithelial-like hiPS cells cultured as either flat 2D colonies, in which cells form sheets with apical-up/basal-down polarity, or as spherical 3D lumenoids, in which a single layer of cells encloses a central lumen and display apical-in/basal-out polarity. In addition to the differences in cell-cell neighbor interactions imposed by these different culture geometries, we also saw differences in the basement membrane environments of 2D and 3D cultures. Visualizing the basement membrane with a human-specific Collagen IV antibody, we observed that 2D hiPS cell colonies deposited very little basement membrane protein, while 3D lumenoids were enclosed in a basement membrane shell. We characterized EMT-related changes in protein expression, the timing of migration onset, the organization of cell junctions, and the integrity of the basement membrane environment in EMT induced in 2D versus 3D hiPS cell culture geometries. While the starting cell culture geometry affected the expression dynamics of some, but not all, EMT-related proteins, the time of migration onset was consistently delayed in EMT induced in 3D lumenoids compared to 2D colonies. To test whether this difference could be attributed to the differences in the basement membrane environments of 2D versus 3D cultures, we perturbed the basement membrane through enhanced or inhibited enzymatic digestion. We found that this shifted the timing of migration onset earlier or later, respectively, than controls, supporting a role for the basement membrane integrity in regulating the timing of cell migration during EMT. Additionally, live-imaging during EMT induced in 3D lumenoids, which have a well-established basement membrane shell, allowed us to observe cell-basement membrane interactions as cells took on migratory potential. We saw that some cells were deformed as they moved through holes established in the basement membrane while other cells were not. This hiPS cell model of EMT allows the study of how differences in the physical arrangement of cells and their extracellular environment affect a state change fundamental to development and disease.

B070/P1198

Direct Differentiation of Primordial Germ Cells from Embryonic Stem Cells via Small-Molecule Screening

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The germ lineage is essential for the generation of new organisms in most animals, transmitting epigenetic information and genetic diversity across generations. One established method to induce primordial germ cell (PGC) fate from pluripotent stem cells (PSCs) involves passing through an intermediate epiblast-like stem cell (EpiLC) stage, followed by in vitro exposure to BMP4, SCF, EGF, and LIF signaling. However, this approach shows a relatively low PGC differentiation efficiency of around 30%. Another hypothesis proposes the direct differentiation of ESCs into germ cells, but this approach faces challenges due to low efficiency and incomplete acquisition of authentic germ cell characteristics, and it remains unclear whether large-scale direct induction of PGCs from ESCs, bypassing the intermediate stage, is feasible. In this study, ESCs were cultured for six days in U-bottom plate under 2i (without LIF) conditions, with small molecules targeting cell cycle regulation, anti-infection, apoptosis, epigenetics, and immunology individually added for screening. Remarkably, addition of cell cycle–related small molecules increased PGC specification efficiency through direct differentiation without the EpiLC intermediate in both female and male ESC lines. In female ESCs, cells co-expressing Blimp1 and Stella reached 18.7% at Day 4 and approximately 60% at Day 6, whereas in male ESCs, Day 4 and Day 6 efficiencies were 35.9% and 44.8%, respectively. These findings suggest that cell cycle–modulating small molecules are key factors promoting direct PGC differentiation from ESCs, regardless of sex. Furthermore, PGC-specific markers

including Blimp1, Stella, Tfap2c, C-kit, Nanos3, Dnd1, Dmc1, Dazl, and Ddx4 were significantly upregulated in Day 6 PGCs compared with ESCs. Overall, this study identifies effective small molecules for directly inducing PGCs from ESCs without passing through an intermediate state, establishing a new paradigm for in vitro reconstruction of the mammalian germ cell lineage.

B044/P1172
Distinct Regulatory Programs Govern Cell Fate Decisions in Development and Regeneration
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How cell fate is determined during development versus regeneration remains a central unresolved question in biology, with broad implications for stem cell biology, tissue repair, and regenerative medicine. Using the mammary gland as a model, we combined classical transplantation assays with single-cell genomics and genetic perturbations to interrogate lineage decisions during basal-to-luminal conversion. We uncovered a transient intermediate state specific to regeneration, revealing that the molecular logic of fate specification in this context is fundamentally distinct from that of development. Comparative analyses further indicate that while developmental differentiation relies on community-driven signaling, regeneration employs alternative programs optimized for rapid tissue repair. Extending our findings to additional epithelial organs, we establish that distinct regulatory strategies underpin cell fate decisions across vertebrate tissues. These results challenge the prevailing view that regeneration merely recapitulates development and provide a new framework for understanding how context shapes stem cell behavior and organ homeostasis.

B052/P1180
Dynamics and regulation of the H19/IGF2 locus during neurogenesis
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The H19/IGF2 locus, containing the lncRNA H19 and growth factor IGF2, are crucial for embryonic development. In most adult tissues this locus is silenced, yet expression persists in stem cell niches with roles attributed to quiescence, proliferation and differentiation. Specific regions of the brain that are enriched for neural stem cells such as the dentate gyrus, pons and floor of the third ventricle show H19 and IGF2 expression, and while IGF2 has been reported to regulate neurogenesis through IGF1R->PI3K/AKT signaling the role of H19 within this neurogenic niche remains misunderstood. The literature describes contradictory roles for H19 as either promoting or inhibiting neurogenesis through various potential mechanisms. Thus, we seek to gain a better understanding of the regulation of the H19/IGF2 locus and determine precise mechanistic and functional consequences of the locus' expression during neurogenesis. Using a tractable induced-neuron (iNeuron) system, created by transducing an inducible Ngn2/Ascl1 vector into the KOLF2.1J iPSC cell line, we created neuron-like cells which express neuronal markers βIII-tubulin, MAP2, NeuN, and Synapsin-1, exhibit calcium signaling and form intricate neurite networks. These neurons were found by RT-qPCR and RNAscope (in-situ fluorescent hybridization) to experience a massive concomitant upregulation of both H19 and IGF2 during neurogenesis, which peaks at day 5 and slowly reduces to baseline throughout maturation. We conclude that suppression of H19 and IGF2 may be crucial to pluripotency and re-expression of H19 and IGF2 occurs during early neurogenesis. We are pursuing the mechanisms governing these observed changes and the functional and mechanistic role of these genes during neurogenesis.

B056/P1184

Electrical stimulation promotes osteogenic differentiation via calcium-mediated ß-catenin site-specific phosphorylation

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Electrical stimulation (ES) has been recognized as a promising approach to enhance osteogenic differentiation by modulating the membrane potential of bone-related cells. Despite its proven beneficial effects, the precise cellular and molecular mechanisms remain incompletely understood. In this study, we aim to elucidate this connection and propose a mechanism underlying ES-induced osteogenesis. The effect of ES (60 V and 18 Hz) on osteogenesis was assessed which revealed a marked increase in calcium deposition in the stimulated human bone marrow stromal cells (hBMSCs). Furthermore, bulk mRNA sequencing identified significant transcriptomic alterations following ES, notably implicating calcium signaling and WNT pathway components as potential mediators of the ES-induced osteogenesis. To further elucidate the underlying molecular events, we performed real-time live-cell imaging, and confirmed that ES induces pronounced calcium oscillations in hBMSCs through activation of voltage-gated calcium channels (VGCCs). Moreover, we identified that the ES-induces intranuclear accumulation of β-catenin and is facilitated by site-specific phosphorylation at the S552 and S675 residues. This site-specific phosphorylation of β-catenin is directly mediated by calcium-dependent kinases, particularly CaMKII and PKC. In summary, this study provides comprehensive mechanistic insight into the ES induced osteogenesis. Our findings establish a clear pathway where the phenomenon is mediated by calcium-directed stabilization of β-catenin through site-specific phosphorylation. These insights deepen our understanding of how bioelectrical stimulation influences osteogenesis, offering potential to improve therapeutic strategies for bone regeneration.

B066/P1194

Elucidating the epigenetic regulatory mechanisms governing mesodermal and myogenic differentiation of stem cells

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Multicellular organisms develop complex body structures through repeated proliferation and differentiation of a single fertilized egg into cells with distinct functions. The differentiation of mature somatic cells, such as neurons and muscle cells, is regulated by various factors, including epigenetic mechanisms. The Prdm gene family, which we focused on in this study, contains a PR domain—a lysine methyltransferase domain—and a ZF domain with DNA-binding capacity. Prdm genes are believed to function as transcription factors that mediate histone methylation.In this study, we aimed to elucidate the role of the Prdm6 gene in regulating mesodermal differentiation, including the myogenic differentiation of stem cells. We generated Prdm6 knockout (KO) cells using the CRISPR-Cas9 genome-editing system in mouse embryonic carcinoma P19 cells. To induce mesodermal differentiation, we employed the hanging-drop method and treated the cells with 1% DMSO for four days. Total RNA was extracted using TRIREAGENT, and gene expression was analyzed by microarray profiling and RT-qPCR according to standard protocols. To investigate the role of Prdm6 during mesodermal differentiation, we compared the differentiation states of Prdm6 KO and wild-type (WT) cells after induction. KO cells failed to undergo myogenic differentiation, suggesting that Prdm6 is essential for muscle cell development. Gene expression profiling revealed that, compared to WT cells, the KO cells showed decreased expression not only of myogenic markers but also of genes active during the early stages of mesodermal

differentiation. These results indicate that Prdm6 functions in the initial phase of mesodermal differentiation. Furthermore, we established a stable cell line carrying a Tet-on–inducible Prdm6 overexpression construct. Doxycycline was added from day 0 to induce Prdm6 expression for rescue experiments. As a result, the expression of skeletal and smooth muscle markers was restored, whereas cardiac muscle markers were not. Taken together, these findings suggest that Prdm6 is indispensable for muscle cell differentiation, while cardiac differentiation may require cooperative action between Prdm6 and other unknown factors. Given its role in gene activation, Prdm6 likely regulates mesodermal differentiation through nuclear histone methylation, such as H3K4me, thereby contributing to the establishment of an active chromatin state.

B067/P1195

Functional analysis of cell adhesion dynamics during muscle cell differentiation

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During development, embryonic stem cells (pluripotent cells) gradually lose their broad differentiation potential and give rise to lineage-specific stem cells, such as neural and myogenic stem cells, which can differentiate into a limited range of cell types. This cell fate restriction is influenced by the surrounding microenvironment, including cell–cell adhesion, extracellular matrix (ECM) adhesion, and secreted factors such as cytokines and growth factors. However, the mechanisms by which these factors regulate differentiation remain poorly understood. Pluripotent stem cells have received significant attention in regenerative medicine and drug discovery due to their ability to generate various cell types. However, directing differentiation into specific lineages and achieving proper tissue organization remain major challenges. This study aimed to elucidate how cell–cell interactions and ECM adhesion regulate muscle cell differentiation from pluripotent stem cells, with the goal of promoting more efficient and targeted differentiation. Mammalian muscle tissues—skeletal, cardiac, and smooth—arise from mesodermal progenitors undergoing dynamic changes in adhesive properties during development. Yet, how these changes influence muscle differentiation is not well understood. Here, we investigated the role of adhesive states during myogenic differentiation using a system in which pluripotent stem cells were induced to differentiate into muscle cells in vitro. We found that altering cell–cell adhesion states influenced commitment toward specific muscle types. For example, dissociation of cell–cell adhesion promoted differentiation into smooth muscle. In addition, we demonstrated that ECM adhesion also plays a crucial role in this process. Gene expression profiling indicated that integrins are involved in promoting smooth muscle differentiation. In conclusion, we show that dynamic regulation of cell–cell and ECM adhesion critically shapes muscle cell differentiation from pluripotent stem cells. Modulating adhesion dynamics may serve as a strategy to guide stem cell fate and improve directed differentiation for regenerative medicine.

B049/P1177

Generating cardiac lineage cells from PLN patient-derived human induced pluripotent stem cells (hiPSCs) for disease modeling and drug discovery to study cardiomyopathic functional and molecular dysfunction

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Background: Heart failure (HF) is a complex clinical condition associated with substantial morbidity and mortality worldwide. The contractile dysfunction and arrhythmogenesis related to HF has been linked to the remodeling of calcium (Ca++) handling. Phospholamban (PLN) has emerged as a key regulator of intracellular Ca++concentration. Of the PLN mutations, L39X is intriguing as it is not been properly characterized. This mutation is believed to be functionally equivalent to PLN null (KO) but contrary to PLN KO mice, L39X carriers develop a lethal cardiomyopathy (CMP). This study aims to establish patient-derived human induced pluripotent stem cells derived cardiomyocytes (hiPSC-CMs) from homozygote L39X carriers (human subjects) as model system, to explain the functional and molecular characterization of the L39X in human pathophysiology related to cardiomyopathy. Hypothesis: The hiPSC-CMs harboring the mutations (L39X carriers) pertaining to the L39 experimental (n =2), L39-KO (n =2), and the WT-KO (n = 2), lines display a specific abnormal cellular distribution, along with irregular transcriptional, translation expression and functional activity compared to the healthy controls (Wild type, WT, n = 2). Results: Compared to the healthy controls (WT-hiPSC-CMs), the PLN, along with SERCA2a, and other calcium handling markers had an abnormal cytoplasmic distribution and formed intracellular aggregates, with the loss of perinuclear localization. Similarly, the functional assays pertaining to the calcium handling (action potential and calcium transient) displayed significant arrythmogenicity. Besides, the PLN-dependent modulation of human cardio myocyte differentiation from days 0, 10, 20, 30 and 40, via the mesoderm or cardiac specification markers like p-Akt, p-GSK3β, MESP1, and WNT3 showed significant differences among the L39X carriers and WT, both transcriptionally and translationally. On the other hand, the PLN-dependent mitochondrial synthase activity for the above time course showed significant differences among different group along with higher mitochondrial membrane potential and oxidative stress. Conclusion: This data confirms an abnormal intracellular distribution of PLN, with the loss of perinuclear accumulation and mis-localization, suggestive of ineffective targeting to or retention of L39X.

B063/P1191

Generation of retinal pigment epithelium (RPE) cells during differentiation of mouse embryonic stem cells in KSR-containing medium

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Retinal pigment epithelium (RPE) cells have huge potential for cell replacement therapy for various retinal degenerative diseases, including age-related macular degeneration. Differentiation of embryonic stem cells (ESCs) under knockout serum replacement (KSR)-supplemented culture conditions results in the predominant generation of neural cells. However, non-neural cell types, including RPE cells, are also generated during the culture with lower efficiencies. In this study, mouse ESC lines- GS-2 and D3 were differentiated under KSR-supplemented culture conditions using the embryoid body (EB) formation protocol. 100% of the EBs showed the presence of neural cells. Many EBs also showed the presence of RPE-like cells, which were recognized based on their cobblestone-like morphology, the formation of tight junctions, and the presence of pigmented granules. These cells were observed from day 7 onwards and were present along the neural cells during the course of differentiation. RPE cells generated from the differentiation of the D3 ESC line were characterized via immunocytochemical analysis of the expression of RPE lineage-associated markers such as CHX10, MITF, CRALBP, BEST1, and ZO1. CHX10 was used as a marker for retinal progenitors, and positive nuclear staining was detected in a few cells derived from the D3 ESC line. MITF, CRALBP, BEST1, and ZO1 served as the markers for RPE cells and were observed in the D3 ESC-derived RPE cells. In the KSR medium, the efficiency of RPE generation was observed to be low. However, in the present study, the EBs were kept in the culture for one month. It is envisaged that long-term cultures can produce RPE cells in large numbers. Also, by using suitable

growth factors, the differentiation can be redirected to generate a large number of RPE cells. Overall, this model system has the potential to generate RPE cells from ESCs for in vitro and in vivo studies.

B072/P1827

Hemidesmosomes regulate epidermal differentiation during embryogenesis

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Epidermal stratification is critical to the skin's function as an environmental barrier and is achieved through careful regulation of basal keratinocyte differentiation. Basal epidermal progenitors adhere to the underlying basement membrane through adhesion complexes like the hemidesmosome, which links the keratin cytoskeleton to the basement membrane through integrin-α6β4 heterodimers. It has been proposed that high expression of integrins is a key component of basal residency, and thus progenitor capacity. However, there is limited evidence that integrins directly contribute to basal identity or play a role in differentiation programs. To address this, we use Lentiviral-mediated Ultrasound Guided Gene-Inactivation and Gene-Expression (LUGGIGE) to generate epidermal-specific, mosaic loss of hemidesmosome adhesion via knockdown of integrin-β4 and its ligand, laminin-α3β3γ2. Importantly, this approach includes a fluorescent H2B-RFP reporter, allowing us to monitor basal cell clonal behavior throughout stratification. We find that Itgb4 and Lama3 knockdown cells are biased towards a differentiative fate, while Scramble controls display equipotent clonal expansion. To determine how hemidesmosome loss biases progenitor cells towards differentiation, we evaluated the two differentiative mechanisms: 1) asymmetric cell division, where one daughter cell is displaced into the differentiated layer following mitosis, and 2) delamination, where basal keratinocytes lose contact with the basement membrane and upregulate a differentiative identity independent of division. Our results indicate that reduced basal adhesion predominantly drives differentiation by promoting delamination, with only mild effects on oriented cell divisions. Furthermore, whole epidermal overexpression of the pro-differentiative Notch1 pathway abrogates integrin-β4 expression, with a similar increase in delamination. These findings demonstrate that hemidesmosomes directly regulate epidermal differentiation, predominantly by promoting delamination, and suggest that canonical differentiative programs may suppress hemidesmosome adhesion as a natural part of epidermal stratification.

B058/P1186

Human Epicardial Adipose Tissue Stem/Progenitor Cells as a Novel Resource for Cardiovascular Disease Modeling in vitro

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Epicardial Adipose Tissue (EAT) is a metabolically active, visceral fat depot directly adjacent to the myocardium. Physiologically, EAT exerts cardioprotective functions, predominantly through its "brown-like" adipocyte status. During aging, this tissue undergoes a 'brown-to-white' transition associated with a pro-inflammatory phenotype. EAT directly affects cardiac functions, contributing to the pathogenesis of cardiovascular disease, plaque formation, atrial fibrillation and fibrosis. As EAT is almost absent in rodents, new models are required to study pathophysiology of this fat depot. The objective of this project is therefore to characterize human EAT samples, isolate Adipocyte stem/progenitor cells (ASCs) and differentiate them into various lineages to obtain a

reproducible source of cells for the in vitro investigation of cardiomyocyte–adipocyte interactions. EAT samples were obtained from patients who underwent cardiac surgery. Each tissue sample was divided to obtain material for histology, RNA/protein- and ASC isolation, respectively. Immunohistochemistry was performed on cryosections to analyze adipocyte morphology. Simultaneous RNA/protein isolation was done using Trizol. A reproducible protocol for isolating ASCs from the stromal-vascular fraction was established using an automated homogenizer. Stemness of ASCs was evaluated through trilineage differentiation assays and FACS surface marker analysis. A cardiomyocyte (CM) differentiation assay was also conducted. Whole-tissue staining using a specific Perilipin1 (PLIN1) antibody showed a characteristic adipocyte-specific pattern and provided the basis for in depth quantification of intracellular lipid droplets. Isolated ASCs from EAT showed strong and consistent viability and proliferation rate, and were able to reliably differentiate into adipogenic, chondrogenic, and osteogenic lineages. This trilineage differentiation was confirmed by RT-qPCR and immunofluorescence. FACS analysis revealed specific adipocyte progenitor- and stemness markers (e.g., CD34, DLK-1, CD45). Preliminary observations indicate the differentiation capability of isolated ASCs into ASC-derived CM progenitor cells, which remain under further investigation. Epicardial ASCs can successfully be isolated and differentiated with our novel, semi-automated protocol. Further validation of the stemness of the identified cell population will be achieved by single-cell sequencing to elucidate their transcriptomic profiles. Their differentiation potential towards cardiomyocytes will be explored more thoroughly with higher differentiation rates towards mature CMs to be expected. Future work will involve co-culture experiments of isogenic in vitro differentiated adipocytes and CMs.

B057/P1185

Ints7 deficiency activates DNA damage response to elicit resurgence of endogenous retrovirus MERVL and anastasis of embryonic stem cells

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The murine endogenous retrovirus MERVL is dynamically activated in a small population of in vitro cultured mouse embryonic stem cells (mESCs) exhibiting totipotent-like features. Yet, the relationship between MERVL activation and cell fate decisions of mESCs is incompletely understood. Through a genome-wide knockout screen, we discovered that MERVL activity is intrinsically linked to DNA damage response pathways. Loss of Ints7, a backbone subunit of the Integrator complex, increased DNA damage and triggered MERVL expression. Mechanistically, Ints7 depletion induced phosphorylation of Kap1, increased chromatin accessibility at MERVL loci, and activated the p53–Dux axis to drive MERVL transcription. Intriguingly, DNA damage-induced MERVL resurgence followed the cleavage of caspase-3, often accompanying a process known as anastasis—cell survival after transient apoptotic signaling. Collectively, our study uncovered that MERVL activation in mESCs is integrated into the cellular circuit for decision-making in response to DNA damage, suggesting that sublethal caspase activation can influence the developmental potential of stem cells.

B053/P1181

Mechanically regulated proteostasis by Hsp27 governs osteogenesis of mesenchymal stromal cells on soft matrices

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Matrix stiffness is a critical biophysical cue that governs mesenchymal stromal cell (MSC) fate, yet the molecular mechanisms by which soft extracellular environments impair osteogenesis remain poorly defined. Here, we established a stiffness-tunable in vitro model to dissect focal adhesion (FA)-mediated mechanotransduction and identified the small heat shock protein Hsp27 as a mechanosensitive regulator enriched in FAs of MSCs cultured on soft matrices. Proteomic profiling revealed that soft matrices reprogram FA composition to favor chaperones and ubiquitin-proteasome-related proteins, with Hsp27 acting as a central effector. Hsp27 promoted proteasome-mediated degradation of the small GTPase Rac1, thereby attenuating lamellipodia formation, cytoskeletal tension, and osteogenic signaling. Genetic deletion or pharmacological inhibition of Hsp27 restored FA maturation, actin stress fiber formation, intracellular traction force, and osteogenic differentiation. In a murine model of aging-associated osteoporosis, we observed elevated Hsp27 expression in femoral bone tissue, accompanied by reduced collagen fiber alignment and increased marrow adiposity, hallmarks of matrix softening. These findings establish Hsp27 as a mechanically responsive regulator of FA proteostasis and identify a stiffness-sensitive degradation pathway that may be targeted to restore osteogenesis in mechanically compromised bone environments.

B045/P1173

Nucleocytoplasmic Shuttling of the Zebrafish Sox21 Orthologs

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The SOX (SRY-related HMG-box) family of transcription factors is highly conserved across metazoans and plays pivotal roles in regulating gene expression during development, stem cell maintenance, and cell fate specification in diverse species. Many SOX proteins contain multiple nuclear localization sequences (NLSs), typically consisting of two clusters of basic residues flanking both ends of the well-conserved high-mobility group (HMG) DNA-binding domain. While SOXB1 subgroup proteins (SOX1/2/3), which promote neural stem cell maintenance, have better characterized nuclear import mechanisms, those of the SOXB2 subgroup (SOX14/21) are less understood. Two closely related SOXB2 paralogs in Danio rerio and orthologs of human SOX21, Sox21a and Sox21b, are essential for balancing neural progenitor proliferation and differentiation. Although both orthologs contain predicted NLS regions in the N-terminus, we could not find experimental evidence in this species demonstrating that these NLS sequences are necessary or sufficient for nuclear localization. Therefore, we generated mCherry-tagged Sox21a and Sox21b truncations, either lacking the N-terminus (including the HMG domain) containing the predicted NLSs or lacking the C-terminus. We then transfected these into HeLa cells. Using both confocal microscopy and nuclear fractionation, we demonstrated that proteins without the N-terminus were cytoplasmic, while those without the C-terminus were nuclear, like the wild-type, full-length proteins. This suggests that the N-terminus is sufficient for nuclear localization, as expected. Using data from human SOX2 as a model, we extended this research by generating single, double, or triple amino acid substitutions of basic lysines and arginines to uncharged alanines within the predicted N-terminal NLSs. Confocal microscopy revealed that these particular point mutations were not sufficient to fully ablate nuclear localization, but there were subtle differences between the two paralogs, with the triple mutant of Sox21a more often cytoplasmic than that of Sox21b. This distinction may be due to the presence of additional leucine-rich nuclear export sequences in the C-terminus of Sox21a, but not Sox21b. We are continuing to investigate the role of specific sequences in the nucleocytoplasmic shuttling of these paralogs. Taken together, these findings provide mechanistic insight into zebrafish Sox21 nuclear import and how paralog-specific localization mechanisms may contribute to distinct developmental roles.

B065/P1193

Reactivation of developmental differentiation programs thwart basal cell carcinoma progression

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Basal cell carcinoma (BCC), the most common human cancer, frequently arises from multipotent hair follicle stem cells (HFSCs), which maintain hair follicle homeostasis and assist epidermal regeneration during wound healing. However, how this inherent plasticity contributes to tumorigenesis remains poorly defined. Using a conditional mouse model of BCC where Sonic hedgehog signaling (Shh) is hyperactivated in HFSCs, we observed that these stem cells undergo atypical fate decisions in a heterogenous manner. Specifically, BCC tumor cells either exclusively express the epidermal stem cell marker Keratin 14 (K14) or co-express Keratin 10 (K10), a differentiation marker normally restricted to the adult interfollicular epidermis and absent from hair follicles. Intriguingly, using in vivo lineage-tracing, we revealed that K10 expression is transiently present during embryonic hair follicle morphogenesis but is silenced postnatally. In BCC, however, this developmental program is reactivated, which we hypothesize is a tumor suppressive mechanism employed to thwart tumor progression. In support of this hypothesis, we discovered that K14/K10 dual-positive tumor cells are significantly less proliferative than K14-only tumor cells, suggesting that derepression of K10 expression in hair follicles is associated with slower tumor growth. Furthermore, acceleration of BCC tumorigenesis via deletion of tumor suppressor P53 was coincident with complete loss of K10 expression. Taken together, these important findings suggest that tumorigenic stem cells can hijack developmental and wound healing plasticity programs to slow tumor growth. Our ongoing work aims to characterize the transcriptional and molecular mechanisms that regulate stem cell fate decisions during BCC initiation and progression, with the long-term goal of exploiting this knowledge to optimize targeted skin cancer therapies.

B046/P1174

Role of Heterogeneous Nuclear Ribonucleoprotein HNRNPU in Maintenance and Differentiation of Pluripotent Stem Cells

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Embryonic Stem cells are distinguished by high pluripotent plasticity, continuous self-renewal, and the ability to generate multilineage cell populations. The perpetuation of pluripotency is intricately orchestration intrinsic gene regulatory networks, metabolic pathways, epigenetic control mechanisms, alternative splicing (AS) machinery. Reports from previous studies suggest that AS is intricately involved in cell fate decisions and embryonic development. In this study, we unveil that some heterogeneous nuclear ribonucleoproteins (hnRNPs) show high expression in pluripotent mouse embryonic stem cells (mESCs) compared to mouse embryonic fibroblasts (MEFs), as evidenced by RT-PCR analysis. We extended our investigation of hnRNP expression in human pluripotent stem cells (TJC8 hiPSC (indiced Pluripotent Stem cells) by qRT-PCR analysis which validated the elevated hnRNP expression in hiPSC cells. To find the inter-association of these hnRNPs and how they are regulated, promoter regions of these hnRNPs were analyzed by in silico approach. We performed PCR validation which revealed their elevated indegenous expression in mESCs as well as hiPSCs . Literature reviews illuminated among the hnRNPs, hnRNPU's pivotal role in important biological process including cranial and cardiac development. To decipher its regulatory significance in stem cell biology, we explored lentiviral-mediated dual-guide RNA CRISPR-Cas9 technology to generate hnRNPU- knockout human

iPSC (icas9) lines. Subsequent molecular analyses demonstrated alterations in pluripotency markers (Oct4, Sox2, C-Myc), muscle progenitor markers (Myogenin, Myf5), and apoptotic gene expression. Future directions aim to unravel the mechanistic intricacies of hnRNPU's influence on pluripotency, lineage commitment, and apoptotic pathways. Through RNA sequencing, organoid model generation, apoptosis assays, splice junction arrays, and overexpression studies, we are moving forward to delineate the molecular underpinnings governing hnRNPU's regulatory landscape in stem cell fate decisions

B062/P1190

Somatic ring canals contribute to the regulation of proliferation and differentiation in the ;Drosophila ovarian follicle stem cell lineage

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In adult stem cell lineages, proliferation and differentiation must be tightly regulated so that new cells are specified at the correct time and place. This process is made more robust through the integration of signal transduction cascades with multiple cell biological inputs, such as metabolic state and mechanical forces. In some cases, cells can go through an alternative form of mitosis in which ingression of the cytokinetic furrow arrests prior to abscission, leaving the sister cells connected by stable intercellular bridges called ring canals. These structures have been well studied in the male and female germline and have also been identified in some somatic tissues, including the Drosophila ovarian follicle epithelium. Follicle cells, similar to germline cells, have been shown to facilitate the intercellular transfer of cytoplasmic contents, although these molecules are smaller in size. Building off of these prior studies, we are investigating how the formation of ring canals and proteins regulating abscission function as a cell biological input into the regulation of proliferation and differentiation in the early follicle stem cell lineage. Consistent with recent studies of germ cell ring canals, our findings suggest that follicle cell ring canals form through a stepwise remodeling of a midbody that contains a kinesin-like protein, Pavarotti (Pav), to a stage in which the midbody is surrounded by a ring of a septin protein, Peanut (Pnut), to a Pav+, Pnut– ring structure, followed by the addition of mucin-like Visgun protein. To further investigate this stepwise process, we developed a computational tool for segmenting 3D confocal images of the follicle epithelium and quantified the number and position of each type of ring canal structure at different stages of the follicle cell lineage. Whereas previous studies found that approximately 89% of cell divisions in the later stages of the follicle stem cell lineage form a stable ring canal, we find that there is much less than one Pav+ ring canal per cell at the early stages. We also find that the composition of ring canals is more heterogeneous at these early stages. In addition, we identified several genes that are important for both the maintenance of proper ring canal number and the regulation of follicle cell proliferation and differentiation. Taken together, our findings identify cytokinesis and ring canal formation as significant points of regulation in the patterning of the follicle epithelium.

B069/P1197

SPRY4-Dependent ERK Negative Feedback Demarcates Functional Adult Stem Cells in the Male Mouse Germline

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Niche-derived growth factors support self-renewal of mouse spermatogonial stem and progenitor cells through ERK MAPK signaling and other pathways. At the same time, dysregulated growth factor-dependent signaling has been associated with loss of stem cell activity and aberrant differentiation. We hypothesized that growth factor signaling through the ERK MAPK pathway in spermatogonial stem cells is tightly regulated within a narrow range through distinct intracellular negative feedback regulators. Evaluation of candidate extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK)-responsive genes known to dampen downstream signaling revealed robust induction of specific negative feedback regulators, including Spry4, in cultured mouse spermatogonial stem cells in response to glial cell line-derived neurotrophic factor or fibroblast growth factor 2. Undifferentiated spermatogonia in vivo exhibited high levels of Spry4 mRNA. Quantitative single-cell analysis of ERK MAPK signaling in spermatogonial stem cell cultures revealed both dynamic signaling patterns in response to growth factors and disruption of such effects when Spry4 was ablated, due to dysregulation of ERK MAPK downstream of RAS. Whereas negative feedback regulator expression decreased during differentiation, loss of Spry4 shifted cell fate toward early differentiation with concomitant loss of stem cell activity. Finally, a mouse Spry4 reporter line revealed that the adult spermatogonial stem cell population in vivo is demarcated by strong Spry4 promoter activity. Collectively, our data suggest that negative feedback-dependent regulation of ERK MAPK is critical for preservation of spermatogonial stem cell fate within the mammalian testis.

B054/P1182
Stepwise Differentiation of Tonsil-Derived Mesenchymal Stem Cells
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Diabetes mellitus (DM) is a chronic metabolic disorder caused by insufficient insulin secretion or impaired insulin action, leading to severe complications. Current therapies, including exogenous insulin, do not fully recapitulate physiological insulin secretion and carry risks of hypoglycemia. Stem cell–based therapies offer a promising alternative by restoring β-cell mass and endogenous insulin production. Mesenchymal stem cells (MSCs) are a potential source due to their ability to differentiate into endodermal lineages, including pancreatic β-cells. Here, we optimized β-cell differentiation of human tonsil-derived MSCs (TMSCs) by comparative evaluation of two signaling-based protocols. TMSCs (passages 5–7) were differentiated in 2D and 3D cultures: Protocol I induced direct differentiation without sequential stages, whereas Protocol II employed a stepwise approach recapitulating embryonic pancreatic development. In 2D culture, Protocol II maintained typical morphology and higher confluency, while Protocol I produced thinner, less confluent cells. Protocol II effectively induced DE differentiation, increasing SOX17, FOXA2, and CXCR4 expression. Both protocols upregulated PP markers (HNF6, NGN3) and PCs markers (Insulin, NKX6.1, PDX1), but Protocol II consistently achieved higher PCs marker expression. In 3D spheroids, Insulin, PDX1, and MafA levels further increased, with Protocol II showing superior expression compared with Protocol I. These results indicate that stepwise, developmentally guided differentiation more effectively generates pancreatic β-cells from TMSCs, highlighting the importance of stage-specific signaling and their potential for stem cell–based diabetes therapy.

B055/P1183
Suppression of Human Antigen R Enhances Myogenic Differentiation of Human Tonsil-derived Mesenchymal

Stem Cells

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Human antigen R (HuR) is a representative RNA-binding protein (RBP) that regulates the stability, translation, and subcellular localization of target mRNAs in diverse cellular processes. In skeletal myogenesis, HuR has been reported to translocate from the nucleus to the cytoplasm, where it stabilizes myogenic mRNAs and promotes the expression of differentiation markers in murine myoblasts and goat skeletal muscle satellite cells. However, its role in mesenchymal stem cell (MSC)-based myogenesis remains unclear. In this study, we investigated the function of HuR during myogenic differentiation using human tonsil-derived MSCs (TMSCs). Knockdown of HuR by small interfering RNA (siRNA) accelerated myogenic differentiation, as evidenced by the transient upregulation of early myogenic regulators—including paired box gene 3 (Pax3), myogenic factor 5 (Myf5), myogenic differentiation 1 (MyoD), and myogenin—at day 3 of differentiation. Moreover, HuR suppression further increased the expression of late myogenic markers, such as dystrophin and skeletal muscle actin, upon completion of differentiation. Interestingly, in contrast to previous reports, immunocytochemistry revealed increased nuclear accumulation of HuR during TMSC-derived myogenesis. Together, these findings demonstrate that HuR functions as a negative regulator of early myogenic gene expression in TMSCs and that its nuclear accumulation represents a distinct regulatory feature compared with other myogenic systems. Our results identify HuR as a previously unrecognized post-transcriptional regulator in MSC-based myogenesis and suggest that its modulation may provide a potential strategy to enhance stem cell–mediated muscle regeneration.

B050/P1178

The CPEB ortholog Orb2 regulates brain size through the TRIM-NHL RNA-binding protein, Brain tumor

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Neural stem cells (NSCs) undergo asymmetric cell division to generate a self-renewing stem cell and a daughter cell fated for differentiation. Impaired NSC self-renewal, proliferation, and differentiation underlie neurodevelopmental disorders like microcephaly, a congenital defect with small head circumference and severe intellectual disability. We identified cytoplasmic polyadenylation element-binding (CPEB) motifs within microcephaly-associated genes and examined the role of the CPEB2-4 ortholog, Orb2, in Drosophila neurodevelopment. While the role of Orb2 in glutaminergic mature neurons in learning and memory is well defined, less is known about its role in development. Unexpectedly, we identified Orb2 as a dose-dependent regulator of brain size, where orb2 loss causes brain hypotrophy and overexpression induces overgrowth. To pinpoint the cells of origin, we employed a tissue-specific depletion strategy. We find Orb2 is required in NSCs, neurons, and optic lobe neuroepithelial cells to protect against microcephaly. We further show that orb2 is required in both neurogenic embryos and larvae to determine larval brain size through a mechanism associated with reduced NSC number and volume. Structure-function analyses indicate Orb2's RNA-binding and translational activation activities promote brain growth, while the poly-Q and ZZ domains limit brain size. Another RNA-binding protein that regulates brain size is the tumor suppressor and translational repressor Brain tumor (Brat). In contrast to orb2 mutants, loss of brat is associated with massive brain overgrowth. We therefore tested whether orb2 and brat genetically interact to maintain balanced brain growth. We identify conserved CPE sites within the brat 3'UTR and show that Orb2 regulates Brat levels in larval brains. Moreover, our data indicate that orb2 is epistatic to brat in the regulation of brain size. Moreover, introduction of a brat transgene is sufficient to rescue orb2-dependent microcephaly, as well as NSC loss and cell size defects. Taken together, our data supports a model whereby interaction between these two conserved RNA-binding proteins is essential for the precise regulation of neural progenitor specification and differentiation throughout

neurodevelopment and important for brain size determination. Our ongoing work will provide further insight into the neurodevelopmental underpinnings of microcephaly.

B061/P1189

The Loss of Emerin Function Disrupts Transcriptional Programming, Chromatin Accessibility, and Genomic Organization

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Emery-Dreifuss muscular dystrophy 1 (EDMD1) is caused by mutations in the EMD gene encoding for integral inner nuclear membrane protein emerin. Emerin is known to regulate nuclear structure and genomic organization, but the precise mechanisms by which EDMD1 mutations affect myogenic differentiation remain poorly defined. To address this, we have performed a multi-omics analysis of differentiating myogenic progenitors lacking emerin, expressing wildtype emerin, or expressing EDMD1 emerin mutations. RNA-sequencing was performed each day for four days of differentiation, which is when mature myotubes form. This analysis revealed widespread disruptions in transcriptional programs during differentiation of emerin-null or EDMD1 mutant myogenic progenitors. In particular, the pathways disrupted in emerin-null or EDMD1 mutant cell lines were related to myogenic commitment and cell cycle withdrawal. We expect these deficiencies arise from dysfunctional genomic reorganization, which is a requirement for myogenic differentiation. To investigate chromatin organization, we performed ChIP-seq to identify lamina associated domain (LAD) composition and organization and ATAC-seq to examine whether changes in genomic organization impacted chromatin accessibility to lead to altered transcriptional programing. Integration of these datasets is expected to reveal disruptions in both chromatin accessibility and LAD dynamics in critical genomic regions important for myogenic differentiation or cell cycle control in both emerin-null and EDMD1 mutant lines. Together, our findings define the transcriptional and genomic organization deficiencies that occur when emerin function is lost, resulting in the inability to coordinate myogenic gene regulatory pathways.

B068/P1196

The role of nuclear myosin 1 in cell fate decision during mouse brain development

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Cell fate decisions are governed by complex developmental processes that rely on dynamic remodeling of chromatin architecture and precise regulation of gene expression programs. In the mammalian nucleus, nuclear myosin 1 (NM1)—an isoform of the Myosin 1c gene—functions as a global transcriptional regulator. Mechanistically, NM1 promotes transcription by binding to the B-WICH chromatin remodeler at DNA promoters and recruiting histone acetyltransferases and methyltransferases, thereby facilitating chromatin opening. Through this mechanism, NM1 regulates gene programs central to cell metabolism and differentiation, including hematopoiesis and adipogenesis. Despite these established roles, the function of NM1 in neuronal development remains unknown. Here we show that NM1 expression peaks between embryonic day 18.5 (E18.5) and postnatal day 0 (P0) in the mouse brain, compared to earlier stages and the adult cerebral cortex. Bulk RNA-seq of wild-type (WT) and NM1 knockout (KO) mouse brains at E10.5 and P0 reveal dysregulation of gene programs related to the vascular system, extracellular matrix, and glial cells. In the absence of NM1, these alterations are associated with the disruption of adult (P120) brain functions, affecting

genes involved in synapse organization, behavior, extracellular matrix, and the development of multiple neural cell types. Single-cell transcriptomics of WT and NM1 KO mice at P0 further demonstrate an increase in vascular cells and a tendency toward glial cell expansion in the absence of NM1. Vascular cells are essential for supplying nutrients that support embryonic neurogenesis, and, in adulthood, they interact with glial cells to maintain a proper neuronal environment. Impairments of vascular integrity and the blood-brain barrier are linked to neurodevelopmental disorders and behavioral dysfunctions. Consistent with this, preliminary behavioral analyses of NM1 KO mice indicate potential motor dysfunctions. Collectively, these findings suggest a crucial role for NM1 in mouse brain development and raise important questions of whether its role during development indirectly contributes to its involvement in central nervous system aging and degeneration.

B060/P1188

Trisomy 12 amplifies proliferative pathways in human iPSCs through dosage-sensitive gene upregulation

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Aneuploidy, the gain or loss of whole chromosomes or chromosome arms, disrupts gene dosage balance and is common in cancer and stem cell genome instability. Trisomy 12 is one of the most frequent chromosomal gains, detected in hematological & germ cells malignancies as well as in cultured human induced pluripotent stem cells (iPSCs). Prior work from our laboratory showed that during culture trisomy 12 iPSCs emerge simultaneously in multiple cells through chromosome bridging of the short p arm of chromosome 12 (chr12). Unlike most trisomies, chr12 trisomic iPSCs gain a proliferation advantage allowing them to persist and eventually dominate. However, the molecular mechanisms by which trisomy 12 provides a proliferative advantage are not understood. We aimed to identify key dosage-sensitive genes and pathways responsible for the growth advantage of trisomy 12, using isogenic iPSCs as a model system. We conducted bulk RNA sequencing and quantitative mass spectrometry-based proteomics on fully disomic and trisomic chr12 iPSCs. Transcriptomic profiling revealed 932 chr12 coding genes in disomic and trisomic iPSCs, with 245 significantly differentially expressed in trisomic chr12 iPSCs when compared to their disomic counterparts (adjusted $p < 0.05$; $|\log_2 FC| > 0.585$). Proteomic analysis identified 218 chr12 encoded proteins, with 11 significantly upregulated ($p < 0.05$, $|\log_2 FC| > 0.585$). Pathway enrichment using Ingenuity Pathway Analysis (IPA), KEGG, and GO highlighted strong activation of Ras, MAPK, Rap1, cAMP, and calcium signaling networks- pathways that regulate cell proliferation, stemness, and survival. Among the candidate drivers, genes such as WNT5B, SLCO1A2, OAS1, NANOG, PXN, CD63, SLC2A3, GDF3, SOX5 emerged as consistently upregulated. We are now developing a CRISPR-Cas9 based functional screen to directly evaluate the contributions of these candidate genes to gain proliferative advantages. In conclusion, our integrative multi-omics analysis reveals that trisomy 12 amplifies dosage of proliferation-associated genes and signaling pathways in trisomic chr12 iPSCs. Ongoing CRISPR-Cas9 screening will establish causal links between gene dosage and growth advantage. Ultimately, this study will help identify therapeutic targets to counter trisomy 12 driven growth in malignancies and enhance the safety for use of iPSCs in regenerative medicine.

Disease Mechanisms and Therapeutics - Cardiovascular and Metabolic Diseases

B367/P1491

A Novel miR-10b-5p/MKI67/CXCL8 Signaling Axis Governs Endothelial Inflammation in Kawasaki Disease

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Kawasaki disease (KD) is a systemic inflammatory disorder of unknown etiology, thought to be triggered by an aberrant immune response to infectious agents. It is characterized by vasculitis, particularly of the coronary arteries, which can lead to life-threatening aneurysm formation. To investigate the molecular mechanisms of KD-associated vasculitis, we performed whole RNA sequencing on samples from KD patients and control subjects with other infectious diseases. This analysis identified 13 microRNAs (miRNAs) that were significantly upregulated in KD patients. We then validated these findings in a murine model of KD using Lactobacillus casei cell wall extract (LCWE). Among the candidate miRNAs, we identified miR-10b-5p as a key regulator; its inhibition with an antagomir significantly reduced aortic valve inflammation in the LCWE model. To delineate the downstream targets of miR-10b-5p, we conducted single-cell RNA sequencing on human coronary artery endothelial cells (HCAECs). This revealed that miR-10b-5p treatment induced a phenotypic shift in HCAECs from a proliferative to a pro-inflammatory state, which was accompanied by the downregulation of the proliferation marker MKI67 (Ki-67). Bulk RNA sequencing confirmed the downregulation of MKI67 in response to miR-10b-5p. Functionally, siRNA-mediated knockdown of MKI67 in HCAECs resulted in increased expression of the key pro-inflammatory chemokine CXCL8. Taken together, our findings reveal a novel miR-10b-5p/MKI67/CXCL8 signaling axis that drives vascular inflammation and represents a potential therapeutic target for managing Kawasaki disease.

B368/P1492

Aberrant phosphorylation downstream of IPP2 initiates arrhythmia through RyR2 signaling

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Cardiac arrhythmias, disorders of the heart's electrical activity, impair ventricular conduction and compromise systemic perfusion. These disturbances cause irregular rhythms that increase mortality and may injure distant organs through embolic events. To enable targeted therapy, a clear understanding of their molecular drivers is essential. Here we identify a link between cardiac overexpression of IPP2 (PPP1R2) under the Myh6 promoter (TgIPP2) and age dependent arrhythmias. TgIPP2 mice exhibit a spectrum of conduction and rhythm abnormalities, including axis deviation, prolonged QRS and PR intervals, absent P waves, atrioventricular dissociation, ventricular tachyarrhythmias, and tall T waves. In young adults, low dose catecholamine challenge (0.2 mg/kg isoproterenol) with surface ECG monitoring provoked more frequent and longer arrhythmic episodes in TgIPP2 mice than in controls. Phosphorylation profiling revealed aberrant phosphorylation of a key ryanodine receptor (RyR2) regulator that governs sarcoplasmic reticulum Ca^{2+} release, which correlated with delayed T wave peaking. Therapeutically, the RyR blocker dantrolene normalized aberrant rhythms in aged TgIPP2 mice, whereas beta blockers or L type Ca^{2+} channel blockers precipitated sudden death. Together these findings indicate that IPP2 driven phosphorylation defects disrupt Ca^{2+} homeostasis and trigger arrhythmias. In conclusion, IPP2 overexpression directly promotes arrhythmia, affecting the AV node, atria, and ventricles, and implicates maladaptive RyR2 regulatory phosphorylation as a targetable mechanism for antiarrhythmic therapy.

B366/P1490

Age-induced expression of the stress response protein REDD1 protects against cardiomyocyte hypertrophy and fibrosis

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Introduction: Progressive fibrosis and myocardial hypertrophy contribute to an increased incidence of heart failure in the elderly. The current study was designed to investigate a role for the stress response protein REDD1 (Regulated in Development and DNA Damage Response 1) in age-related cardiac remodeling. REDD1 is an important stress response protein involved in cellular energetics and homeostatic mechanisms, yet little is known regarding its potential role in heart disease. Methods: Hearts were collected from young (6 months) and aged (12 months) wild-type (REDD1+/+) and REDD1 knockout (REDD1-/-) mice. Heart weights were measured and normalized to body weight. Fixed heart tissue was paraffin embedded, sectioned (5µm), and visualized. Cardiomyocyte cross-sectional area was quantified in H&E-stained sections using ImageJ. Fibrosis was evaluated in heart sections using Masson's trichrome staining. REDD1, collagen I, and the mesenchymal marker α-smooth muscle actin (αSMA) were evaluated in cardiac sections by immunofluorescence. Results: REDD1 protein abundance was increased in the hearts of aged mice as compared to young controls. Mortality was increased in aged REDD1-/- mice as compared to REDD1+/+ controls. Heart weights and cardiomyocyte cross-sectional area were also increased in aged REDD1-/- mice as compared to aged REDD1+/+ mice. Markers of cardiac fibrosis, including collagen I and αSMA expression, were increased in the hearts of aged REDD1-/- mice, as compared to controls.Conclusions: The findings support that an age-related increase in REDD1 acts to moderate the development of cardiac remodeling. Increased cardiac fibrosis and hypertrophy in REDD1-deficient mice supports a model in which REDD1 helps preserve myocardial structure during aging. The findings support the importance of future work to investigate REDD1-dependent signaling events as a potential therapeutic targets for age-related cardiac dysfunction.

B369/P1493

Cardiac-Specific Deletion of Fragile X-Related Protein 1 Induces Dilated Cardiomyopathy Through Impaired Contractile Regulation

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Fragile X-Related protein 1 (FXR1) is an RNA-binding protein essential for post-transcriptional regulation of gene expression in cardiac muscle, and its dysregulation has been linked to dilated cardiomyopathy (DCM), a disorder characterized by ventricular dilation and impaired systolic function. Although FXR1 is recognized as a key regulator of cardiac homeostasis, the molecular mechanisms by which it contributes to DCM pathogenesis remain poorly understood. To define the role of FXR1 in regulating cardiac muscle contractility and its contribution to DCM, we employed molecular, cellular, and in vivo approaches. RNA immunoprecipitation assays identified contractility-associated mRNAs as FXR1 targets, supporting a direct role in regulating the expression of proteins critical for cardiac muscle contraction. To further study disease progression, we examined the relationship between FXR1 levels, expression of its downstream targets, and muscle dysfunction using RT-qPCR and western blot analysis and tested whether restoration of FXR1 or its targets could improve contractility in cardiac-specific Fxr1 knockout (cKO) mice. Functional assessments demonstrated that loss of FXR1 in mice resulted in impaired cardiac performance, as evident from reduced ejection fraction and increased ventricular dilation by echocardiography. Consistent with these findings, isolated cardiomyocytes from Fxr1 KO hearts exhibited reduced sarcomere shortening, force production, and contraction velocity, underscoring the requirement of FXR1 for normal contractile function. Together, these results establish that FXR1 regulates cardiac muscle contraction by controlling the expression and translational efficiency of mRNAs

encoding essential contractile proteins, and its loss disrupts sarcomere organization, alters target expression, and accelerates DCM pathology. These findings provide new mechanistic insight into how RNA-binding proteins control cardiac physiology and highlight FXR1 as a potential therapeutic target for the treatment of DCM and related forms of heart failure.

B359/P1483

Cytomegalovirus exacerbates atherosclerosis through viperin-mediated inflammation

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Object Atherosclerosis, a chronic inflammatory disorder, is a leading cause of mortality worldwide. While classical risk factors are well recognized, accumulating evidence indicates that viral infections may also accelerate disease progression. Cytomegalovirus (CMV), a widespread pathogen, has been implicated in promoting atherosclerosis. However, the underlying mechanisms remain to be fully elucidated. It has been reported that viperin, an interferon-inducible protein with diverse biological functions, has proviral activity in HCMV infection, modulates inflammatory responses, and, importantly, is expressed in atherosclerotic lesion. This study aimed to determine whether viperin mediates CMV-driven atherogenesis and contributes to vascular inflammation. Method Apolipoprotein E-knockout (ApoE KO) mice are a well-established model for studying atherogenesis. In this study, we used ApoE KO and ApoE/Viperin double knockout (DKO) mice. These mice were fed a Western diet and infected intraperitoneally with murine CMV (MCMV) for 12 weeks. After the infection period, atherosclerotic lesions were quantified in Oil Red O-stained aortas, and inflammatory cytokine mRNA expression in aortic tissue was assessed Result ApoE KO mice exhibited a significantly increased atherosclerotic plaque burden compared to their DKO counterparts. Additionally, the aortic tissue from ApoE KO mice showed increased expression of inflammatory cytokine mRNAs relative to those from DKO mice. Conclusion These findings suggest that CMV exacerbates atherosclerosis through viperin-mediated inflammatory responses, supporting a role for viperin in CMV-driven vascular inflammation. Further investigations are underway to delineate the molecular pathways governing the viperin-CMV interaction.

B362/P1486

Dietary fiber and fructose remodel the gut microbiota to alter glucose tolerance in a lipid-dependent manner

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The human diet is complex, and combinations of dietary components shape susceptibility to metabolic disease. Yet, physiological effects of individual nutrients are typically studied in isolation. Here, we investigated how combinations of dietary fiber and high-fructose corn syrup (HFCS) differentially affect glycemic control. Through the intraperitoneal and oral glucose tolerance tests, we found that low fiber and HFCS exerted additive effects to impair glucose tolerance, and these differences were suppressed upon depletion of the gut microbiota with an antibiotic cocktail. Using microbial metagenomics in combination with untargeted metabolomics, we identified Dubosiella newyorkensis, a recently identified murine commensal, as selectively

enriched in mice consuming a diet low in fiber and high in HFCS and associated with lipid metabolic signatures that correlated with glucose intolerance. Monocolonization of germ-free mice with D. newyorkensis was sufficient to promote glucose intolerance. Remarkably, administration of conditioned medium from cultures of D. newyorkensis to specific-pathogen free (SPF) mice impaired glucose metabolism, an effect that was blocked by lipid depletion. Furthermore, we found that a human-colonizing species taxonomically related to D. newyorkensis, Clostridium innocuum, recapitulated glucose intolerance in murine models after monocolonization. Human datasets corroborate the link between low fiber, C. innocuum expansion, and diabetic risk. Our findings establish that combinatorial effects of specific dietary nutrients remodel the gut microbiota in a manner that regulates glucose tolerance through effects on bacterial lipid metabolism, providing potential targets for therapeutic intervention.

B361/P1485

Effect of high glucose on oxidative stress-related gene expression in HUVECs, a model of the human vascular endothelium.

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Endothelial dysfunction is the pathogenic basis of vascular complications in diabetes and is modulated by factors such as oxidative stress, caused by the overproduction of reactive oxygen species associated with high extracellular glucose concentrations. Although therapies exist that reduce the risk of developing vascular complications, their protective effect is limited, as they don’t reverse pre-established endothelial damage. This highlights the importance of further understanding the molecular pathways involved in this process to develop more effective therapeutic strategies. In this context, the objective of this project is to evaluate the in vitro effect of sustained high glucose concentrations on the expression of genes related to oxidative stress in human umbilical vein endothelial cells (HUVECs). As part of the project’s progress, several oxidative stress markers were quantified using colorimetric methods, including glutathione production via the Ellman reagent and nitrite concentration using the Griess assay, using HUVECs cultured in Dulbecco’s Modified Eagle Medium at glucose concentrations of 5.5 mM, 10 mM, and 35 mM for 24 hours. Results showed significant differences in glutathione production between 5.5 mM and 35 mM ($p \leq 0.05$), whereas no significant differences were observed in nitrite quantification between 5.5 mM and 10 mM. Furthermore, to select key genes for future gene expression analysis, 122 protein-coding genes associated with diabetes, hyperglycemia, endothelium, and oxidative stress response were identified. From this dataset, a protein–protein interaction network was constructed using the STRING v12.0 platform, generating 117 nodes and 335 edges with a confidence score of 0.9. Using the CytoHubba tool and integrating information from the literature, the genes GSR, PRDX1, NOS3, NQO1, NFE2L2, and CAT were selected as key regulators of oxidative stress under hyperglycemic conditions in endothelial cells. In conclusion, elevated glucose didn't significantly alter nitrite production but decreased glutathione levels in a concentration-dependent manner, suggesting an impact on the cellular redox balance during the first 24 hours of exposure. Additionally, the selection of the guide genes GSR, PRDX1, NOS3, NQO1, NFE2L2, and CAT could provide crucial insights into how glucose concentration affects, at the molecular level, the main metabolic pathways related to oxidative stress in the endothelium, thereby justifying future investigations of their gene expression as part of the ongoing project.

B364/P1488

ESCRT III-Driven Repair of Nuclear Rupture Reverses Transcriptional Arrest in Lamin-Related Cardiomyopathy

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Loss-of-function mutations in the nuclear lamina protein Lamin A/C (LMNA) cause heart arrhythmia, chamber dilation, and fatal cardiac arrest in LMNA-related cardiomyopathy. A lack of understanding of its pathogenesis has hampered the development of treatment strategies. Previously, we generated a mouse model of this disease with inducible, cardiomyocyte-specific Lmna knockout (LmnaCKO). Nearly half of all cardiomyocytes in LmnaCKO hearts developed localized rupture of the nuclear envelope before any other deficits, suggesting that nuclear rupture is a disease-initiating event (En et al., 2024; PMID: 38814785). Here, we report that the ESCRT-III membrane remodeling complex repairs nuclear ruptures, restores rupture-associated transcriptional defects, and counteracts disease progression in LmnaCKO mice. We found that LmnaCKO cardiomyocytes lost RNA Polymerase II (Pol II) from ruptured nuclei and globally downregulated transcriptional activity. However, nearly half of ruptured nuclei eventually restored nuclear compartmentalization, indicating endogenous repair. Repaired nuclei resumed transcriptional initiation but did not fully restore transcriptional elongation, likely due to frequent re-rupture. We found that ESCRT-III complex proteins accumulated at nuclear envelope rupture sites and that this accumulation was driven by BANF1 binding to the rupture sites. Inhibiting the ESCRT-III ATPase VPS4 increased rupture frequency, reduced repair, and accelerated progression of cardiomyopathy in LmnaCKO mice, suggesting that endogenous rupture repair counteracts disease progression. A heart from an LMNA-DCM patient similarly exhibited frequent nuclear envelope rupture and its association with BANF1, suggesting rupture repair activity in this disease. Collectively, our data implicate rupture-associated transcriptional arrest in the pathogenesis of LMNA-related cardiomyopathy and suggest that enhancing rupture repair may be a therapeutic strategy. More broadly, this study sheds light on the etiology of other conditions associated with nuclear envelope rupture, including myocardial infarction, neurodegenerative disorders, cancer, and senescence.

B356/P1480

Functional Characterisation of Genetic Variants Affecting Adipocyte Maturation and Insulin Activity in SGBS Cells

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Insulin resistance (IR) is a pathological condition where cells are unable to respond to insulin, resulting in elevated blood glucose levels. It increases the risk of developing cardiometabolic diseases such as type 2 diabetes, a significantly prevalent disease as the global population gradually becomes more obese. Triangulation of three hallmark traits of IR (higher fasting insulin, higher triglycerides, and lower HDL cholesterol) identified 282 loci with joint genome-wide significant association implicated in dysfunctional peripheral adipose storage capacity and adipocyte function. Using several methods of gene prioritisation and differential expression quantitative trait locus in the adipose tissue, we selected LAMB1, TNFAIP8 and TWEAK for functional characterisation in SGBS preadipocytes. LAMB1 is an essential subunit of laminin and TNFAIP8 and TWEAK are part of the TNFα superfamily, with contrasting anti- and pro-apoptotic roles, respectively. We hypothesise that the LAMB1 locus impairs adipocyte expansion due to its function as an extracellular matrix attachment protein, leading to ectopic storage of excess lipids. Similarly, we postulate that TNFAIP8 and TWEAK have complementary outcomes in adipocyte function through their inflammatory response. We

observed that knockdown (KD) of TNFAIP8 significantly reduced the ability of SGBS cells to differentiate, demonstrating an elongated cell morphology early during differentiation that does not undergo lipid accumulation despite being viable. TWEAK KD accelerates differentiation with lipid droplet formation during the early commitment stage. Mature cells exhibit significantly larger and more lipid droplets and increased insulin-stimulated glucose uptake. Inflammatory cytokines in mature cells were reduced in TWEAK KD, whilst levels were elevated in TNFAIP8 KD, suggesting that the inflammatory response is preventing proper development of adipocyte function. We will further validate our hypothesis with a double KD to determine the interaction between both genes for SGBS differentiation, with a focus on lipid accumulation, as the loci were implicated in adipose storage capacity. LAMB1 KD shows a similar morphology to TWEAK KD cells, with lipid accumulation early during differentiation. Glucose uptake is increased in LAMB1 KD before differentiation initiation, but not during differentiation. Our current study provides insights into three novel IR loci and their associated genes to understand the underlying mechanisms that lead to the development of IR.

B357/P1481

Hydroxycitrate, a Caloric Restriction Mimetic, Prevents Alcohol-Induced Microtubule and Organelle Motility Defects

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Caloric restriction has emerged as a promising therapeutic strategy for mitigating various metabolic diseases, including alcohol-associated liver disease. This study investigates hydroxycitrate, a caloric restriction mimetic, as a novel therapeutic intervention for cellular dysfunctions. This mimetic is a competitive inhibitor of ATP citrate lyase, an enzyme that converts citrate to oxaloacetate and acetyl-CoA. Because acetyltransferases transfer the acetyl group from acetyl-CoA to the ε-amino group of lysines, its inhibition is predicted to decrease cellular acetyl-CoA levels, thereby promoting global protein deacetylation. Decreased acetyl-CoA levels have also been linked to decreased lipid biosynthesis, suggesting that hydroxycitrate also has the potential to prevent alcohol-induced fatty liver disease at a cellular level. For these studies, we used polarized, hepatic WIF-B cells. These cells exhibit endogenous alcohol metabolism, mimicking liver in situ. Cells were treated with hydroxycitrate in the absence or presence of alcohol to determine its capacity to prevent selected alcohol-induced hepatic cellular alterations. We determined that hydroxycitrate treatment prevented alcohol-induced tubulin acetylation in a dose-dependent manner, consistent with its proposed function as an acetyltransferase antagonist. It also prevented alcohol-induced peroxisome proliferation and alcohol-induced oxidative stress (as observed by decreased ROS (reactive oxygen species) accumulation), suggesting it might additionally function as an anti-antioxidant at the cellular level. Furthermore, when alcohol-treated WIF-B cells were additionally treated with oleic acid (to mimic the Western diet), the addition of hydroxycitrate ameliorated lipid accumulation as observed by decreased numbers of lipid droplets of reduced size, consistent with its proposed function in reducing cellular fat. We further determined that hydroxycitrate addition prevented known alcohol-induced defects in microtubule plus-end and minus-end directed protein trafficking due to tubulin acetylation. Finally, live cell imaging revealed that hydroxycitrate prevented alcohol-induced defects in microtubule-based organelle motility. Together, these results reveal that hydroxycitrate affects several key cellular processes impacted by alcohol, including protein acetylation, organelle dynamics, and cytoskeletal function. These findings suggest that hydroxycitrate, used alone or with aldehyde scavengers, could be a potential supplemental therapy with cellular therapeutic effects in preventing and/or attenuating alcohol-induced hepatic cellular injury.

B358/P1482

Meclizine rescues cardiac function and mitochondrial ultrastructure by ATP- and glycolysis-independent mechanisms in a genetic model of mitochondrial energy dysfunction

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Primary mitochondrial cardiomyopathies are an unmet clinical challenge, as there are no therapies that directly address the underlying mitochondrial dysfunction. We previously reported that the cardiomyocyte-specific deletion of the mitochondrial phosphate carrier (SLC25A3), which imports phosphate required for ATP synthesis, produces a model of mitochondrial cardiomyopathy in which total cardiac ATP levels are preserved despite defective mitochondrial ATP production. This was accompanied by increased glycolytic activity and reduced mitochondrial flux, leading us to hypothesize that pharmacologically enhancing glycolysis might be protective when the mitochondrial energy machinery is intrinsically impaired. To test this, we turned to meclizine, an FDA-approved antihistamine previously shown to shift metabolism toward glycolysis. Chronic meclizine treatment in SLC25A3-deficient mice attenuated cardiac hypertrophy, improved systolic function, and restored mitochondrial ultrastructure. Unexpectedly, meclizine suppressed glycolytic enzyme expression and reduced lactate accumulation, suggesting that meclizine does not induce a glycolytic shift in SLC25A3-deleted hearts. Instead, proteomic and functional analyses revealed preservation of mitochondrial cristae architecture via MICOS upregulation and improved NAD+/NADH homeostasis through uncoupled electron flux and NAD+regeneration. Together, these findings identify meclizine as a clinically approved compound that promotes cardioprotection in mitochondrial disease not by driving glycolysis, but by preserving mitochondrial membrane organization and redox balance, highlighting mitochondrial quality and NAD+ redox homeostasis as therapeutic targets for primary mitochondrial cardiomyopathies.

B371/P1495

Nuclear envelope rupture in cardiomyocytes orchestrates early transcriptomic changes and immune activation in LMNA-DCM that are reversed by LINC complex disruption2 in Spermatogenesis and Oogenesis

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Mutations in the LMNA gene, encoding nuclear envelope proteins lamin A/C, cause a spectrum of diseases known as laminopathies, frequently affecting striated muscle. Among these laminopathies, LMNA-related dilated cardiomyopathy (LMNA-DCM) is the most prevalent and severe disease. Despite evidence of nuclear defects, DNA damage, and p53 activation in LMNA-DCM tissues, the causality and timing of these events remain unclear, limiting therapeutic development. Although preclinical models have targeted altered signaling via pharmacological and genetic interventions, their limited success, often modest and non-curative, may reflect a lack of mechanistic understanding of the disease pathogenesis, as most studies focus on late-stage disease when severe phenotypes are already present and irreversible. To identify early molecular drivers of LMNA-DCM, we developed a tamoxifen-inducible, cardiomyocyte-specific Lmna knockout mouse model (Lmna-cKO) and performed multi-level transcriptomic analyses at early (11-days post injection, dpi) and late (25-dpi) disease stages. This model enables distinction between primary effects of lamin A/C loss and secondary consequences of tissue deterioration and overall declining health. Lmna-cKO mice exhibited progressive fibrosis and cardiac enlargement, mirroring LMNA-DCM progression. Bulk RNA-seq identified emerging differentially expressed genes (eDEGs), whose disease-contribution intensified over time, and which enriched

for inflammation, innate immunity, as well as cytoplasmic DNA sensing signaling pathways. Single-nucleus RNA-seq revealed that eDEG expression was largely driven by disease-specific cardiomyocyte subpopulations (CM3 and CM5). Our observation of nuclear envelope (NE) rupture in Lmna-cKO cardiomyocytes, coupled with activation of cytoplasmic DNA sensing pathways, suggests a mechanistic link to widespread transcriptional dysregulation. Despite normal lamin A/C levels in non-myocytes, cell-cell communication analysis and spatial mapping revealed aberrant interactions between the CM3/CM5 cardiomyocyte subpopulations, pro-fibrotic fibroblasts, and infiltrating immune cells. Cardiomyocyte-specific disruption of the LINC complex, which transmits cytoskeletal forces to the nucleus, substantially reduced NE ruptures, normalized expression of over half of the dysregulated genes, and dramatically improved cardiac function and extended survival from 3-5 weeks to over 1 year in Lmna cKO mice. These findings implicate mechanical nuclear fragility in Lmna-deficient cardiomyocytes as a central driver of LMNA-DCM, whose downstream activation of cytosolic DNA sensing pathways initiates a cascade of maladaptive cell-cell communication, leading to fibrosis and inflammation.

B360/P1484

Pathogenic Mutations in Leiomodin 2 Trigger Nonsense-Mediated mRNA Decay and Protein Loss, Which Can Be Suppressed by Steric-Blocking Oligonucleotides.

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Leiomodin 2 (Lmod2), a cardiac-predominant actin-binding protein, is essential for thin filament assembly and normal heart function. Loss-of-function mutations in LMOD2 cause severe neonatal dilated cardiomyopathy. Although most pathogenic alleles are predicted to encode truncated proteins, little to no mutant LMOD2 protein is detected in patient tissue. We found that nonsense-mediated mRNA decay (NMD), which degrades transcripts carrying premature termination codons, underlies loss of mutant protein for multiple LMOD2 disease-causing mutations. To restore protein expression, we developed steric-blocking oligonucleotides that interfere with exon junction complex deposition, thereby suppressing NMD and stabilizing mutant LMOD2 mRNA. Functional studies demonstrated that expression of a disease-relevant truncation (Lmod2[W405*]) in Lmod2 knockout mice attenuates progression of cardiac pathology, indicating that the truncated protein retains partial activity. However, homozygous Lmod2[W405*] mice eventually develop dilated cardiomyopathy and impaired contractility, establishing that Lmod2[W405*] is not sufficient to support normal cardiac function. Together, these findings identify NMD as the primary mechanism driving loss of mutant LMOD2 protein, provide evidence that antisense oligonucleotides can restore mutant LMOD2 protein expression, and highlight that even partially functional LMOD2 variants may confer therapeutic benefit in LMOD2-associated cardiomyopathy.

B363/P1487

Regulation of post transcriptional expressions adaptation by tRNA-derived RNA fragments (tRFs) in metabolic dysfunction associated steatotic liver diseases

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Despite emerging evidence implicating transfer RNA-derived fragments (tRFs) in metabolic dysfunction-associated steatotic liver disease (MASLD), their cellular role in disease progression remains poorly understood. These small non-coding RNAs may drive post-transcriptional dysregulation by altering codon usage bias or reshaping the tRNAome, potentially influencing key pathways in MASLD progression. To address this we focussed a simplified qPCR-based workflow for systematic tRFs profiling with disease severity and analyzed the expression patterns of PDGFB/GATA4 and FUNDC2 across histopathology graded MASLD stages to identify potential regulatory networks involving tRFs. 116 patients were recruited at the All India Institute of Medical Sciences, New Delhi, after obtaining institutional ethical clearance in accordance with Helsinki guidelines. The cohort comprised four patient groups: 36 MASLD, 25 diabetes (T2DM), 30 metabolic syndrome and diabetes Mets +T2DM and 25 metabolic syndrome (MetS). Liver biopsy and plasma samples were collected exclusively in the MASLD group, while the other groups (T2DM, MetS + T2DM, and MetS) including healthy subject (n=25) were evaluated using plasma samples only. Differentially expressed miRNA targets were predicted using RNAhybrid, which computes minimum free energy for miRNA-target binding, including non-canonical sites. High-quality small RNA-seq reads (17-40nt) were adapter-trimmed and perfectly aligned to tRNA references. tDRmapper classified fragments by cleavage position (5′/3′/internal tRFs; tRNA halves), quantifying counts and mapping locations. This stringent pipeline ensures accurate detection of tRFs while minimizing false positives through perfect-match alignment. Our findings reveal that the most significantly upregulated tRFs during cirrhosis and hepatocellular carcinoma progression are associated with interactions involving PDGFB and FUNDC2. Such advancements may reduce the need for liver transplantation, which currently accounts for 8–9% of global liver transplants. Our integrated approach reveals tRFs as key regulators in MASLD-to-HCC progression, modulating PDGFB/FUNDC2 and GATA4 pathways. The optimized qPCR and tDRmapper pipeline (17-40nt, Bowtie-aligned) robustly quantifies tRFs, while RNAhybrid and GO analysis decode miRNA-mRNA/lncRNA networks. Using this novel methodology, we observed that tRFs significantly reprogram the hepatic tRFome, potentially enabling the identification of novel fragments that could serve as therapeutic targets for MASLD. These advancements may offer substantial diagnostic and therapeutic possibilities that could ultimately decrease the necessity for liver transplantation in advanced MASLD cases.

B370/P1494

The Effects of Coffee Fruit Extract and Its Bioactive Components on Fat Metabolism in Caenorhabditis elegans

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Coffee fruit dry extract (CFE) is a standardized powdered extract derived from the whole Coffea arabica fruit, containing approximately 40% phenolic acids, such as chlorogenic acid (CGA), caffeic acid (CA), and gallic acid (GA). These natural compounds are known for their role in lipid metabolism, with potential health benefits or therapeutic applications. Obesity, a major public health challenge, is characterized by excess fat accumulation and unbalanced lipid metabolism, making the study of natural compounds with lipid-modulating properties highly relevant. This study aims to analyze the influence of CFE and its bioactive components on fat accumulation in Caenorhabditis elegans, a widely used genetic model for investigating metabolic processes and pathways. L1-stage nematodes were treated for two days at 20 °C with 0.03–0.06% CFE, CGA, CA, GA, or 0.4% DMSO (vehicle control). The coffee fruit extract reduced fat accumulation, likely due to gallic acid or its combination with other phenolic acids. Since the treatments did not impact the growth rate of nematodes, the reduction in fat was not due to decreased growth. In fact, 0.03% CGA increased the nematode length by 61 µm, while 0.06% GA reduced length by only 40 µm. RNA sequencing was performed on CFE-treated nematodes and compared to the control. CFE significantly regulated 75 genes, 67 of which were upregulated and 8 were downregulated compared to the control. The differentially expressed genes were clustered into gene ontology (GO) terms. The most significant GO terms were chitin biosynthetic process and the negative

regulation of endopeptidase activity. Among the genes regulated by CFE were chs-2, gmeb-2, mlt-10, nas-6, nas-14, nep-1, phg-1, and pqn-67. These genes are associated with the nematode's pharyngeal pumping and pharynx development, which may be linked to food intake. Altogether, this study suggests that CFE and some of its bioactive components, such as GA, regulate fat storage likely by modulating food intake in C. elegans. These findings offer potential applications for obesity-related metabolic research.

B365/P1489
Transcriptomic Changes in the Colon of Juvenile Pigs with Diet-Induced Metabolic Dysfunction-Associated Liver Disease
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Background: Metabolic dysfunction-associated liver disease (MASLD) is linked to an increased risk of colon pathologies, with an unbalanced diet being recognized as one of the critical factors affecting the gut-liver axis. We have previously established a pediatric animal model of MASLD by feeding juvenile Iberian pigs a diet high in fat and fructose for 10 weeks. The aim of this work was to study the initial molecular imbalance in the colon of diet-induced juvenile MASLD pig model and determine whether these changes are associated with colonic mucosal thickening. Methods: Eighteen 15-day-old Iberian pigs were assigned to receive for 10 weeks either a standard diet (SD) or a Western diet (WD). Colon tissue was collected for immunohistochemistry, transcriptomics, and protein expression analyses. Functional enrichment analyses were performed on differentially expressed genes to identify Gene Ontology (GO) terms using the Database for Annotation, Visualization, and Integrated Discovery. Data were analyzed by multivariate and univariate statistics. Results: All WD-fed pigs developed MASLD. Immunohistochemistry of colon tissue showed increased crypt depth, increased total number of cells per crypt, and increased number of proliferating Ki67+ cells per crypt in WD compared with SD ($P \leq 0.05$). Cellular component GO terms associated with focal adhesion - essential for maintaining epithelial barrier integrity, and for external side of plasma membrane – linked to immune defense and interaction with the gut microbiota, were downregulated in WD-fed pigs ($P \leq 0.05$), whereas basolateral plasma membrane was increased in WD compared to SD ($P \leq 0.05$). Biological processes GO terms associated with cell-matrix adhesion, collagen fibril organization, and extracellular matrix were downregulated in WD-fed pigs ($P \leq 0.05$). In addition, biological processes GO terms associated with translation and immune response were downregulated in WD-fed animals ($P \leq 0.05$) suggesting a suppression of protein synthesis and immune activity. There were several other significantly modulated pathways including calcium-mediated signaling, cholesterol metabolic process, ion homeostasis, and synaptic structure. In conclusion, Western diet led to colonic mucosal thickening in MASLD pigs and dysregulated gene expression pathways governing epithelial integrity, extracellular matrix remodeling, and immune signaling, all pathways which promote colon tumorigenesis.

Disease Mechanisms and Therapeutics - Cell and Gene Therapy

B429/P1553
Increased adiponectin by placental stem cells triggers AdipoR/PPARa axis activation in ovarian metabolic disorders
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Adiponectin is a protein secreted by fat cells. It plays a crucial role in regulating blood sugar levels by improving insulin resistance and promoting glucose and lipid metabolism. Especially, their receptors, AdipoR1/R2, are known to promote fatty acid oxidation and participate in glucose uptake. Abnormal excess lipid accumulation in the ovaries causes metabolic syndrome such as polycystic ovarian syndrome (PCOS), which interferes with steroid production and causes ovarian dysfunction. While current treatments, such as hormone replacement therapy, have limited efficacy and potential risks, there has been increasing interest in developing PCOS treatment based on mesenchymal stem cells (MSCs). This study investigated whether placenta-derived mesenchymal stem cells (PD-MSCs) could restore ovarian function by modulating adiponectin signaling in a thioacetamide (TAA)-induced rat model with ovarian lipid metabolic disorder. PD-MSCs (2×10^6 cells) were intravenously transplanted, and ovarian tissues were analyzed four weeks later Following PD-MSC transplantation (Tx), we assessed the expression of markers for lipid metabolism, follicular development, and steroidogenesis. Our results showed that PD-MSC Tx significantly improved metabolic parameters, including insulin sensitivity and lipid profiles, otherwise, reduced both the size and number of ovarian lipid droplets in ovarian tissues. PD-MSC Tx also upregulated the expression of adiponectin, PPARα, and CPT1a, enhancing lipolysis and fatty acid oxidation. Interestingly, balanced lipid metabolism by PD-MSC Tx induce to increase the number of primordial follicles accompanied by elevated expression of steroidogenic markers such as StAR, CYP19A1, and ERα. Additionally, we observed decreased serum levels of LH and the LH/FSH ratio in ovarian dysfunction rat model with PD-MSC Tx, whereas increased serum levels of AMH and E2. In vitro assays, TAA treatment increased the number of lipid droplets in granulosa and theca cells, but increased lipid droplets were significantly decreased when co-cultured with PD-MSCs ($p<0.05$). Notably, siRNA knockdown of adipoR1 and adipoR2 abolished this effect, confirming the essential role of the adiponectin–AdipoR1/R2–PPARα axis in metabolic recovery. These findings suggest that PD-MSCs alleviate metabolic stress and support ovarian function restoration via adiponectin/ AdipoR1/R2 axis, offering a promising therapeutic approach for lipid metabolic diseases including PCOS.

B430/P1554

Utilization of cell-penetrating peptide adaptors to enhance delivery of charged protein cargos

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Cell-penetrating peptides (CPPs) are promising tools for delivering therapeutic proteins and biomolecules (“cargos”) into cells. However, most CPP-linked cargos are retained in endosomes and trafficked back to the cell surface or targeted for degradation rather than reaching the cytoplasm or other subcellular destination as intended. To improve endosomal escape, our group developed CPP-adaptors in which a CPP is fused to calmodulin (CaM) rather than the cargo molecule. The prototype adaptor, TAT-CaM, consists of the CPP moiety of HIV transactivator of transcription fused to human calmodulin. Cargos are linked to a CaM binding site (CBS), allowing for noncovalent, reversible, Ca2+-dependent coupling to adaptors. During internalization and endosomal transport, CBS-cargos dissociate from adaptors and more readily escape endosomes than covalently fused CPP-cargos, facilitating cytoplasmic delivery, efficiently bypassing endosomal entrapment. Our study focused on the effect of intrinsic cargo charge on internalization. We investigated whether supercharged GFP-based adaptors could increase the internalization of variably charged GFP-cargoes, hypothesizing that a more positive charge would enhance internalization. We found that our CPP adaptors are capable of internalizing variously charged cargoes. We observed that higher concentrations (200-400nM) of the adaptor can induce a maximum saturation point. The saturation effect is charge-dependent at lower concentrations. Ultimately, we concluded that at higher concentrations, supercharged adaptors modestly increased cargo

internalization. This finding led us to use lower concentrations (25-100 nM) to better observe how highly charged adaptors affect cargo internalization. At lower concentrations ranging from 25 to 100nM, we found that the supercharged adaptors significantly increased the internalization of cargos with various charges.

Disease Mechanisms and Therapeutics - Cellular and Molecular Biomarkers

B351/P1475
Aromatase Interacting Partner in Breast (AIPB) at the MAM Increased Expression with a Reduction in Metabolic Activity by Pyrvinium Pamoate
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Endoplasmic reticulum and mitochondria associated-ER membrane (MAM) proteins play a crucial role in metabolizing enzymes that are associated in one compartment but finally localized in a different compartment as a final translocation. The metabolic activity depends on their specific state either at the intermediate or final state of translocation. The protein can be fully active in the intermediate state, where it is not completely folded. Aromatase Interacting Partner in Breast (AIPB) is a newly identified, 22-kDa, protein localized at the mitochondria associated-ER membrane. AIPB is responsible for the synthesis of estradiol in breast tissue. During the development of breast cancer, steroid hormone estradiol is significantly increased. While specific therapies have been developed to treat hormonally regulated breast cancers including Estrogen (Er+), Progesterone (Pr+) and Human Epidermal Growth Factor Receptor (Her2-), there are currently no definitive treatment modalities for Triple-negative breast cancer (TNBC). Estradiol is catalyzed by aromatase from testosterone, but this key enzyme is absent in TNBC cells, suggesting that estradiol in TNBC is synthesized by a different mechanism. Therefore, we determined the mechanism of anthelminthic drugs including Pyrvinium Pamoate (PvR), as a possible tool to determine regulation of AIPB expression and its metabolic capacity to synthesize estradiol. We have developed MDA-MB-231-AIPB stable cells under the control of tetracycline (Tet) promoter uniformly expressing AIPB upon induction with tetracycline. These cells have a doubling time of 22 hours. We hypothesize that PvR might interact with AIPB and the catalysis of estradiol synthesis. Therefore, we induced cells with a 50,000-fold concentration (1.0 ng - 50,000 ng) PvR for 48 hours and sequentially analyzed activity change by measuring estradiol synthesis. We observed the PvR concentration range had no toxicity with cell growth for 96 hours. Immunoblotting of the PvR induced cells increased AIPB expression, which resulted in decreased estradiol synthesis by approximately 3-4-fold. We also observed that AIPB-PvR complex is present in the MAM fraction, suggesting the estradiol catalytic activity is likely associated with AIPB at the MAM region. In conclusion, PvR could be stabilizing AIPB’s expression associated at the MAM for further change in translocation and therefore reducing estradiol levels.

B352/P1476
Effects of Developmental Alcohol and Nicotine E-Cigarette Co-Exposure on Physiological Measures
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Alcohol and nicotine are the most widely used licit drugs during pregnancy, and while the risks of prenatal alcohol exposure are well established, nicotine use has shifted with the rapid increase in e-cigarette consumption. E-cigarettes can elevate systemic drug concentrations relative to traditional delivery methods, raising concern given the frequent co-use of alcohol and nicotine among pregnant individuals. Despite this, the developmental and physiological consequences of such co-exposure remain poorly characterized. This study

employed mouse and cell culture models to evaluate whether combined alcohol and e-cigarette nicotine exposure alters hepatic function. Neonatal C57BL/6J mice and their dams were exposed once daily to vaporized alcohol (2.5 h at 1% EtOH, air flow 10 L/min) and/or e-cigarette nicotine (0.5 h at 18 mg/mL, seven 6-s puffs) from postnatal days (PD) 4–9, a developmental window equivalent to the human third trimester brain growth spurt. Alcohol exposure resulted in blood alcohol concentrations of ~200 mg/dL in dams, mimicking a moderate binge dose commonly reported in pregnant individuals. At PD40, offspring displayed no significant differences in serum triglyceride levels, serum amyloid A, TNF-α expression in liver tissue, or alcohol dehydrogenase protein levels compared to air or vehicle controls and single-drug groups, suggesting that acute hepatic alterations and inflammation may dissipate by adolescence. In contrast, Hepa 1-6 mouse hepatoma cells exhibited a concentration-dependent increase in metabolic activity following acute nicotine exposure, with effects observed in both nicotine-alone and co-exposure conditions, but not from ethanol exposure alone. Overall, these findings indicate that while developmental alcohol and nicotine co-exposure via e-cigarettes may not yield lasting hepatic changes detectable by adolescence, nicotine produces acute effects on liver cell metabolism, highlighting the importance of nicotine exposure and teratogenic risk.

B350/P1474

Evidence for a cryptic red cell alloantigen showing intra-substrain variability in sprague-dawley rats

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Background: Blood group antigens shape host–pathogen interactions, immune clearance, and erythrocyte turnover. In Sprague–Dawley (SD) rats, red blood cell (RBC) antigen systems are poorly defined, creating hidden variables for transfusion, inflammation, and infection studies. We aimed to characterize intra-substrain variability of RBC antigens in SD rats and to define biochemical and immunologic features of an uncharacterized alloantigen. Methods: Forty-one male SD rats underwent sequential plasma–RBC crossmatching by gel card agglutination. Based on reactivity, animals were stratified as antigen-positive, antibody-positive (naturally occurring hemagglutinins), or double-negative. Antigen-negative rats were alloimmunized by repeated intraperitoneal injections of antigen-positive RBCs. Induced antisera were evaluated by direct antiglobulin test, indirect immunofluorescence, and flow cytometry. Antigen dependence was probed with trypsin and neuraminidase treatment, periodate oxidation, and lipid extraction. For molecular identification, immunoprecipitation was followed by LC–MS/MS. Results: Crossmatching resolved three phenotypes within the same substrain, and alloimmunization of incompatible pairs generated RBC-specific antisera that restored agglutination and yielded surface-localized staining. Lipid extraction had no effect on reactivity. In contrast, trypsin treatment and periodate oxidation reduced or eliminated binding, whereas neuraminidase or oxidation sometimes unmasked reactivity in samples previously testing negative. These patterns are consistent with a glycoprotein epitope whose accessibility depends on sialic acid and/or neighboring protein complexes. Ex vivo incubation led to gradual signal loss, indicating lability of the determinant. Conclusions: SD rats harbor intra-substrain antigenic heterogeneity that includes a putative glycoprotein alloantigen with regulated surface accessibility (antigen unmasking). Alloimmunization yields diagnostic antisera suitable for functional typing. Recognizing cryptic antigen variability is necessary for experimental design and interpretation in preclinical models; next steps are to define prevalence across broader SD populations and identify the cognate molecule.

B354/P1478

Investigating the role of S1PR1-3 in the process of post-traumatic angiogenesis

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Angiogenesis is a multistep, tightly regulated process characterized by the growth of new capillaries that is mediated by angiogenic factors, extracellular matrix components, and endothelial cells. Angiogenesis is essential during tissue repair, which is highly dependent on adequate blood supply and on functional and temporally regulated angiogenesis. However, a better understanding of post-traumatic angiogenesis during tissue repair is still needed. Sphingosine 1-phosphate (S1P) is derived from cell membrane sphingolipids and binds to G protein-coupled receptors (S1PR) to regulate a wide range of critical biological processes, including fibrosis. COX-2, a key enzyme in prostaglandin biosynthesis, attenuates fibrosis, regulates angiogenesis, and is upregulated after injury. We hypothesized that the S1P-COX-2 signaling plays a role during angiogenesis after injury. First, we investigated the expression of angiogenic genes using an in vitro injury model consisting of human bone-marrow mesenchymal stem cells (BM-MSCs) treated with transforming growth factor beta 1 (TGFβ) for 24 h. Gene expression analysis for the angiogenic genes ANGPTL2, FGF2, VEGFA, and COX2 was performed by q-RT-PCR. GAPDH was used as a housekeeping gene. Significance was calculated by a two-tailed Student's t-test. BM-MSCs treated with TGFβ demonstrated a trend towards the upregulation of ANGPTL2 (Control: 1.0 + 0.0; TGFβ: 1.5 + 0.3; p=0.17), FGF2 (Control: 1.0 + 0.0; TGFβ: 1.8 + 1.2; p=0.41), VEGFA (Control: 1.0 + 0.0; TGFβ: 2.5 + 1.2; p=0.15), and COX2 (Control: 1.1 + 0.1; TGFβ: 3.1 + 2.3; p=0.26). Following these results, we investigated whether knockdown of S1PR1-3 in human BM-MSCs, followed by treatment with TGFβ for 24 h, would have any effects on the expression of the angiogenic markers. S1PR1-3 knockdowns (S1PR1-KD, S1PR2-KD or S1PR3-KD) were confirmed by q-RT-PCR and compared to a scramble control. Following S1PR1-3-KD, a trend towards the upregulation of ANGPTL2 (Control + TGFβ: 1.2 + 0.4; S1PR1-KD + TGFβ: 2.3 + 1.6; p=0.28; S1PR2-KD + TGFβ: 2.3 + 0.1; p=0.24; S1PR3-KD + TGFβ: 2.2 + 1.4; p=0.25), FGF2 (Control + TGFβ: 1.0 + 0.0; S1PR1-KD + TGFβ: 1.6 + 0.9; p=0.36; S1PR2-KD + TGFβ: 3.4 + 1.7; p=0.29; S1PR3-KD + TGFβ: 1.6 + 0.6; p=0.24), VEGFA (Control + TGFβ: 1.0 + 0.0; S1PR1-KD + TGFβ: 1.0 + 0.3; p=0.78; S1PR2-KD + TGFβ: 1.4 + 0.0; p=0.06; S1PR3-KD + TGFβ: 1.4 + 0.6; p=0.41), and COX2 (Control + TGFβ: 1.3 + 0.4; S1PR1-KD + TGFβ: 2.4 + 1.2; p=0.35; S1PR2-KD + TGFβ: 2.7 + 1.5; p=0.54; S1PR3-KD + TGFβ: 2.0 + 0.9; p=0.43) was still observed. Additional studies are ongoing to validate these results and the biological significance of these findings. Altogether, our results support the notion that our in vitro model is a useful system for post-traumatic translational studies.

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B353/P1477

Mechanically compressed airway epithelial cells release mediators that influence differential gene expression in a differentiation state dependent manner

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The airway epithelium is a protective barrier for the respiratory tract, defending the body from external insults such as allergens, injuries, and infections. When the airway is exposed to these external stimuli, airway narrowing, or bronchoconstriction, can be induced. Previous studies have found that bronchoconstriction, and the consequential mechanical compression of airway epithelial cells, results in pathophysiological responses, specifically, airway remodeling. Here, we investigate the effect of mechanical compression on the

differentiating airway epithelium and the resultant biological effects of paracrine and autocrine signaling in human bronchial epithelial (HBE) cells using an in vitro air liquid interface (ALI) culture system. We discuss the secretion of mechanosensitive mediators and their resultant effect on the differential expression of genes (DEGs) at progressive stages of differentiation. Our study demonstrates that the differentiation state of HBE cells influence their biological response to mechanical compression. First, we show that treatment of HBE cells with mechanosensitive mediators results in differential expression of inflammation, differentiation, and airway disease signature genes, and that the effect varies depending on differentiation state. Further, we demonstrate that the released mechanosensitive mediators overlap with previously identified airway disease-related cytokines. Our findings may provide further insight into a mechano-inflammatory feedback loop whereby mechanical compression of epithelial cells lead to the release of inflammatory response and disease mediators, further exacerbating disease pathology. Thus, these findings may provide a novel framework to understand the interplay between bronchoconstriction, inflammation, and airway remodeling in the progression and persistence of airway diseases such as asthma, chronic obstructive pulmonary disease (COPD), and idiopathic pulmonary fibrosis (IPF). Identification of the key biological players in this vicious mechano-inflammatory feedback loop could provide insight into new targets for therapeutic action in airway disease identification, prevention, and treatment.

Disease Mechanisms and Therapeutics - Drug Discovery and High throughput Screening

B434/P1558
Defining chemical modulators of RPE epithelial-to-mesenchymal transition in age-related macular degeneration
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With an aging population, age-related diseases and disorders are of increasing commercial and scientific interest. One area that is susceptible to dysfunction is the sensitive macula tissue within the human eye, which is responsible for detailed sight. This tissue includes cell types of photoreceptors and the cells that support them, the retinal pigment epithelia (RPE). To support photoreceptors of the eye, the RPE is highly metabolically active. Thus, through prolonged life, RPE cells are subjected to metabolic disruption, such as oxidative stress and chronic inflammation. The damage and degeneration of RPE cells are characteristic of age-related macular degeneration (AMD). The dysfunction of the RPE in AMD is characterized by the RPE undergoing a transition from an epithelial to a mesenchymal state (EMT). Previous work within AMD is limited to clinical case studies and EMT research conducted in other model systems, which have been applied to AMD. Furthermore, research to date has performed viability and protective assays to determine the effects of any chemical or target of interest within AMD. This leads to a gap in understanding the targeting of the molecular players in RPE dysfunction by EMT. This study aims to identify chemical modulators of the epithelial-to-mesenchymal and mesenchymal-to-epithelial transitions within RPE-1 cells by a high-content imaging, cell painting high-throughput screen. To establish EMT within RPE-1, treatments were performed that induced epithelial and mesenchymal states, as assessed by immunoblot detection of state-specific markers. Then, RPE-1 cells were subjected to an unbiased cell painting high-throughput screen with chemical libraries across the specific RPE-1 cell state populations. Deep learning image analysis was employed to identify features of interest, and z-score statistics were applied to morphological, texture, and intensity metrics. Selected hits are verified through further inhibition experimentation with immunoblotting and fixed fluorescent microscopy. We have established and optimized the unbiased high-content cell painting high-throughput screen, capable of

identifying chemical modulators essential for RPE-1 function and the dysregulation of EMT. This study aims to elucidate the molecular players involved in RPE-1 dysfunction through EMT, thereby aiding in the therapeutic treatment of AMD.

B431/P1555

Discovery of a Selective Cytochrome P450 4A Inhibitor for the Treatment of Metabolic Dysfunction-associated Steatotic Liver Disease liver(MASLD)

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Metabolic dysfunction-associated Steatotic liver disease (MASLD) is characterized by hepatic steatosis related with obesity, type II diabetes, or metabolic abnormalities, and involves fat accumulation, lipotoxicity, oxidative stress, and ER stress. Cytochrome P450 4A (CYP4A), a fatty acid hydroxylase producing reactive oxygen species, has emerged as a potential therapeutic target. Through in silico screening the compound C418 was identified, and derivatives C4181 (C1) and C4182 (C2) were synthesized and shown to strongly inhibit CYP4A without cytotoxicity while displaying favorable metabolic stability and permeability. In lipid-uptaked HepG2 cells, C1 and C2 enhanced glucose uptake, reduced lipid accumulation and ROS, and normalized gluconeogenesis, lipogenesis, and ER stress markers. In diet-induced T2DM mice and db/db mice, these inhibitors lowered body weight, improved glucose and insulin tolerance, reduced serum glucose and insulin, and ameliorated hepatic steatosis, triglycerides, oxidative stress, and liver injury while restoring adiponectin and regulating metabolic gene expression. In MASH models they improved serum glucose, hepatic triglycerides, fibrosis, and inflammatory and fibrotic gene expression. Human-relevant 3D liver organoids and HepaRG models further confirmed that CYP4A inhibition reduced lipid accumulation, triglycerides, and CD36 expression while enhancing glucose utilization and attenuating stress-related pathways. Transcriptomic analyses revealed broad downregulation of steatosis, diabetes, inflammation, and ER stress networks. Overall, selective CYP4A inhibitors C1 and C2 effectively alleviated MASLD features in vitro and in vivo by suppressing oxidative and ER stress, reducing lipotoxicity, and limiting hepatic inflammation and fibrosis, highlighting CYP4A inhibition as a promising therapeutic strategy for MASLD.

B435/P1559

First-in-Class Small-Molecule Modulators of the CD28-B7 Pathway: Discovery via SEC-ASMS Screening with Biophysical, Functional, and PK Validation

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Immune checkpoint blockade has transformed cancer immunotherapy, yet many patients relapse due to compensatory T-cell co-stimulatory signals. The CD28–B7 axis represents the archetypal co-stimulatory pathway and plays a key role in sustaining antitumor immunity, autoimmunity, and transplant rejection. While biologics such as CTLA4-Ig validate CD28 as a therapeutic target, no small-molecule inhibitors of CD28 have previously been reported, largely due to the shallow, protein–protein interface. Here we describe the first discovery and characterization of small molecules that directly bind and functionally inhibit CD28. A library of 6,336 compounds was screened using size-exclusion chromatography–affinity selection mass spectrometry (SEC–ASMS), yielding 52 preliminary binders and 25 reproducible hits. Follow-up triage with TRIC-based high-throughput assays and microscale thermophoresis (MST) identified three validated binders, with 5MS and 19MS demonstrating functional blockade of CD28–B7 engagement in ELISA. A catalog-based SAR expansion

yielded optimized analogs 5MS-5 and 19MS-5, which displayed low micromolar affinities (Kd = 24.1 ± 6.3 μM and 12.5 ± 3.7 μM, respectively). Both compounds inhibited CD28-driven luminescence in a cellular blockade reporter assay (IC50 = 10.3 ± 3.3 μM and 5.2 ± 1.3 μM) and suppressed IFN-γ, IL-2, TNF-α, and sCD69 release in PBMC and mucosal tissue co-cultures, while maintaining >90% T-cell viability up to 100 μM. Molecular docking and 50 ns molecular dynamics simulations revealed stable engagement of Asn107, Lys95, and Asp106 at the CD28–B7 interface, correlating with experimental potency and highlighting a restricted druggable hotspot. In vitro pharmacokinetic profiling demonstrated favorable solubility (PBS 97–>100 μM; FaSSIF 89–124 μM), moderate lipophilicity (LogD7.4 = 3.31 and 1.87), good permeability (Papp 1.5–2.1 × 10^{-6} cm/s), and high stability in simulated gastric and intestinal fluids (>85–90%). Microsomal stability was consistent across species (t½ ~90–110 min), with high plasma stability (>150 min) and strong plasma protein binding (91–98%). Off-target assays revealed no cytotoxicity in WI-38 or HS27 cells (>100 μM), no hERG liability (>50 μM), and minimal CYP inhibition at 10 μM. Collectively, these findings establish a new chemical entry point for targeting CD28, demonstrate the feasibility of drugging a co-stimulatory receptor with small molecules, and provide promising scaffolds for developing resistance-proof therapies in autoimmunity, transplantation, and cancer immunotherapy.

B437/P1561

Investigating the biology of sirtuins in Bacillus subtilis using novel inhibitors

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Sirtuins are NAD^+-dependent enzymes conserved across all domains of life and known for their roles in deacetylating and deacylating lysine residues. In Bacillus subtilis, the sirtuin SrtN exhibits multiple deacylase activities including deacetylase, lipoamidase, and deformylase functions and regulates acetyl-CoA synthetase (AcsA). AcsA is inactivated by acetylation of a conserved lysine residue when acetyl-CoA levels are high. SrtN, along with the Zn^{2+}-dependent deacetylase AcuC, reactivates AcsA by removing this acetyl group. Under low acetate conditions, both SrtN and AcuC are essential for optimal bacterial growth; loss of either result in slower growth and reduced yield. To investigate SrtN's deacylase activity further, a high-throughput homogeneous time-resolved fluorescence (HTRF) screen identified 11 potent SrtN inhibitors. Follow-up dose-response analyses using MALDI-mass spectrometry revealed two lead compounds that inhibit SrtN deacetylase activity at low micromolar concentrations (3.43 μM and 4 μM). Western blot analysis confirmed that compound A inhibits SrtN activity in vivo. Cytotoxicity testing in HEK 293 cells showed that compound A has an IC_{50} of 3.02 μM, indicating notable toxicity, whereas compound B is less toxic. Additionally, compound A inhibited the growth of B. subtilis and Staphylococcus epidermidis, and slowed growth of Acinetobacter baumannii at 30 μM. These findings suggest that SrtN inhibitors can serve as valuable tools for probing bacterial deacylation pathways and may have potential as leads for developing new antimicrobial agents targeting bacterial sirtuins.

B433/P1557

K-RAS Molecular Glue Degrader: Coopting Ubiquitin Ligases for Discovery of Molecular Glues

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Despite the launch of new inhibitors that target K-RAS and variants, it remains a major challenge for oncologists. Targeted Protein Degradation (TPD) by molecular glues overcome the ADME PK issues. Chemical space for cereblon and VHL driven TPD is overcrowded. There is a need to develop effective degraders for K-RAS. LifeSensors has leveraged its TUBE (Tandem Ubiquitin Binding Entities) platform to discover the K-RAS MGs via an in vitro biochemical ubiquitination assay. We have performed HTS screening with new E3 ligases that promote ligand dependent ubiquitination of K-RAS in vitro using microtiter plates that are embedded with poly-ubiquitin binding TUBE. The MGs are subsequently validated in mutant K-RAS cancer cell lines. Application of the TUBE mediated approaches to discover novel ligands that promote ubiquitination overcomes artifacts observed with luciferase reporter assays. The K-RAS selective MGs also downregulate MAPK pathway. The MG mediated KRAS degrader employs a novel E3 ligase that is present in tumor tissues. Demonstration of novel MG degradation mechanism for K-RAS supports application of TUBE-based discovery of MGs that coopt new ligases for broader therapeutic applications and highlights the power of molecular glues in targeted protein degradation. We will show data how poly-ubiquitin chain selective TUBE (K48 or K63 poly-ubiquitins) are used to analyze MGs mechanism of action operate via autophagy (K63) or proteasomal degradation (K48). Gross, P.H., et al., Accelerating PROTAC drug discovery: Establishing a relationship between ubiquitination and target protein degradation. Biochem Biophys Res Commun, 2022. 628: p. 68-75. Ali S.M et al., "Unraveling chain specific ubiquitination in cells using tandem ubiquitin binding entities." Scientific Reports 15, no. 1 (2025): 22961. https://doi.org/10.1038/s41598-025-07242-9

B436/P1560

ShK Toxin Diversity in Sea Anemones: A Source of Novel Therapeutic Leads

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Venoms are complex bioactive cocktails of proteins that have evolved independently in diverse animal lineages. Targets for these venoms include medically important therapeutics, biocontrol, and biomanufacturing applications. The pharmaceutical potential for venoms is tremendous and has transformed the landscape and capabilities of medicine, with over a dozen venom derived pharmaceuticals currently on the market (e.g. Wegovy and Ozempic8). Among these, neurotoxins found in venoms which regularly target ion channels, are suitable starting points to design peptide derived therapeutics to combat autoimmune disorders and multiple sclerosis. One of the best studied neurotoxins currently being used as a pharmaceutical therapeutic is the Shk toxin, which was identified in the sea anemones Stichodactyla helianthus. This and other ShK-like peptides have the potential to inhibit the voltage-gated potassium (Kv1.3) channels, serving as the inspiration for synthetic analogs currently in clinical trials for combating autoimmune diseases. Despite this pharmacological potential, the diversity, regulation, and evolutionary context of ShK-like genes across sea anemones remain poorly understood as pharmaceutical approaches have focused on this one toxin, while evolution has resulted in dozens of ShK analogs with unexplored pharmaceutical potential across sea anemones. Here, we examined transcriptomic datasets from sea anemones to identify and characterize ShK candidates. Using a series of bioinformatic pipelines incorporating Trinity, TransDecoder, MAFFT, and iCn3D, we assembled transcriptomes, annotated mature peptides, reconstructed phylogenies, and predicted three-dimensional structure of ShK analogs across this group. We identified 216 ShK-like candidates with conserved cysteine domains. Structural predictions further highlight subsets of these peptides with pharmaceutical promise, including those clustering with previously validated drug leads. Our findings expand the catalog of ShK-like peptides beyond the well-characterized S. helianthus toxin and underscore the potential of sea anemones and potentially other venomous animals as a source of novel Kv1.3 channel inhibitors. By integrating the evolutionary comparative method, with molecular and structural analyses, this work strengthens the link between toxin evolution and

therapeutic innovation, positioning sea anemone-derived peptides as an underexplored frontier for drug discovery.

B432/P1556
Validating an evolutionary platform for therapeutic discovery using diverse organisms
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At Arcadia Science, we're leveraging evolution to accelerate the discovery and testing of therapeutics by integrating target identification, organism selection, and biologics design in a single connected platform (www.arcadiascience.com/platform). It's essential to demonstrate that computationally generated hypotheses — such as protein designs or target–disease pairings — can be tested and validated across diverse biological contexts. We're addressing this need by developing scalable, informative, and biologically relevant validation strategies using diverse cell and organismal biology. As an early test case, we focus on enzyme replacement therapies for lysosomal storage disorders, due to their well-defined molecular causes and known therapeutic mechanisms. For this validation, we selected five human enzymes and paired them with multiple genetically tractable organisms: Saccharomyces cerevisiae, Schizosaccharomyces pombe, Chlamydomonas reinhardtii, and Caenorhabditis elegans, which we selected using Zoogle (zoogle.arcadiascience.com), our phylogenetics-based organism ranking tool. With high-throughput phenotyping, we identified organism-specific mutant phenotypes and established assays to assess rescue by wild-type and computationally designed protein variants. By testing design predictions in living systems, this pipeline establishes a closed loop between computational design and experimental validation. More broadly, it demonstrates how cell biology can be used to build rigorous, multi-system strategies for biological validation, enabling efficient data-driven mining of the tree of life for the design and development of effective therapeutics.

Disease Mechanisms and Therapeutics - Infectious Diseases and Host Pathogen Interaction

B374/P1498
Antibodies disrupt bacterial adhesion by ligand mimicry and allosteric interference
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A critical step in infections is the attachment of microorganisms to host cells using lectins that bind surface glycans, making lectins promising antimicrobial targets. Upon binding mannosylated glycans, FimH, an adhesin in E. coli, undergoes an allosteric transition from an inactive to an active conformation that under tension acts as a catch-bond. Distinct monoclonal antibodies that alter FimH glycan binding are available, but the mechanisms of action remain unclear. Here, we use cryoEM paired with mass spectrometry, adhesion assays, and molecular dynamics simulations to determine the structure-function relationships underlying antibody-FimH binding. Our studies show four mechanisms of action, including an antibody containing a variable-loop glycan that mimics a FimH ligand and binds directly in the binding pocket of FimH. Hypervariable loop

glycosylations occur in 15% of circulating immunoglobulin G antibodies, and while several hypotheses have been proposed to explain why they exist, none included the direct, specific engagement of the glycans with the target antigen. The antibody mAb475 presents a previously unreported, yet likely common, mechanism of interaction between antibodies and lectin domains via a glycan. Several B-cell malignancies are characterized by high-mannose or mixed type glycosylations on the hypervariable loops of Fabs produced from IgGs or B-cell receptors of these cells. Specifically, the glycosylated position on mAb475 has previously been identified on a Fab from the B-cell receptor of lymphocytes from a patient with diffuse large B-cell lymphoma. N-linked glycosylations on hypervariable loops have also been described in follicular lymphoma and Burkitt's lymphoma. Our data directly visualize the interaction between a glycosylated Fab and a bacterial lectin, supporting the hypothesis that microbial lectins may contribute to the progression of a subset of B-cell lymphomas. In addition, this work shows that high-mannose glycans on B-cell receptors may be selected during an infection by a microbe that uses mannose-binding lectins as part of its repertoire. This finding supports the hypothesis that FimH and other mannose-binding lectins trigger initial steps in the development of B-cell malignancies like diffuse large B-cell lymphomas that present with high-mannose glycans on B-cell receptors.

B377/P1501

Cellular Mechanisms of PARP Inhibition in Restricting Hepatitis B Virus Replication

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Chronic hepatitis B virus (HBV) infection is sustained by covalently closed circular DNA (cccDNA) that persists in hepatocyte nuclei. Since current nucleos(t)ide analogues cannot eliminate cccDNA, its eradication is essential for achieving a functional cure. Our previous screening with an HBV NanoLuc reporter virus (HBV-NL) identified poly (ADP-ribose) polymerase 1 (PARP1) as a host factor required in the late phase of HBV infection. As PARP1 is a key DNA repair enzyme implicated in cccDNA formation, this study aimed to identify and characterize PARP inhibitors with anti-HBV activity. Hepatitis B virus (HBV) is a hepatotropic DNA virus that chronically infects over 250 million people worldwide and can lead to cirrhosis and hepatocellular carcinoma. Chronic hepatitis B virus (HBV) infection is sustained by covalently closed circular DNA (cccDNA) that persists in hepatocyte nuclei. Since current nucleos(t)ide analogues cannot eliminate cccDNA, its eradication is essential for achieving a functional cure. Our previous screening with an HBV NanoLuc reporter virus (HBV-NL) identified poly (ADP-ribose) polymerase 1 (PARP1) as a host factor required in the late phase of HBV infection. As PARP1 is a key DNA repair enzyme implicated in cccDNA formation, this study aimed to identify and characterize PARP inhibitors with anti-HBV activity. We assessed the impact of PARP inhibitors on HBV replication in Hep38.7-Tet and HBV-infected HepG2-NTCP cells by qPCR analysis of cccDNA, RNA, and pgRNA, and by Western blotting of HBV proteins (HBc). PARP inhibitor AZD2461 was found to significantly reduce the expression levels of HBV cccDNA in infected hepatocytes, and this effect was observed without inducing measurable cytotoxicity in host cells, indicating that the compound acts specifically on viral processes rather than compromising cell viability. In addition to its impact on cccDNA, treatment with AZD2461 also resulted in a marked decrease in the levels of HBV RNA and pgRNA, suggesting that viral transcription and replication intermediates are strongly suppressed. Consistent with these observations, the expression of HBV proteins, including both structural and non-structural components, was also substantially diminished. Taken together, these findings indicate that the antiviral activity of AZD2461 extends beyond the initial establishment of cccDNA and instead interferes with downstream steps of the HBV life cycle, including RNA transcription, pregenomic RNA synthesis, and protein production. Therefore, AZD2461 appears to exert its inhibitory mechanism at a stage following the formation

of cccDNA, making it a promising candidate for therapeutic intervention aimed at limiting HBV persistence and replication.

B381/P1505

Dopamine Receptors Expression in Peripheral Blood Mononuclear Cells: Opening the Pandora's Box of Inflammation in COVID-19 Survivors?

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The coronavirus disease 2019 (COVID-19) is an inflammatory systemic condition caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Despite intense research resulting in improved management of COVID-19, mortality and long-term sequelae still occur. The dysfunctional immune response is strongly linked to disease severity and poor outcomes although its regulation and time course are largely unknown. Emerging evidence suggests that neuro-immune interactions, potentially mediated by dysregulated dopaminergic pathways, may be associated with the cytokine storm. Apart from its key role as a neurotransmitter, dopamine (DA) is synthesized by immune cells and modulates their function through DA receptors (DR D1-5). We aimed to study the expression of DR in peripheral blood mononuclear cells (PBMCs) and plasma inflammatory markers of COVID-19 survivors from 6 months till 2 years after hospital discharge. This cohort study comprises 209 adult patients who were hospitalized with SARS-CoV-2 infection confirmed by RT-PCR testing of nasopharyngeal swab samples during the typical peak periods of the pandemic: October to March 2020/2021 [cohort I; n=78; age (years) 64±9 (mean±SD)] and October 2021 to March 2023 [cohort II; n=131; age (years) 60±11 (mean±SD)]. Data collection occurred from January 2023 till March 2024 concerning peripheral blood tests that included the measurement of the expression of DRD1-5 by semiquantitative real-time PCR in PBMCs and determination of inflammatory markers. The expression of DRD1 (mean±SD) (2.97 ± 0.11) x 10-8 vs (2.68 ± 0.44) x 10-8 [t(156.45) = 7.166, p < .001, d =0.816], DRD2 (2.08 ± 0.07) x 10-8 vs (2.13 ± 0.23) x 10-8, [t(169.27) = -2.375, p = .019, d =-0.275], DRD4 (3.52 ± 0.40) x 10-6 vs (3.22 ± 0.56) x 10-6, [t(200.36) = 4.424, p < .001; d =0.582] and DRD5 (1.62 ± 0.09) x 10-7 vs (1.67 ± 0.20) x 10-7 , [t(196.33) = -2.547, p = .012; d = -0.308] were significantly different between cohorts I and II, respectively. The Neutrophil-to-Lymphocyte Ratio (NLR), the number of neutrophils and plasma level of D-dimer were lower in cohort I in comparison with cohort II. This cohort study suggests that the inflammatory process is lower in the cohort with a higher time-lapse between SARS-CoV-2 infection and study evaluation. The observed different patterns of DRD1, DRD2, DRD4, and DRD5 expression in PBMCs point to a dysregulation of dopaminergic signaling within the immune system, which may influence the chronic inflammatory state after SARS-CoV-2 infection. These findings support the concept that neuro-immune interactions, mediated through dopamine receptors pathways, are integral to the long-term immunopathology of COVID-19.

B380/P1504

Functional Characterization of SLFN11 Mouse Orthologs Reveals Broad Antiviral Activity

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The expression of Schlafen (SLFN) family proteins is regulated by type I interferons. Several SLFN proteins have demonstrated broad antiviral activity. Notably, SLFN11 restricts the replication of HIV-1 and flaviviruses such as West Nile virus (WNV). However, some viruses, including Vesicular Stomatitis virus and Rift Valley Fever virus, can evade this restriction. Since SLFN11 is not expressed in mice, its in vivo antiviral function cannot be studied using murine models of WNV infection. To overcome this limitation, we investigated the antiviral activity of Slfn8 and Slfn9, the murine orthologs of SLFN11.Here, we present data showing that both Slfn8 and Slfn9 inhibit WNV and HIV-1 in human cell-based models. The antiviral activity of these proteins' maps to their N-terminal domain. Interestingly, although Slfn8 is expressed at lower levels than Slfn9, both exhibit comparable antiviral potency, suggesting that Slfn8 may be intrinsically more effective. Furthermore, the low expression of Slfn8 was consistent across various promoters, indicating a post-transcriptional or translational regulation. Notably, this reduced expression was not observed in Slfn8 variant 2, which contains the N-terminal region followed by a unique, truncated C-terminal sequence.

B373/P1497

Hepatitis C virus modified E2 mRNA-LNP candidate vaccine promotes B cell helper CXCR5+T cells

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A comprehensive strategy to control hepatitis C virus (HCV) infection due to the significant global burden must include vaccine development for the successful prevention of liver disease. Spontaneous clearance of HCV requires strong, multiepitope-specific, and sustained T-cell immune responses from both CD4+ helper T cells and CD8+ cytotoxic T cells, along with a supportive role of B cells. However, this remains a challenge as infected humans often fail to clear HCV and progress to chronic liver disease. T-cell help for B cells is essential for a high-affinity antibody response and the development of memory B cells. T follicular helper cells (Tfh) specialize in providing cognate help to B cells and are required for the generation of T cell-dependent B cell responses. We investigated the role of the modified soluble E2 antigen (sE2F442NYT) of HCV in healthy human peripheral blood mononuclear cells (PBMCs) and separately on immune cells from immunized mice to elucidate the mechanisms underlying immune regulation. HCV parental sE2 and sE2F442NYT displayed a role in inducing T-helper 17 (Th17) cell phenotype, as indicated by interleukin-17 (IL-17) expression and signal transducer and activator of transcription 3 (Stat3) phosphorylation. The spleen cells from sE2-mRNA-lipid nanoparticles (LNP) or sE2F442NYT-mRNA-LNP immunized mice exhibited similar IL-17A mRNA level, and Th17 (CXCR3-CCR6+) cells in CD4+CD44+ spleen cells, supporting both sE2 and modified sE2F442NYT induced Th17 polarization. Immunohistochemical and multiplex immunofluorescence imaging studies revealed abundant CD4+CXCR5+T cells co-localized with BCL6 in sE2F442NYT-mRNA-LNP immunized mouse spleen cells compared to unmodified sE2-mRNA-LNP immunized animals, suggesting sE2F442NYT induces stronger Tfh generation. We previously demonstrated increased total IgG production and isotype switching from IgG1 to IgG2a and IgG2b in sE2F442NYT immunized mice. The stronger B and T-cell responses observed following sE2F442NYT immunization support the overall in vivo outcome, indicating a higher generation of B cell helper T cells compared to the unmodified sE2-mRNA-LNP. Thus, our study contributes to the rational design of HCV vaccine strategy by the selection of sE2F442NYT antigen for helper CXCR5+T cell induction.

B378/P1502

Herpes Simplex Virus Remodels Golgi and Endosomes into a Stably Associated Complex in Neuronal Cells

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Herpes simplex virus type 1 (HSV-1) is an enveloped, double-stranded DNA virus that infects epithelial cells and establishes latent infection in trigeminal ganglia, recurrently reactivating throughout life. Viral egress requires the envelopment of capsids and tegument proteins with host-derived membranes and viral glycoproteins, in the cytoplasm. During this process, HSV-1 induces extensive remodeling of host organelles. In neuronal cells, the virus forms perinuclear clusters of viral proteins and host membranes, termed the cytoplasmic viral assembly compartment (cVAC). The trans-Golgi network, early endosomes (EEs), and recycling endosomes (REs) are prominent candidate membrane sources for viral envelopment and are consistently found within the cVAC; however, the mechanisms by which HSV-1 reorganizes these compartments remain unclear. To investigate this, we first examined the association between membranes within the cVAC. Nocodazole, a microtubule-depolymerizing agent, was added to infected CAD cells at 3 hours post-infection (hpi) to disrupt retrograde transport–dependent cVAC clustering. At 12 hpi, high-resolution immunofluorescence imaging was used to observe colocalization of associated membranes. We observed that the Golgi apparatus was fragmented during infection but retained mini-stack organization, indicated by colocalization of GalT and GM130 even in dispersed puncta. We then analyzed endosomal components. Although nocodazole dispersed EEs and REs, these compartments appeared more clustered in infected cells compared to uninfected cells. Importantly, these endosomes showed significantly increased colocalization with GalT, suggesting that HSV-1 promotes stable associations between endosomes and Golgi mini-stacks within the cVAC. To determine whether endocytosis contributes to cVAC formation, dynamin-dependent internalization was inhibited using dynasore from 3 hpi. Dynasore treatment reduced single-step viral replication up to 3 logs. Nevertheless, perinuclear recruitment of fragmented Golgi and viral glycoprotein gE, a hallmark of cVAC formation, still occurred. EEs were absent from the cVAC, while REs remained detectable. These findings suggest that although cVAC formation is independent of endocytosis, its functionality as a viral envelopment compartment may be partially impaired under endocytosis inhibition. Finally, to assess the role of REs, Rab11 knockout (KO) CAD cells were generated. Viral replication in Rab11 KO cells was comparable to wild-type CAD cells, and perinuclear clustering of gE and Golgi persisted. This suggests that REs are consistently recruited to the cVAC but are dispensable for cVAC formation and productive HSV-1 replication.

B383/P1507

High resolution multiomics analysis reveals temporally-resolved host responses to flavivirus infection

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Flaviviruses, including Zika virus (ZIKV) and Dengue virus (DENV), pose a significant global health threat, causing diseases that range from mild symptoms to severe, life-threatening conditions. Upon infection, flaviviruses induce extensive intracellular remodeling that both supports and responds to viral replication. At the cellular level, infection can lead to diverse outcomes; cell death, productive replication, persistent infection, or resistance, depending on the complex interplay between the host cell environment and state, and viral determinants. Despite advances in virology, we still lack the ability to predict how individual cells within a heterogeneous population will respond to flavivirus infection. To address this gap, we employed a high temporal resolution multiomics approach to study A549 lung adenocarcinoma cells during the first 24 hours of infection. By integrating proteomics with both bulk and single-cell transcriptomic data, we identified a core host response to flavivirus infection at both the RNA and protein levels. Comparative analysis revealed an early ZIKV-specific response, characterized by the upregulation of genes related to cell adhesion, lipid metabolism,

and NF-κB signaling. Single-cell RNA sequencing allowed the classification of “infected” and “bystander” cells with specific transcriptional signatures, and uncovered a distinct subpopulation (~5%) of late infected cells exhibiting heightened expression of immune and inflammatory pathways—which we called "hyper-responders.” Ongoing work is focused on dissecting how the initial cellular state, along with virus-specific and conserved host responses to ZIKV and DENV, influences viral replication and cell state trajectories. Through the integration of multimodal molecular data and use of large language models in the A549 infection model, our goal is to build a comprehensive framework of post-infection cellular dynamics and robust pipeline for identification of gene candidates for host-directed therapeutics.

B379/P1503
Interaction of SARS-CoV-2 NSP14 and human CTRP6 in viral infection
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The limited vaccine effectiveness, need for booster doses, and suboptimal vaccine efficacy among different age groups and populations underscore the need to investigate the underlying mechanisms of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Specifically, interactions between viral and host cell proteins enable SARS-CoV-2 to hijack cellular processes for viral replication and immune evasion. Nonstructural protein 14 (NSP14) of SARS-CoV-2, with its 3'-to-5' exoribonuclease and N7-methyltransferase activities, ensures accurate viral genome replication and facilitates immune evasion. Human C1q/TNF-related protein 6 (CTRP6), a regulator of metabolism and inflammation, has been implicated in various diseases. Our preliminary findings demonstrated an interaction between NSP14 and CTRP6 in human A549 and HEK293 cells, confirmed through confocal microscopy and co-immunoprecipitation assays. Notably, higher NSP14 expression significantly suppresses translation in HEK293 cells to halt production of antiviral proteins. Intriguingly, this suppression was reversed by CTRP6 co-expression. This suggest that CTRP6 modulates NSP14 activity, and additional functional assays will further investigate how the NSP14–CTRP6 interaction regulates viral replication and COVID-19 progression, with the aim of determining a potential role of CTRP6 in viral infection and as a therapeutic target.

B382/P1506
IRTKS stabilizes Tir-mediated bacterial attachment and pedestal formation
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Enterohemorrhagic Escherichia coli (EHEC) is a foodborne pathogen that breaches the intestinal epithelium and causes frequent outbreaks of bloody diarrhea and hemolytic uremic syndrome. Once in contact with enterocyte epithelial cells, EHEC secretes Translocated Intimin Receptor (Tir), a bacterial effector, into the host cytoplasm to initiate intimate bacterial attachment and a signaling pathway for cytoskeletal host protein recruitment. EHEC then reorganizes the host’s cytoskeleton to form dynamic actin-rich structures known as “pedestals”, which facilitate bacterial cell-to-cell spread and colonization of the intestine. Several gaps remain in our understanding of EHEC’s unique infectious mechanism, particularly how Tir gets delivered to and inserted into the membrane for intimate bacterial attachment and subsequent pedestal assembly. During infection, Tir also binds to the inverse BAR (I-BAR) domain of Insulin Receptor Tyrosine Kinase Substrate (IRTKS), a host protein involved in cell membrane curvature and previously shown to play a role in EHEC

pedestal assembly. Using an imaging-based approach, we unexpectedly found that overexpression of IRTKS and specifically its I-BAR domain led to an increase in bacterial attachment and the mislocalization of Tir. We also observed the mislocalization of EspFU, another bacterial secreted effector essential for pedestal assembly, which corresponded with the disruption of pedestal formation. In addition, IRTKS knockout in both HeLa and CACO-2BBE cells resulted in decreased levels of Tir and EspFU at the host-pathogen interface. Although IRTKS has previously been shown to play a role in EHEC pedestal actin assembly downstream of bacterial attachment, our results suggest that IRTKS may also contribute to bacterial attachment and the enrichment and stabilization of Tir in the apical plasma membrane. Future work will focus on loss of function experiments and live imaging to understand the dynamics of Tir insertion into in the host cell plasma membrane preceding for bacterial attachment. Overall, this research extends our understanding of how IRTKS promotes bacterial attachment and EHEC pathogenesis.

B384/P1508
Nsp6 hijacks host insertases into viral replication centers
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β-coronaviruses evade host immune detection by replicating their genomes at replication centers (vRC) formed within the membrane of the endoplasmic reticulum (ER)1,2,3. For example, SARS-CoV-1 and CoV-2 encode three non-structural membrane proteins (Nsp 3, 4, and 6) which can remodel the ER membrane to form their vRCs4. While these Nsps are sufficient to remodel ER membranes, it is not known whether Nsps also function to recruit the host machinery required for viral replication and assembly into these vRCs. Here, we use mouse hepatitis virus (MHV) as a model system to study how β-coronaviruses assemble viral vRCs on the ER in murine hepatocytes. We discovered that the Nsp6 protein is sufficient to recruit and sequester host ER insertases into Nsp6-remodeled ER membrane domains. Canonical and prominent insertases sequestered by Nsp6 include the Sec61 translocon, ER-membrane complex (EMC), and the GEL complex. FRAP and FLIP experiments confirm that Nsp6 domains remain continuous with the ER and do not restrict membrane protein diffusion, except for those insertases that are sequestered there by Nsp6. Together these data demonstrate a dual role for Nsp6 in remodeling ER membranes and sequestering host insertase machinery away from the general ER and into vRCs.

B386/P1510
Role of listeriolysin O and phospholipases C in Listeria monocytogenes intercellular protrusion dynamics, resolution, and autophagy avoidance
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Listeria monocytogenes is a facultative intracellular bacterium that infects a large diversity of host cells and directly spreads from cell to cell to avoid the extracellular immune defenses while disseminating within infected tissues and causing a systemic infection. In this work, we characterized in real time by fluorescence microscopy the host cell plasma membrane dynamics during L. monocytogenes cell-to-cell spread. The plasma membranes of donor and recipient cells were labeled with distinct fluorochromes and 3 dimensional movies were acquired for 7 hours to monitor fluorescent L. monocytogenes and establish the role its major secreted virulence factors listeriolysin O (LLO) and phospholipases C (PLCs) at each cell-to-cell spread stage. We characterized, for the first time, the dynamics of the canonical cell-to-cell spread pathway in which intercellular

protrusions resolve into double-membrane vacuoles, followed by the successive disruption of donor and recipient membranes. We found that LLO controls protrusion resolution into a double-membrane vacuole and the disruption of both donor and recipient membranes, whereas the phospholipases C (PLCs) control donor membrane disruption and prevent bacterial entrapment into autophagosomes. Furthermore, we identified a second, more efficient “non-canonical” cell-to-cell spread pathway strictly requiring LLO/PLCs cooperation, which resolves into a single-membrane vacuole that is rapidly disrupted. This study provides the first detailed analysis of membrane dynamics within intercellular protrusions and vacuoles, establishing an advanced model for L. monocytogenes cell-to-cell spread.

B375/P1499

Role of Urolithin-A in Alleviating Mitochondrial Dysfunction and Inflammation in Sepsis-Induced ALI

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Sepsis is a major global health threat, often leading to acute lung injury (ALI) driven by inflammation and mitochondrial dysfunction. The cellular process of mitophagy, which selectively clears damaged mitochondria, is a key protective mechanism during sepsis. We investigated if Urolithin-A, a potent mitophagy activator, could serve as a therapeutic agent to alleviate sepsis-induced ALI. In LPS-stimulated macrophages, Urolithin-A treatment rescued mitochondrial health by reducing reactive oxygen species (ROS) and apoptosis. We found Urolithin-A enhanced mitophagy by upregulating key proteins like PINK1 and Parkin, supporting mitochondrial quality control. In two distinct mouse models of sepsis and ALI, Urolithin-A significantly reduced lung injury, evidenced by improved lung injury scores and decreased wet/dry weight ratios. The treatment also mitigated the inflammatory response, as shown by lower levels of inflammatory markers (iNOS, IL-1β, and MPO) and improved epithelial barrier integrity. Our findings demonstrate that Urolithin-A is a promising therapeutic candidate for sepsis-induced ALI, acting by reducing mitochondrial dysfunction, enhancing mitophagy, and modulating the inflammatory response.

B376/P1500

Surgical revision of S. aureus infection increases virulence factor expression and activates a multi-tissue inflammatory response

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This study investigates the disruption of the host-pathogen interface of a biofilm-associated orthopedic infection using surgical revision. While these types of procedures are considered essential, the cellular and molecular mechanisms by which the immune system, musculoskeletal system, and bacteria coordinate their response to this secondary injury remains unclear. To investigate this, we performed histology, bulk RNA sequencing, and a cytokine array to define the dynamic interplay between host and microbe. Our results show that immunological niches already compromised with infection – such as the bone marrow, lymph nodes, and circulating blood – further upregulate pro-inflammatory programs in response to revision surgery. Following revision surgery, bone-resorbing osteoclasts increase in number at the site of injury and lead to overall bone volume losses in infected rats. Lymph nodes expand significantly in response to revision with increases in CD3+ and CD4+ T cells, but this expansion appears to restrict lymph flow and hinders long-term T cell activation.

Circulating pro-inflammatory cytokines increase with surgical revision, and some levels remain elevated for up to 14 days, leading to increased systemic inflammation in infected rats. This enhanced inflammation at the incision site in response to revision has no effect on reducing bacteria numbers. Instead, it leads to increased expression of virulence factors, enhanced tissue remodeling, and damage. Some tissue types appear better equipped to recover from this inflammatory insult. Muscle tissue, for example, expresses significant increases in innate and adaptive immune cell transcriptional signatures including neutrophils, macrophages, T cells, and B cells 4-days post-revision, but these signatures are far less prominent 14-days post-revision. This finding corresponds with physiological improvements in muscle tissue morphology that are present on day 14. Conversely, bone appears poorly equipped to recover from the inflammatory insult of surgical revision. Revision leads primarily to increases in bone resorption and a worsening of bacterial infiltration and morphology within the bone marrow. These findings show that revision surgery negatively impacts all tissues within the surgical site, but how long these negative impacts are present is tissue specific. Understanding the timing and tissue changes associated with revision of infected orthopedic sites will help inform the design of additional interventions to minimize tissue damage and maximize bactericidal effects.

B385/P1509

Synaptopodin is required for efficient intercellular spread by bacterial pathogens.

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The virulence of many intracellular pathogens requires an ability to spread between cells of the epithelium. Here, we identify the short-chain fatty-acid butyrate promotes the spread of Shigella flexneri between epithelial cells in a manner dependent on the actin-binding protein synaptopodin. Synaptopodin is recuited to bacteria by the S. flexneri proteins IcsA and Spa15. Its recruitment is most abundant as the bacteria begin to deform the plasma membrane into structures known as protrusions, where it enhances protrusion formation and the kinetics of protrusion resolution. At the bacteria, synaptopodin stabilizes polymerized actin filaments and promotes the generation of forces that drive protrusion formation. Altogether, these data establish synaptopodin promotes efficient intercellular spread by bacterial pathogens.

B387/P1511

Vancomycin coupled with cold plasma treatment inhibits osteoclast activity in Staphylococcus aureus-infected bone

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Staphylococcus aureus (S. aureus) not only readily adheres to bone and implant surfaces, forming a biofilm, but also infiltrates the bone through canaliculi. Additionally, inflammation associated with S. aureus infection drives bone loss through osteoclast activity. This project analyzes femoral bones in a rat model of S. aureus infection, comparing no treatment, treatment with vancomycin, and a combination of vancomycin + cold plasma treatment. Cold plasma is known to be microbicidal through generation of reactive oxygen and nitrogen species, ions, electrons, heat, and UV radiation. Initially, our rat model undergoes surgical hardware implantation and infection with S. aureus (MSSA 25923). A second revision surgery is performed one week later to replace infected hardware and administer treatment. At 4- or 14-days post-revision, tissues and hardware are collected for CFU counts, microCT scans, and histology. Bacterial colonization of hardware

steadily increases after revision up to day 14 when untreated. Vancomycin or combination of vancomycin + cold plasma decreases hardware CFU at end term. Cortical bone volume (BV/TV) is decreased by infection as measured by micro-CT. Interestingly, only vancomycin without cold plasma attenuates bone loss at day 14. TRAP staining of osteoclasts shows increased numbers along the endosteum of the diaphysis in infected compared to uninfected rats. Osteoclast number and size are decreased with vancomycin + cold plasma treatment. Brown and Hopps staining shows bacterial presence in both bone and marrow with infection. The femoral bone appears ill-equipped to respond to S. aureus infiltration, as bacterial number increases drastically after revision surgery, with significantly more bacterial clumping and a higher overall infection score observed by day 4 and maintained at day 14. However, vancomycin + cold plasma significantly reduced bacteria numbers and infection score in the bone. While vancomycin treatment with and without cold plasma decreased hardware colonization, only vancomycin alone reduced osteolysis. Cold plasma, as an additional treatment with vancomycin, does not preserve bone, but may mitigate bacteria within the bone. Taken together, these findings show vancomycin treatment is effective at slowing osteolysis, but other treatments such as cold plasma could synergize to provide better clinical outcomes.

B388/P1512
YAP1 Localization Dynamics Contribute to Epstein-Barr Virus Lytic Reactivation
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Epstein-Barr virus (EBV) infects approximately 95% of the population, establishing a lifelong infection through dormancy, or latency, in B lymphocytes. This persistence contributes to the development of degenerative diseases, such as multiple sclerosis, and cancers, such as gastric carcinoma. Crucially, we lack a molecular understanding of the viral mechanisms that manipulate host pathways, impeding therapeutic options for EBV related diseases. Here, we investigated the role of Hippo pathway effectors YAP and TAZ in EBV reactivation. Previous studies have shown that YAP and TAZ, transcriptional co-activators that regulate TEAD-dependent gene expression, are important for viral reactivation. Furthermore, YAP and TAZ localization resets, characterized by depletion in the nucleus followed by an enrichment in the nucleus, allow for bursts of transcription of target genes. We hypothesize that it is these localization resets that promote expression of EBV gene BZLF1, the master switch for viral reactivation. Using live-cell time-lapse imaging of EBV-infected gastric carcinoma cells with an endogenous YAP-eGFP knock-in, we monitored expression of a BZLF1 reporter and YAP localization resets to determine whether the two events are related. Chemical regulation of YAP localization was achieved through thapsigargin and lysophosphatidic acid, which we used to shift YAP localization to the cytoplasm or nucleus, respectively. Importantly, inhibition of YAP/TAZ activity with verteporfin or siRNA significantly reduced expression of the EBV gene BZLF1. Furthermore, we observed that YAP/TAZ expression and localization dynamics are altered following viral induction, suggesting that EBV both influences and depends on YAP/TAZ signaling during reactivation. These developing conclusions reinforce YAP-mediated transcription as a critical regulatory step in EBV reactivation and a potential target for therapeutic intervention.

Disease Mechanisms and Therapeutics - Neurodegenerative disorders

B405/P1529
Alpha-Synucleinopathy Induces Neuronal Senescence In vivo and Senolytic Treatment Attenuates Alpha-Synucleinopathy.
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Aging is a universal risk factor for the major neurodegenerative diseases, including Parkinson's Disease (PD). In this regard, cellular senescence is thought to be responsible for the deleterious effects of aging and emerging as a pathological factor in neurodegenerative diseases. Because α-synuclein (αS) pathology and neurodegeneration is mechanistically linked to the pathogenesis of PD and other α-synucleinopathies, we examined the pathological relationship between α-synucleinopathy and cellular senescence. We studied a mutant αS transgenic mouse model (TgA53T) of α-synucleinopathy to determine if the disease in the model was associated with the induction of senescence. We used immunohistochemistry, RNAscope, and qRT-PCR. Our results show that α-synucleinopathy is associated with increased levels of senescence markers and signs of DNA damage response (DDR; γH2Ax, Lamin B1, HMGB1), p16Ink4A, p21Cip1, and SASP factors. Nanostring-nCounter transcriptomic analysis also show enrichment for senescence gene sets. Cellular localization of senescence markers show that multiple cell types exhibit increased p16 and/or p21, but neurons with αS aggregates are first to show increased senescence markers. Similarly, neurons with αS pathology exhibit DDR early during the process of α-synucleinopathy while the glial cells do not show any alternations until the late stage of the disease. Analysis of human PD cases show also show that DDR and senescence markers are selective increased neurons with αS pathology but not in glial cells. To determine the pathologic significance of senescence in α-synucleinopathy, TgA53T mice were treated with a senolytic cocktail [Dasatinib (12 mg/kg) and Quercetin (50 mg/kg) (D+Q); once a week]. Behavior analysis show that D+Q treatment attenuated preclinical motor and cognitive dysfunction in TgA53T mice. More importantly, D+Q treatment significantly delayed the onset of α-synucleinopathy and reduced neuropathology, including αS pathology, neuroinflammation, and neurodegeneration. D+Q treatment also reduced senescence markers in TgA53T mice. To determine if increased senescence promotes α-synucleinopathy, we used Ercc1-deficient mice to increase senescence burden in TgA53T model. We show that TgA53T/Ercc1-/Δ mice develop aS pathology sooner following aS PFF inoculation compared to TgA53T/Ercc1+/Δ mice, showing that increased senescence accelerates aS pathology. Our data show that cellular senescence is induced in neurons with α-synucleinopathy first and secondarily in glial cells. Further, targeting senescent cells using senolytics may provide neuroprotection from α-synucleinopathy.

B402/P1526

Defining the role of Matrin 3 in m^{6}A RNA modification and ALS pathogenesis

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Amyotrophic Lateral Sclerosis (ALS) is a progressive and fatal neurodegenerative disease leading to the loss of upper and lower motor neurons (MN) in the central nervous system (CNS). MN degeneration can be caused due to dysregulation of a multitude of molecular pathways, of which RNA metabolism plays a crucial role. Matrin 3 is an RNA binding protein (RBP) that regulates RNA metabolism, and its mutations have been linked to familial ALS. While Matrin 3 pathology has been observed in ALS through nuclear overabundance and cytoplasmic accumulation and aggregation, it remains ambiguous how Matrin 3 abnormalities contribute to ALS pathogenesis. We hypothesize Matrin 3 regulates m6A modification of RNA in neuronal cells, and that dysregulation can lead to pathological outcomes. N6-Methyladenosine (m6A) is a dynamic post-transcriptional RNA modification and has been implicated in ALS through either hypo- or hypermethylation of RNA. In this pathway, mRNA is methylated via the writer complex consisting of methyltransferases METTL3 and METTL14, and demethylated via erasers such as FTO or ALKBH5. Previously, other RBPs including Matrin 3 had been shown to regulate this process. Here, we aim to identify if Matrin 3 contributes to ALS pathogenesis by

regulating m6A RNA methylation in neuronal cells. This study took an in vitro approach by utilizing the MN-like cell line NSC-34. Upon knocking down the MATR3 gene with siRNA, we found that while m6A levels decreased, the methyltransferase responsible for m6A RNA methylation, METTL3, was unaffected at both the protein and transcript levels. However, upon silencing the METTL3 gene, Matrin 3 protein decreased, m6A levels decreased, but MATR3 transcript levels remained unchanged. To determine whether MATR3 mRNA was unable to be translated due to lack of m6A methylation, we performed a tagged ribosome affinity purification assay and confirmed that MATR3 mRNA was not in the translatome. Future studies will further evaluate the specific molecular mechanisms in which Matrin 3 and the m6A transcriptome interact with each other.

B390/P1514

Designed Ankyrin Repeat Proteins as tools to investigate the role of Leucine Rich Repeat Kinase 2 autoinhibition in Parkinson's Disease linked mutants

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Leucine Rich Repeat Kinase 2 (LRRK2) is one of the most commonly mutated genes that leads to Parkinson's Disease (PD). PD-linked LRRK2 mutations cause increased phosphorylation of LRRK2's Rab GTPase substrates. In the transient autoinhibited conformation, where the N-terminal domains of LRRK2 drape across the kinase domain, Rab substrate cannot access the kinase active site to be phosphorylated. Due to a lack of tools to study LRRK2 conformation in cells, little is known about the mechanism of LRRK2 autoinhibition or the effects of PD-associated mutations on conformational autoinhibition. This study uses novel Designed Ankyrin Repeat Proteins (DARPins) to investigate how LRRK2's conformation affects activation and substrate phosphorylation, and how PD-associated mutations affect autoinhibition. DARPins are small proteins selected for high binding affinity to target proteins. Here we use the DARPin C12 to test how conformation can affect LRRK2 behavior. Using cryogenic electron microscopy, we found that DARPin C12 promotes the activated conformation of LRRK2 by interfering with the autoinhibitory interaction interface. In cells, DARPin C12 promotes increased substrate phosphorylation similarly to PD associated LRRK2 mutants. This suggests that promoting the active conformation of LRRK2 results in increased substrate phosphorylation by increasing the accessibility of the kinase domain. We hypothesize that this mimics how PD linked mutations outside the kinase domain increase substrate phosphorylation. When co-expressed with kinase domain mutant Gly2019Ser LRRK2, DARPin C12 induces an increase in LRRK2 Rab phosphorylation over baseline, but Arg1441His LRRK2, a mutation outside of the kinase domain, does not show altered Rab phosphorylation in the presence of C12. This suggests that mutations in the kinase domain affect substrate phosphorylation via a different mechanism than mutations outside of the kinase domain. LRRK2 double mutants containing both mutations show additive increases in Rab phosphorylation over baseline of either individual mutant. Our work shows that DARPin C12 induces conformational changes in LRRK2 that lead to relief of autoinhibition. Our initial analysis of LRRK2 PD-linked mutations shows that our assay could be expanded to profile any PD-linked LRRK2 mutation to determine if Rab phosphorylation is increased by relief of autoinhibition.

B403/P1527

Different toxicant exposures related to Gulf War Illness converge on tau pathology

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One third of veterans who were deployed in the first Gulf War developed Gulf War Illness (GWI), a multi-symptom disease. CNS symptoms include impairments in sleep, attention/concentration, as well as learning and memory. GWI is hypothesized to arise due to toxicant exposures combined with battlefield stress. GWI animal models include a stress component (restraint or corticosterone injections) but vary significantly with regard to the toxicant regimen. We previously showed an elevation in tau (a microtubule-associated protein vulnerable in many diseases and injuries) in a cognition-relevant region of the brain as well as in human brain organoids following exposure to cortisol/corticosterone and DFP, a sarin analog. In many neurodegenerative diseases, tau first rises in the brain to produce mild cognitive impairment (MCI) and then forms pathological aggregates and filaments that lead to dementia. On this basis, we previously speculated that GWI is a prolonged early phase tauopathy. Because veterans who acquired GWI were exposed to a variety of different toxicants, we explored our hypothesis using primary cultures of rat hippocampal neuron exposed to two very different toxicant regimens. The first was based on an organophosphate, namely DFP, and resulted in reduced synapse numbers. The other was based on non-organophosphates, namely permethrin, DEET, and pyridostigmine bromide, and resulted in only subtle changes in synapses. Despite these differences, both displayed marked elevations in tau, suggesting that different GWI toxicant regimens converge on tau pathology. In a cohort of GWI veterans, tau was not only elevated in the blood but more pathologically phosphorylated (Tau 217 and Tau 181), which, together with MCI indicate potential risk for dementia. We propose that providing tau-based therapy for these veterans may help ameliorate MCI to delay or prevent dementia. We are exploring tau antisense oligonucleotides (ASOs) as potential therapeutic intervention.

B399/P1523

Effect of histone deacetylase inhibition on aggresome formation in a ß-methylamino-L-alanine-induced cytotoxicity model of Amyotrophic Lateral Sclerosis

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Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease characterized by the progressive loss of motor neurons, leading to muscle weakness, atrophy, and eventual loss of motor function. While familial ALS (fALS) arises from known genetic mutations, over 90% of ALS cases are sporadic and idiopathic. The environmental neurotoxin β-methylamino-L-alanine (BMAA) has been implicated in sporadic ALS through its formation of the BMAA-carbamate complex, an analog of glutamate, which promotes oxidative stress and protein misfolding. Histone deacetylase inhibitors (HDACis) such as Vorinostat (pan-HDACi) and Tubastatin-A (specific HDAC6 inhibitor) may provide neuroprotective benefits by enhancing protein degradation pathways and reducing cellular stress. To evaluate these HDACis, ACS-5006 neuroprogenitor cells (NPCs) and NSC-34 motor neuron-like cells were treated with varying concentrations of BMAA, Vorinostat, or Tubastatin-A, and viability was quantified using an AlamarBlue assay. Aggresome formation was assessed via fluorescence microscopy using Aggresome Detection Reagent and Hoechst 33342 nuclear stain. Integrated fluorescence intensity (IFI) was used to quantify relative aggresome accumulation. Vorinostat exhibited an IC50 of 9.0 µM and 2.6 µM and Tubastatin-A exhibited an IC50 of 49.4 µM and 10.8 µM for ACS-5006 and NSC-34 respectively. For the NPC group, BMAA treatment alone significantly increased aggresome accumulation compared to untreated controls. Co-treatment with Vorinostat reduced BMAA-induced aggresome formation ($p < 0.01$, $n = 3$), suggesting a neuroprotective effect. In contrast, Tubastatin-A did not significantly reduce aggresome formation levels and in some cases increased them compared to the control. HDAC6 regulates aggresome formation and cell viability during proteotoxic stress by binding polyubiquitinated misfolded proteins and

dynein motors for transportation along microtubules to aggresomes. Its inhibition decreases canonical aggresome formation; however, may promote compensatory aggregation via inclusion bodies or non-canonical inclusions.These findings suggest that pan-HDAC inhibitors like Vorinostat mitigate BMAA-induced proteotoxic stress, whereas selective HDAC6 inhibition alone may be insufficient. Future studies will employ flow cytometric analysis and mitochondrial ROS assays to further elucidate mechanisms of HDACi-mediated neuroprotection in BMAA-induced ALS models.

B391/P1515

Harnessing PINK1 Kinase Molecular Glues to Trigger Mitophagy in Parkinson's Therapy

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Progenra has identified a new class of small molecules that act as molecular glues for PINK1. By stabilizing and activating PINK1, these compounds enhance phosphorylation of ubiquitin and activate the parkin E3 ligase. This PINK1–parkin activation cascade drives robust mitophagy at nanomolar concentrations in both cell-based and animal models. Beyond promoting mitochondrial clearance, the PINK1 glues stimulate mitochondrial biogenesis. Multi-omics approaches—including proteomics, metabolomics, and Seahorse bioenergetic profiling—demonstrate that treatment restores and augments mitochondrial performance under disease-relevant conditions. To monitor PINK1 activity in cells and tissues, Progenra employed Tandem Ubiquitin Binding Entity (TUBE) affinity platforms. A next-generation tool, the phospho-ubiquitin–specific TUBE (pTUBE), enabled mapping of the phospho-ubiquitome induced by PINK1 glues. While the role of PINK1 and phospho-ubiquitin signaling is well established in Parkinson’s disease, emerging evidence also points to broader functions in skeletal muscle physiology and other systemic processes, which will be highlighted.

B407/P1531

HDL-bound S1P affects the subventricular niche and early neuropathological features of Alzheimer's disease

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Circulating blood factors are critical for homeostasis of the adult ventricular-subventricular (V-SVZ) and subgranular zones, which contain neural stem cells (NSCs) crucial for sustained neurogenesis. Circulating sphingosine-1-phosphate (S1P) bound to apolipoprotein M (ApoM), a principal component of high-density lipoproteins, is involved in various biological processes, but its role in neurogenic niches is poorly understood. Herein, using Apom-/- mice, we show that blood ApoM-S1P deficiency impairs the SVZ-NSC pool, neurogenesis, ependymal cell polarity, and cerebrospinal fluid flow, leading to olfactory dysfunction and ventricular enlargement, early neuropathological features of Alzheimer’s disease (AD). Enhancing the complex significantly rescues these defects by activating S1P1 receptor signaling in SVZ-NSCs. Consistently, blood ApoM-S1P levels are reduced in early AD patients and correlate with olfactory deficits and ventricular enlargement. Similar abnormalities are recapitulated in young APP/PS1 mice and reversed by restoring blood ApoM-S1P levels. Thus, these data reveal pathogenic mechanisms underlying early neuropathological features of AD and identify the blood ApoM-S1P complex as a potential diagnostic and therapeutic target.

B394/P1518
Histone Modifications in Neurodegeneration: H3S10ph in ALS/FTD
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An aging population has led to a drastic increase in the prevalence of neurodegenerative diseases. Among these, frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) impact different neuronal types causing motor and behavioral symptoms, respectively. Bearing significant overlap in disease mechanisms, these two disorders comprise two ends of a disease continuum. Several genes have been linked to ALS/FTD pathology, including FUS, TDP-43, and C9orf 72. A hallmark of ALS/FTD is the cytoplasmic mislocalization and aggregation of the FUS, TDP-43, and c9orf72 proteins. As genetics alone fails to explain the etiology of ALS/FTD, recent work has explored epigenetic mechanisms in this context. Epigenetic channels include the post-translational modification (PTM) of histones. DNA wraps around the core histone proteins H2A,H2B, H3, and H4, which can undergo acetylation, phosphorylation, and mono-, di-, or trimethylation among many other modifications on specific residues. We reveal unique histone PTM landscape aberrations are connected to distinct ALS/FTD proteinopathies in yeast models. Several of these findings are recapitulated in human cellular disease models. Furthermore, we show that chemical and genetic manipulation of proteins that install and remove histone PTMs leads improved cell survival in yeast models, highlighting epigenetic mechanisms as a potential target for therapeutic development in neurodegeneration.

B400/P1524
Immunofluorescence array tomography of neurofilament accumulations in a mouse model of Charcot-Marie-Tooth disease type 2E
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Neurofilaments are abundant polymers in axons and are required for the growth of axon caliber, influencing conduction velocity. Neurofilaments assemble in the nerve cell body and are transported into axons along microtubule tracks. For decades, it has been known that many neurodegenerative diseases exhibit axonal swellings packed with neurofilaments. However, little is understood about the prevalence, location or mechanism of formation of these accumulations and their significance for disease progression. Intriguingly, such accumulations are also observed in Charcot-Marie-Tooth disease type 2E (CMT2E), which is caused by mutations in neurofilament protein L (NFL), and also in a genetically authentic knock-in mouse model of CMT2E heterozygous for the N98S mutation in NFL (N98S/+). We are using immunofluorescence array tomography to characterize the neuronal neurofilament distribution in dorsal root ganglia (DRG) of these mice. DRGs are dissected from adult N98S/+ and +/+ mice and embedded in hydrophilic resin. Ribbons of 200 nm-thick serial sections are immunostained and imaged using widefield microscopy. Rendering of the aligned images yields volumes with approximately isometric diffraction-limited resolution in three dimensions. In wild type DRGs, the neurofilament content of the cell bodies is uniformly low and all myelinated axons contain abundant neurofilaments along their entire length. In CMT2E DRGs, we observe elevated neurofilament in ~5% of cell bodies. Within any single plane of section, ~8% of the myelinated axonal profiles have elevated neurofilament including some (~3%) with balloon-like spheroidal swellings and thinned myelin. In contrast, a majority (~75%) of myelinated axonal profiles in the N98S/+ DRGs lack neurofilaments, and outside the DRGs in the spinal nerves, all myelinated axons lack neurofilaments. These observations indicate that neurofilaments in CMT2E DRGs are sequestered within cell bodies and/or proximal axons and fail to exit the DRG, which

suggests an impairment of their transport within or close to the cell body. We speculate that the axonal spheroids represent the site of this transport impairment, analogous to a log jam in a river. If this is true, then every axon that lacks neurofilaments distally should have a neurofilament accumulation proximally, and neurofilaments should be present in axons upstream (proximal) to the spheroids and absent downstream (distal). We are performing volumetric reconstructions of the axons to test these hypotheses.

B392/P1516

Impact of DnaJB1 Expression on Proteostasis in a Human Cellular Model of a-Synuclein Toxicity

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Parkinson's disease (PD) is defined by the buildup of misfolded α-synuclein (α-syn) aggregates, which drive dopaminergic neuron loss. The purpose of this study was to examine whether the co-chaperone DnaJB1, a component of the Hsp70 system, can reduce α-syn–associated toxicity in human cells. To test this, plasmids encoding fluorescently tagged α-syn and DnaJB1 were generated by restriction enzyme cloning. PCR amplification introduced restriction sites for α- syn and DnaJB1. The amplified fragments were verified by agarose gel electrophoresis, digested, purified, and ligated into expression vectors. Following transformation into competent Escherichia coli, plasmids were purified by midi prep and confirmed by restriction digest and DNA quantification. These steps successfully produced expression constructs for both α-syn and DnaJB1. This work establishes a platform for transfection into HEK293T cells, where the effect of DnaJB1 on α-syn–induced cytotoxicity will be evaluated. Our results demonstrate the feasibility of generating and validating these constructs, providing a basis for future experiments that probe the therapeutic potential of enhancing proteostasis in models of neurodegenerative disease.

B393/P1517

Investigating DnaJB Chaperone Interactions with Neurodegenerative Protein Aggregates Using a Yeast Two-Hybrid Assay

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Correct protein folding is essential for maintaining structural integrity and biological function, whereas misfolded proteins can form toxic aggregates driving disease. Recent research has identified that DnaJB (Hsp40) family of chaperone proteins have potent disaggregation ability in conjunction with Hsp110/Hsp70 to counteract this aggregation. These proteins have high potential for therapeutic intervention of neurodegenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis (ALS). This study utilizes yeast as a model organism to investigate these interactions using a Yeast Two-Hybrid (Y2H) Assay. DnaJB proteins are cloned into pGADT7 plasmids as activating domain (AD) fusion proteins and amplified in E. coli, while neurodegenerative-prone proteins are cloned into pGBKT7 plasmids to serve as binding domain (BD) fusion proteins. This design will yield the study of protein-protein interactions between DnaJB isoforms and neurodegeneration-linked aggregates in yeast, facilitating further investigation between chaperone protein and its counterpart neurodegenerative protein for potential therapeutic application in human cells.

B406/P1530

Linking metabolic irregularities to neurodegenerative phenotypes in AOA2: Insights from Senataxin knockout cells

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Ataxia with oculomotor apraxia type 2 (AOA2) is a rare neurodegenerative disease that has no cure and virtually no therapeutic options for patients. This disease is caused by mutations in the gene Senataxin. Senataxin is a helicase responsible for resolving RNA:DNA hybrids that form during transcription and plays roles in DNA repair and other cellular processes. Genetic knockout (KO) of Senataxin has several downstream consequences including decreases in genome stability, increased levels of DNA damage, and elevated innate immune activation. We have recapitulated these findings in Senataxin KO (Setx-/-) mouse embryonic fibroblasts (MEFs), which are derived from the AOA2 mouse model. We also observe an increase in reactive oxygen species (ROS), a novel phenotype in these KO cells. These phenotypes are known drivers of neurodegenerative diseases and markers of inflammation and oxidative damage, however, their role in AOA2 remains unclear. In our study we aimed to fill this gap in knowledge by elucidating the molecular causes of DNA damage, innate immune activation, and ROS production in Setx-/- MEFs. Our initial results indicate that mitochondrial function and morphology are dysregulated and abnormal, which may be leading to increased ROS production. We also have identified an irregular glycolytic rate phenotype in these cells. Combined these findings indicate a previously unidentified phenotype in which Setx-/- cells display metabolic difference compared to wildtype cells, indicating a potential link between metabolic dysregulation and AOA2 disease related phenotypes. Future work will dissect if these phenotypes are recapitulated in our AOA2 mouse model and AOA2 patient-derived cells. By understanding the molecular mechanisms of these phenotypes and validating them in both in vitro and in vivo models we can identify biomarkers of AOA2 which is vital for developing potential therapeutic targets for patients.

B389/P1513

Mapping the cellular interaction landscape of endogenous LRRK2 using designer small molecules

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Hyperactivation of the Rab kinase Leucine Rich Repeat Kinase 2 (LRRK2) is a common cause of both sporadic and familial forms of Parkinson's Disease (PD), a neurodegenerative condition affecting over 10 million people worldwide. As a result, significant efforts have been made to design compounds which specifically inhibit LRRK2, with a view to the development of new treatments for PD. Recently, we designed and characterized the first type-II kinase inhibitors specifically developed to target LRRK2. These inhibitors differ from previously described type-I LRRK2 inhibitors by targeting the autoinhibited state of the protein rather than the active-like conformation. We have leveraged this distinction in the design of new tools to specifically study LRRK2 in its different conformational states. Based on existing type-I LRRK2 inhibitors and our new type-II compounds we have developed a toolkit of designer small molecules for studying specific populations of endogenously expressed LRRK2 in disease relevant cell types. We have derivatized inhibitors with either biotin (for affinity-based isolation of endogenous LRRK2 and its protein interactors) or fluorophores (for visualizing different

populations of endogenous LRRK2 in fixed and live cells). These cell-permeable tools have allowed us to characterize the protein interactors and localization of different populations of LRRK2 in its native cellular context. This study sheds new light on LRRK2's cellular interaction landscape and the molecular mechanisms behind previously described LRRK2-related phenotypes. In turn, we are developing a detailed, molecular-level understanding of the cellular consequences of therapeutic LRRK2 inhibition with conformation-specific drugs in different PD relevant cells. Ongoing work aims to extend this analysis to the characterization of conformation specific post-translational modifications of LRRK2. Modifications specific to active LRRK2 have the potential to be used as novel biomarkers to aid diagnostics and the design of targeted therapeutics. As such, this study is highly significant for the PD field in informing the design strategy for future LRRK2 targeting compounds for PD treatment.

B404/P1528

Microtubule polarity flaws as an early driver of neurodegeneration in SPG4 Hereditary Spastic Paraplegia

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Dieback degeneration of the corticospinal tracts underlies the gait defects that characterize Hereditary Spastic Paraplegia (HSP). Mutations in the SPAST gene give rise to the most common form of HSP, which is called SPG4-HSP. Symptoms progress over the lifetime of the patient and severity of symptoms has been linked to the extent of axonal degeneration. Understanding the earliest events in axonal pathology is important for developing therapies that can prevent disease progression. In neurons derived from the motor cortex of early postnatal transgenic mice expressing one copy of human mutant spastin (SPAST-C448Y+/-), we found little or no synapse loss at four-weeks in culture but a substantial reduction in neuronal activity, as measured with a multi-electrode array. Previous studies suggested alterations in molecular motor proteins responsible for organelle transport as a potential cause of axonal degeneration in SPG4-HSP. While it is conceivable that slower overall rates of organelle movements may account for neuronal activity deficits and culminate over the years in the degeneration of the axon, we wondered if there might be something even more consequential involved related to the microtubule tracks along which the organelles are transported. We found that the normally uniform plus-end-out polarity pattern of the axon's microtubules is significantly corrupted in the axons of the disease model. Curiously, inhibitors of kinesin-5, a motor protein that tamps down the sliding of microtubules in the axon, corrected the reduced neuronal activity. We posit that greater microtubule sliding enabled by inhibition of kinesin-5 clears the axon of incorrectly oriented microtubules, and that hyperactivation of kinesin-5 might be an early driver of axonal degeneration in SPG4-HSP.

B398/P1522

Sphingolipid dysregulation contributes to ER stress sensitivity in a yeast model of ALS8

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that results in progressive loss of upper and lower motor neurons, leading to death typically within 3-5 years of diagnosis. Numerous genes are linked to familial ALS, including VAMP-associated protein B (VAPB), which encodes an ER-resident protein that functions at membrane contact sites with organelles including the plasma membrane and mitochondria. A P56S mutation in VAPB is linked to ALS8, and results in protein misfolding, ER collapse and sensitivity to ER stress. We previously developed a fungal model for ALS8 in S. cerevisiae by deleting the yeast VAP homologs,

SCS2 and SCS22, and expressing humanized mutant scs2P51S, P58S that bears the analogous ALS8-linked mutation (P58S). Yeast ALS8 cells phenocopy ER collapse and stress sensitivity seen in mammalian cells expressing VAPBP56S; thus, we used our model to perform a high-copy suppressor screen to identify genes whose overexpression reduced sensitivity to ER stress. We find that numerous components of the sphingolipid biosynthetic pathway, including the sphingosine kinase Lcb4, the mannosylinositol phosphorylceramide synthase subunit Sur1, and a fragment of the ceramide synthase Lac1, improve cell growth under ER stress conditions. Moreover, we show that pharmacological manipulation of sphingolipid biosynthesis in the yeast ALS8 model reduces ER stress sensitivity. Taken together, we propose that ceramide biosynthetic enzymes may be a relevant therapeutic target in ALS8.

B396/P1520
UFMylation Hyperactivation Drives Tau Propagation via the TRAPPC8-CLN8 Axis
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Alzheimer’s disease causes memory loss and decline, with growing evidence that the buildup and spread of a protein called tau contributes significantly to the process. Our research has uncovered a new pathway—called UFMylation—that disrupts the cell’s recycling system and fuels tau buildup. We found that two other proteins, TRAPPC8 and CLN8, are central to this process. By studying human brain cells and mouse models, we aim to show how this pathway drives disease and test ways to restore cell health and slow Alzheimer’s progression.

B395/P1519
Understanding variant-specific vulnerability of cerebellar granule neurons in TUBB4A-related leukodystrophy
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Microtubules (MTs), composed of α-β tubulin heterodimers, are essential for neuronal function, intracellular trafficking, and myelination. Mutations in tubulin genes cause a range of neurological disorders, including leukodystrophies—rare genetic diseases involving abnormalities in brain white matter. Heterozygous missense mutations in the TUBB4A gene result in TUBB4A-related leukodystrophy (TUBB4A-LD), with a clinical spectrum from isolated hypomyelination to severe Hypomyelination with Atrophy of the Basal Ganglia and Cerebellum (H-ABC). Milder TUBB4A-LD variants result in isolated hypomyelination with no cerebellar atrophy. Conversely, the recurrent TUBB4AD249N variant causes H-ABC, resulting in severe dystonia, loss of previously acquired motor skills, and early childhood mortality. H-ABC impacts oligodendrocytes, striatal neurons, and cerebellar granule neurons (CGNs). While CGN loss is a hallmark feature of H-ABC, the impact of the Tubb4aD249N variant on CGN death remains unclear. Our studies employ novel mouse models to examine the cellular impact of specific TUBB4A-LD variants, revealing differences in neuronal vulnerability. Using high-resolution microscopy and biochemical approaches, we assess alterations in MT dynamics and organelle trafficking. Although a previously developed antisense oligonucleotide therapy effectively mitigates certain pathological features, it fails to prevent CGN loss—highlighting the need for more targeted interventions. To address this, we will test whether CGN–specific gene suppression can mitigate CGN loss and improve functional outcomes. By defining cell-type–specific mechanisms and testing targeted therapies, my work aims to advance treatment strategies for TUBB4A-LD and related tubulinopathies.

B401/P1525
Validating a patient-derived induced pluripotent stem cell model for studying neurofilament accumulations in Charcot-Marie-Tooth disease type 2E
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Charcot-Marie-Tooth (CMT) is a progressive hereditary disease of the peripheral nervous system which results in axon degeneration, muscle weakness and atrophy. CMT type 2E (CMT2E) is caused by mutations in neurofilament protein L (NEFL), a critical subunit of neurofilaments. Neurofilaments are necessary polymers of the neuronal cytoskeleton that provide structural support and increase axonal caliber. These polymers are synthesized and assembled in the nerve cell body and transported into axons by the mechanisms of axonal transport. Nerve biopsies of people with CMT2E have revealed that some axons exhibit massive swellings packed with neurofilament polymers whereas others lack neurofilaments entirely. Similar findings have been reported in the NEFLN98S/+ mouse model of CMT2E, and neurofilament accumulations have been reported in motor neurons differentiated from patient-derived NEFLN98S/+ induced pluripotent stem cells (iPSCs). We are using these iPSC-derived NEFLN98S/+ neurons to investigate how the accumulations form. As a control, we are using an isogenic cell line in which the N98S mutation was corrected using gene editing. These lines were generously provided by Luke Judge at UCSF. Neurofilament content was quantified in cell bodies and axons using immunofluorescence microscopy. Sample size was determined using a power analysis and the experimenter was blinded to the culture identity during the experimentation and analysis to avoid bias. CMT2E neurons exhibited statistically significant accumulations of neurofilaments in the cell bodies and in swellings along the axons, but the overall neurofilament content of the axons was depleted in spite of these axonal swellings. Since neurofilaments are synthesized in the cell body and transported into and along axons, this suggests that there is a disruption in the mechanism by which the neurofilaments leave the cell body and move along the axon in the diseased neurons. Time-lapse imaging revealed that the axonal swellings often form at growth cones and are surprisingly dynamic, forming and dispersing and sometimes even moving along the axons, reminiscent of motile varicosities described previously in primary cultures. Swellings also form in control cultures but are smaller and fewer in number. These experiments validate patient-derived iPSCs as a model to study neurofilament accumulation in CMT2E in cell culture, but the relevance of the axonal swellings to the swellings observed in vivo remains to be established.

Disease Mechanisms and Therapeutics - Other cellular mechanisms of disease and therapeutic targeting

B325/P1449
A systematic review of a functional cure for HIV: Block and Lock Strategies
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This systematic review of a functional cure for Human Immunodeficiency Virus (HIV) aims to examine all block and lock strategies that scientists have been studying for the past two decades. Since the discovery of antiretroviral drug therapy (ART), HIV has transformed from a fatal disease to a manageable chronic condition. However, it remains incurable, as latent HIV infected cell reservoirs evade ART and can reactivate and spread infection upon discontinuation of ART. Block and lock strategies target or "block" cellular factors, viral factors or cellular signaling pathways, and modify the structure of the HIV promoter region to "lock" it into a state of

deep latency, thereby preventing any reactivation. An early and one of the most advanced approaches uses didehydro-Cortistan A (dCA) to target Tat, a viral protein central to HIV transcription. dCA binds to Tat, disrupts Tat-TAR interaction, and inhibits transcription. Other strategies use NullBasic, a mutant Tat protein that outcompetes and disrupts Tat, and Integrase-LEDGF Allosteric inhibitors, molecules that disrupt Integrase-LEDGF/p75 interaction to prevent viral integration and maturation. Since then, a whole host of small molecules have been screened to target critical transcription proteins and pathways like Bromodomain containing protein 4 (BRD4) and Cyclin-Dependent Kinase 9 (CDK9) binding, Mechanistic target of rapamycin (mTOR) protein, Janus kinase/signal transducer and activator of transcription pathway (JAK/STAT), Facilitates Chromatin Transcription protein complex (FACT), Heat Shock Protein 90 (HSP90), kinases and other targets. Beyond small molecules, RNA based strategies promote latency by modifying HIV promoter region through small interfering and long non-coding RNA and CRISPR/CAS gene-editing technology attempts to cleave parts of or the entire viral genome out of the human genome, rendering the infection benign. Pursuing a cure for HIV is quite challenging as our understanding of viral and host factors and their interactions, and signaling pathways is still evolving. Adding to the complexity is heterogeneity of host cell latent reservoirs and successful and targeted delivery of treatment. Research so far varies widely in measurement of outcomes related to measuring latency, therapy duration, viral rebound and toxicity. Standardizing experimental outcome framework, optimizing combination of therapies and improving treatment delivery methods are critical to advancing HIV functional cure research. Additionally, establishing a consistent and comprehensive checkpoint of the global research on cell and HIV interactional biology is equally important, ensuring that new insights and potential corrections to existing research are captured.

B336/P1460

Acute and subchronic atrazine exposure causes prostatic tissue damage, endocrine disruption, and oxidative stress in gerbils

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Atrazine is the second most widely used herbicide worldwide for weed control. Its environmental persistence and endocrine-disrupting properties have been associated with disorders in hormone-dependent organs, including the reproductive system. Regulatory agencies such as the World Health Organization (WHO) and the Environmental Protection Agency (EPA) consider doses ≤ 0.02 mg/kg/day to be without lifetime health risks. This study aimed to characterize the effects of acute and subchronic low-dose atrazine exposure on the ventral prostate of adult gerbils. All procedures were approved by the Ethics Committee on Animal Use of the Federal University of Goiás, Goiânia, Brazil (CEUA-UFG no. 112/23). Adult male gerbils (Meriones unguiculatus, 90 days old) received 0.02 mg/kg/day of atrazine diluted in 0.2 mL saline by gavage for 3 days (3ATZ) or 21 days (21ATZ), while control groups received only vehicle for 3 days (3C) or 21 days (21C). Ventral prostates were analyzed by histopathology, immunohistochemistry, and oxidative stress assays. Kruskal–Wallis and ANOVA tests were applied, with Dunn's or Tukey's post hoc tests for nonparametric or parametric data, respectively ($p \leq 0.05$). Atrazine exposure produced time-independent tissue damage characterized by epithelial hyperplasia and stromal inflammatory foci. Androgen receptor immunolabeling decreased (3C = 95.7% vs. 3ATZ = 66.9%; 21C = 93.4% vs. 21ATZ = 61.9%; $p \leq 0,0001$), whereas estrogen receptor alpha (3C = 3.2% vs. 3ATZ = 8.3%; 21C = 2.9% vs. 21ATZ = 9.2%; $p \leq 0,0001$) and proliferating cell nuclear antigen (3C = 2.1% vs. 3ATZ = 5.3%; 21C = 2.4% vs. 21ATZ = 6.9%; $p \leq 0,0001$) increased. Additionally, the ventral prostates showed an increased immunolabeling area for the pro-inflammatory cytokine tumor necrosis factor-alpha (C = 468.1 μm^2 vs. 3ATZ = 1004.2 μm^2; 21C = 327.9 μm^2 vs. 21ATZ = 1443.4 μm^2). Oxidative stress assays revealed reduced catalase (3C =

14.4 vs. 3ATZ = 8.6; 21C = 12.5 vs. 21ATZ = 4.9 U CAT/mg protein; $p \leq 0,01$) and superoxide dismutase activity (3C = 38.9 vs. 3ATZ = 25.7; 21C = 35.8 vs. 21ATZ = 16.8 U SOD/mg protein; $p \leq 0,01$), along with increased malondialdehyde levels (3C = 6.8 vs. 3ATZ = 11.9; 21C = 7.3 vs. 21ATZ = 16.4 nmol MDA/mg protein; $p \leq 0,001$). Thus, it can be concluded that the acceptable daily intake of atrazine recommended by regulatory agencies is not safe for the prostate. Acute and subchronic exposures to atrazine triggered harmful effects in the gland, such as prostatic hyperplasia and inflammation. These morphological changes are related to hormonal dysregulation, increased cellular proliferation, and oxidative stress in the prostate.

B328/P1452

Characterization of novel gene variants associated with male infertility in Qatar

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Male infertility is a complex, multifactorial condition with diverse presentations, from absent spermatozoa to specific abnormalities in sperm quality such as reduced sperm motility. Genetic causes account for 15–30% of cases, but at least 40% remain idiopathic due to a lack of diagnostically relevant genes. Whole genome sequencing has advanced gene identification in male infertility over the past years. In this study, we investigated three brothers with infertility who presented with normal semen parameters and hormone profiles, but defective sperm motility. Whole genome sequencing of these individuals identified two missense variants in MTIF3 and PRR14. To explore the functional impact of the mutations, we transfected U2OS cells with wild-type and variant MTIF3 and PRR14. By using co-localization microscopy studies, we found that the variant did not affect the localization of MTIF3 to mitochondria, nor PRR14 to the nucleus. We then began to explore the effects of the MTIF3 variant on mitochondrial function, and the PRR14 variant on cell cycle. Preliminary data indicate that transfection of the PRR14 variant did not have overt effects on heterochromatin, nuclear size or cell cycle distribution. Interestingly, transfection of the MTIF3 variant into MTIF3-knockdown U2OS cells impairs ATP production, represented by an increase in the levels of phosphorylated AMP-protein kinase (pAMPK). By contrast, expressing wild-type MTIF3 leads to an increase in ATP levels, represented by a decrease in the levels of pAMPK. To further analyze the effect of this variant on mitochondrial function, we evaluated oxygen consumption rate using the Oroboros instrument. This study proposes a critical role of MTIF3 in mitochondrial energy production, particularly in relation to sperm motility. Ultimately, our work aims to help unravel the underlying mechanisms behind male infertility.

B326/P1450

Combined treatment with Metformin and Cinacalcet Reverses Mitochondrial Dysfunction and Cyst Growth in Human ADPKD Cells

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Autosomal Dominant Polycystic Kidney Disease (ADPKD) is an inherited renal disorder caused by mutations primarily in the PKD1 or PKD2 genes. It is characterized by dysregulated epithelial cell proliferation, abnormal fluid secretion, and extracellular matrix remodeling, which drive the progressive formation and enlargement of renal cysts, leading to a gradual decline in renal function and end-stage kidney disease. Therapeutic strategies targeting renal cystogenesis in ADPKD have highlighted the potential of metformin and cinacalcet. Metformin, a widely used AMPK activator, counteracts the suppressed AMPK signaling observed in ADPKD. Cinacalcet, an allosteric activator of the calcium-sensing receptor, has been shown to reduce cyst growth and epithelial proliferation in ADPKD models. Here we provide evidence that combined treatment with metformin (200 μM) and cinacalcet (1 nM) for 3 days fully restores the altered bioenergetic profile in immortalized human ADPKD renal cyst cells harboring a homozygous PKD1 mutation, including improved mitochondrial ATP production and correction of multilevel oxidative phosphorylation defects. Moreover, the dual treatment significantly reduced forskolin-induced cyst growth of human 3D cells derived from ADPKD cyst-lining epithelia. With respect to the single administration, the combined treatment had a synergistic effect in reducing S6 ribosomal protein phosphorylation (marker of mTORC1), that is early increased in ADPKD. Collectively, these findings indicate an additive therapeutic benefit of combined metformin and cinacalcet treatment, supporting its potential for further preclinical investigation in ADPKD.

B347/P1471

CYP4A Inhibition as a Therapeutic Strategy for Metabolic dysfunction-associated steatotic liver disease (MASLD) : Targeting Metabolic Dysfunction, Inflammation, and Fibrosis

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Metabolic dysfunction-associated steatotic liver disease (MASLD), formerly known as nonalcoholic fatty liver disease (NAFLD), is characterized by hepatic steatosis accompanied by metabolic comorbidities such as obesity, type 2 diabetes mellitus (T2DM), and hypertension. Due to its multifactorial pathogenesis, MASLD remains a challenge for therapeutic development, highlighting the need for novel treatment strategies. Cytochrome P450 4A (CYP4A), an enzyme involved in oxidative lipid metabolism, has been implicated in disease progression and represents a potential therapeutic target. In this study, we aimed to identify and evaluate novel CYP4A inhibitors to expand the MASLD treatment pipeline. Using in silico screening, we identified two candidate compounds, C86 and C87, that significantly reduced CYP4A expression and enzymatic activity. These compounds were tested across multiple MASLD models, including in vitro hepatocyte systems, transgenic and diet-induced mouse models, and 3D liver organoids. Treatment with C86 and C87 reduced lipotoxicity, oxidative stress, endoplasmic reticulum stress, and apoptosis, while also improving insulin resistance. Moreover, in models of hepatic inflammation and fibrosis, both compounds ameliorated liver injury as evidenced by improved histological and biochemical markers. Based on these findings, our study highlights C86 and C87 as promising CYP4A inhibitors that effectively regulate genes associated with hepatic steatosis, inflammation, type 2 diabetes mellitus (T2DM), and metabolic dysfunction-associated steatotic liver disease (MASLD) in diabetic models. These inhibitors represent valuable candidates for further research aimed at addressing metabolic dysfunction-associated steatotic liver disease (MASLD).

B330/P1454

Developing and Mature Photoreceptors Respond Differently to Chronic Stress and Inflammation

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Chronic inflammation and elevated cortisol are the hallmark of numerous conditions, including diabetes and heart disease. These conditions are compounded by damage to the blood-retina barrier that can lead to blindness. While the various organs associated with each of those conditions have been studied in the context of stress and inflammation, less is known about how the neural retina responds to the same insults. Basigin gene products are expressed within the neural retina and are known to be important for supporting healthy metabolic coupling between Müller glial cells and photoreceptor neurons. Specifically, Basigin variant-1 (Bv1) on the surface of photoreceptors binds to Basigin variant-2 (Bv2) on the surface of Müller cells to ensure that the neurons receive adequate amounts of metabolic substrates to support their cellular functions. The purpose of the present study was to assess Basigin gene expression in response to chronic inflammation and stress to assess health of the neural retina under those conditions. Neural retinas were isolated from a mix of male and female mice at post-natal day 7 (P7) and P180 and were incubated in culture medium containing lipopolysaccharide (LPS, 10 μg/mL), cortisol (10 μg/mL), or saline for 24 hours. RNA was isolated, quantified, and subjected to q-RT-PCR using primers specific for Bv1 and Bv2. It was determined that developing and mature mouse retinas respond to stress and inflammation differently. For the P7 animals, both LPS and cortisol exposure resulted in a significant decrease in Bv2 but had no significant effect on Bv1. For the P180 animals, LPS exposure resulted in a significant decrease in expression of Bv1 and Bv2, whereas cortisol exposure resulted in a significant increase in Bv2. The data suggest that chronic inflammation in adults directly affects the photoreceptor neurons, in addition to the Müller cells, setting up a scenario for vision loss. Prolonged elevated cortisol likely causes Müller cells to become reactive, which can lead to additional damage of the adult neural retina. Developing neural retinas do not respond the same way, protecting the photoreceptors and preventing Müller cell reactivity. It is evident that chronic inflammation and elevated cortisol directly affect the mature neural retina in a negative way.

B342/P1466

Dominant role of VAMP8 in airway mucin secretion

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Airway mucus protects the lungs by trapping inhaled particles and pathogens, but its malfunction drives diseases such as asthma, cystic fibrosis, and COPD. While mucin production and hydration have been studied extensively, the exocytic machinery that controls secretion remains incompletely understood. Mucins are released at two rates: a low baseline rate and a high stimulated rate, and for Munc18 proteins, involving distinct molecular components (18a in baseline and 18b in stimulated). We investigated the role of vesicle-associated membrane proteins (VAMPs), a family of SNARE proteins critical for exocytosis, in controlling these two rates of secretion. Analysis of public single-cell RNA-seq data revealed that VAMP8 is the most abundant isoform in human airway secretory cells. Using airway-epithelium–specific deletant mice, we found that VAMP8 is required for both baseline and stimulated mucin secretion. VAMP2 is the second most abundant isoform, partners with Munc18a in neurons, and localizes to mucin granules by IF microscopy. However, combined deletion of VAMP2 and VAMP3 produced no detectable defect in baseline secretion. Future work will be directed to determining whether VAMP2 has a role in baseline secretion despite the dominant effect of VAMP8.

B331/P1455

Effects of native Hawaiian noni extracts in high-risk neuroblastoma

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The aim of this project is to explore the efficacy of Noni extract in treating High-Risk Neuroblastoma. High-risk Neuroblastoma (HRNB) is an extracranial pediatric cancer in which up to 65% of patients are resistant to therapies. While recent advances in medicine have improved treatment options for patients with HRNB, there are no effective treatments for relapsed HRNB patients who have undergone treatment and experienced tumor recurrence. Noni (Morinda citrifolia) is an evergreen tree found in the Pacific Islands, Southeast Asia, Australia, and India with large leaves and yellow fruit. Noni contains vitamin C, vitamin A, antioxidants and many other chemicals that might help repair damaged cells in the body which make it a suitable candidate for potential therapeutic applications for HRNB. In order to assess the efficacy of noni extract we assessed its; cell viability using Sulfo-rhodamine B (SRB) assay, its antioxidant content using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, its protein content using Western Blot, and the epigenetic regulation of NB gene expression using the Illumina Infinium Human Methylation 450K BeadChip microarray. In SRB experiments, noni extract shows significant cell growth inhibition in a dose-dependent manner. In DPPH experiments, noni extract shows significant antioxidant content, emphasizing its potential antioxidant capacity. Western blot analysis showed that the noni extract induced apoptosis and reduced the expression of DNA methyltransferases, DNMT1 and DNMT3a suggesting that it may regulate DNA methylation and gene expression. Analysis of DNA methylation and gene expression showed that noni extract resulted in hypomethylation of key genes that may be involved in the mechanism of action of the extract. Native Hawaiian preparations of Noni had potent antioxidant and anti-cancer effects in high-risk NB. The Noni extract induced apoptosis and decreased the expression of DNA methyltransferases, DNMT and DNMT3a. Furthermore, Noni extract caused hypomethylation of key genes that may be involved in the mechanism of action of Noni, including genes involved in regulating numerous processes in the mitochondria including those involved in autophagy and apoptosis.

B349/P1473

Exploring Nuclear p21 as a Potential Modulator of Cyst Initiation and Expansion

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Autosomal dominant polycystic kidney disease (ADPKD) is characterized by progressive cyst formation, leading to end-stage renal failure. While cyst initiation is known to involve epithelial dedifferentiation, proliferation, and injury responses, the molecular events that trigger and sustain cyst growth remain incompletely defined. The cyclin-dependent kinase inhibitor p21 has emerged as a context-dependent regulator of both proliferation and senescence, but its role in PKD pathogenesis remains unclear. The objective of this study was to determine whether nuclear p21 expression and localization contribute to cyst initiation and progression in PKD. To test this, we used inducible Pkd2 deletion mouse models (systemic Cagg-CreER) and performed analyses at early (6 weeks post-induction, wpi), cyst initiation (12 wpi), and late (16 wpi) disease stages. Immunofluorescence staining was used to localize p21 in kidney tissue and quantify p21 expression as it correlates to cyst progression. Proliferation was also quantified using both Ki67 immunostaining and EdU incorporation assays. Our results revealed that p21 expression was markedly increased in epithelial cells, and importantly, its

localization was predominantly nuclear rather than cytoplasmic. Nuclear p21 upregulation correlated with an increased frequency of irregularly shaped nuclei, suggesting cellular and nuclear remodeling as an early cyst-associated change. While both Ki67 and EdU labeling demonstrated increased proliferation, the proliferative response was observed after p21 expression and in a lower total percentage of cells. These findings suggest that nuclear p21 is not a response to proliferation and may be a driver of epithelial remodeling. Nuclear expression was significantly detected before overt cyst expansion, indicating that p21 may also act as an early biomarker of cyst initiation. p21 was also noted in liver cysts in Pkd mutants, suggesting that p21 may have an overarching role in all cystic tissues. We have developed a p21, Pkd2 double knockout and a p21 overexpression mouse line to directly investigate the role of p21 in cystogenesis. In conclusion, our findings identify nuclear p21 as a novel marker and possible promoter of cystic epithelial remodeling in PKD. Rather than only a cell-cycle inhibitor, nuclear p21 associates with structural alterations in cystic kidney models that precede proliferative changes, thereby facilitating cyst initiation and progression. This work provides new mechanistic insight into connections between nuclear p21 accumulation and cyst initiation. Future genetic studies where we induce or inhibit p21 expression will help define this relationship and determine whether it is a potential therapeutic target and early biomarker of disease trajectory.

B340/P1464

Extracellular matrix stiffness reduces pirfenidone inhibitory effects on myosin light chain phosphorylation in lung fibroblasts

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Pulmonary fibrosis is a progressive and debilitating respiratory disorder characterized by extracellular matrix (ECM) deposition and stromal stiffening. Current clinically approved antifibrotic drugs including pirfenidone and Nintedanib only modestly affect the trajectory of disease and are not curative for this disorder. Thus, deciphering the pathophysiological mechanisms that contribute to pulmonary fibrosis and therapeutic strategies that arrest or even reverse aberrant ECM remodeling remain top research priority. Stromal stiffening in fibrosis is thought to be a secondary consequence of tissue inflammation. However, increasing evidence supports that the mechanical properties of tissues are integrated by cells into biochemical signals that affect cell fate and function, a process termed mechanotransduction. Here, we investigated how ECM stiffness influences lung cell therapeutic response of primary fibroblasts from pulmonary fibrosis patients. Employing mechanically tunable hydrogels conjugated with relevant ECM ligands, our studies demonstrate that primary lung fibroblasts are sensitive to changes in ECM stiffness. We also discovered that pirfenidone robustly inhibits levels of phosphorylated myosin light chain, a core component of mechanotransduction. Importantly, inhibitory effect of pirfenidone on mechanical tension was prominent on physiological relevant normal soft matrix (400 Pa) but this effect is diminished along with increased ECM stiffness (pathophysiological stiffness). In summary, our work showed that pirfenidone inhibits cellular mechanical tension and ECM stiffness compromises inhibitory effects of pirfenidone in myosin light chain phosphorylation. Our work also suggests that inhibition of stiffness sensing ability of the cells may help maximize pirfenidone effect in mechanotransduction.

B348/P1472

Extracellular Vesicles Derived from Intravenous Immunoglobulin Therapy Serving as Decoys to Suppress IFNγ-

Activation of JAK/STAT1 Signaling

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Immunoglobulin (Ig) replacement therapy (IgRT), which includes intravenous (IVIg) and subcutaneous (SCIg), is a rigorously purified and concentrated pool of antibodies (>95% IgG) isolated from the plasma of up to 15,000 healthy donors. Mechanistically, IgRT boosts the immune system by providing a diverse repertoire of antibodies in immunocompromised patients or by blocking pathogenic antibodies in patients with autoimmune diseases. However, the mechanism of action in treating chronic inflammatory diseases is not well understood. Extracellular vesicles (EVs) are membrane-bound nanoparticles secreted by virtually all cells carrying bioactive molecules such as lipids, proteins, and nucleic acids, that play a critical role in intercellular communication. To date, the presence of EVs and their role in mediating the anti-inflammatory effects of IgRT has not been shown. Here, we isolated EVs from IVIg and unprocessed normal human plasma (UHP) using size exclusion chromatography (SEC) or ultracentrifugation. EVs were characterized by size, number, protein amounts, and surface markers. By nanoparticle tracking analysis, IVIg and UHP EVs were within the expected exosomal size range of ~100 nm and transmission EM showed membrane-bound vesicles. Bead-based multiplex analysis by flow cytometry (MACSPlex) of known EV surface immunooncology markers showed that IVIg EVs displayed significantly reduced platelet markers and enriched in stemness markers compared to UHP EVs. Luminex multiplex cytokine immune profiling revealed that the levels of 22 pro-inflammatory cytokines were dramatically reduced in IVIg EVs compared to UHP EVs. t-SNE algorithm analysis shows shared and distinct clusters of IVIg and SCIg compared to UHP cytokines and further confirms systematic, biologically meaningful differences across the fractionation spectrum, dramatically impacting the treatment of acute and chronic inflammatory disease. Interestingly, interferon-gamma (IFNγ), a pleiotropic cytokine with both pro- and anti-inflammatory functions, was elevated in IVIg EV-rich fractions. Here, we demonstrate that IFNγ binds to the surface of EVs, most likely through circulating anti-IFNγ antibodies, and IFNγ-EVs can inhibit IFNGR-mediated JAK/STAT1 activation. In conclusion, IVIg EVs can function as decoys to suppress inflammatory signaling pathways.

B338/P1462

Fucoxanthinol Delivered from Microalgae to Prevent Inflammation in Lung Epithelial Cells.

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Microalgae are a great source for natural products beneficial for human health. One such product is fucoxanthin—a carotenoid pigment with anti-inflammation properties. Fucoxanthin can be extracted and hydrolyzed to form fucoxanthinol, which is the bioactive form and shows great medical promise. However, fucoxanthinol currently is rarely available for researchers. We have developed a sustainable and cost-effective method to extract, hydrolyze, and purify fucoxanthinol from a microalga. Our method yields about 2 mg of pharmacological grade (purity > 95%) fucoxanthinol from 10 g of dried microalgal biomass. Here, we present an overall method to produce fucoxanthinol and the anti-inflammatory effects of the purified fucoxanthinol on lung epithelial cells. Using Air-Liquid Interface cultured BEAS-2B cells, various concentrations of fucoxanthinol (0.3, 1, 3, 10, 30 µM), were administered 24 hours before exposure to copper oxide nanoparticles. As controls, we administered fucoxanthin (30 µM), resveratrol (100 µM), or dimethyl sulfoxide (<0.1%). Cell viability was assessed using an Alamar blue assay. Future experiments will be conducted to determine the efficacy of fucoxanthinol in preventing inflammation.

B346/P1470

GP130 promotes sorafenib resistance in hepatocellular carcinoma through the inhibition of BECN1-mediated aberrant mitophagy

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Sorafenib resistance (SR) poses a significant challenge in the treatment of advanced hepatocellular carcinoma (HCC). Although mitophagic cell death—the hyperactive and non-selective degradation of mitochondria leading to cell death—is implicated in cancer, its underlying mechanisms in the context of SR are unclear. GP130 not only promotes HCC development in vitro and in vivo but is also highly overexpressed in patients with advanced HCC and is significantly upregulated in sorafenib-resistant tumors, correlating with poor patient prognosis. Mechanistically, GP130 enhances SR by suppressing mitophagic cell death, which is characterized by sustained mitophagy and mitochondrial damage. In SR patients, a negative correlation between GP130 and BECN1 expression was observed, and GP130 deletion enhanced BECN1-PARKIN interaction, facilitating their recruitment to healthy mitochondria and ultimately inducing mitophagic cell death. GP130 mediates BECN1-dependent mitophagic cell death via phosphorylation of STAT3 at tyrosine 705, independent of mTORC1 activation, thereby contributing to SR. Based on these findings, the therapeutic potential of GP130 inhibition in combination with sorafenib was investigated. This combination significantly suppressed cell proliferation and tumor growth in vitro and in vivo by promoting BECN1-mediated mitophagic cell death. Furthermore, increased PARKIN and BECN1 expression positively correlated with overall survival in both HCC and sorafenib-treated patients. Collectively, our findings establish the GP130-BECN1 axis as a critical regulator of therapeutic response and mitophagic cell death. This positions GP130 as a key driver of SR and a promising therapeutic target for innovative SR-HCC treatment.

B316/P1440

Inhibition of ROR1/YAP/TAZ Signaling Restores Ferroptosis in Radioresistant Triple-Negative Breast Cancer Cells

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Triple-negative breast cancer (TNBC) remains one of the most difficult subtypes of breast cancer to treat due to its high metastatic potential, lack of targeted therapies, and frequent resistance to radiotherapy. While ferroptosis—a non-apoptotic, iron-dependent form of regulated cell death—has recently gained attention as a promising approach to bypass treatment resistance, the underlying mechanisms of ferroptosis resistance in TNBC are still largely unclear. In this study, we established a radioresistant TNBC cell line (MDA-MB-231-RR) and found that these cells display a marked resistance to ferroptosis. This phenotype is associated with elevated intracellular glutathione (GSH) content, reduced lipid peroxidation, and increased expression of the antioxidant regulators SLC7A11 and GPX4. Transcriptomic profiling and molecular analyses revealed that the non-canonical Wnt receptor ROR1, together with its downstream effectors YAP and TAZ, plays a pivotal role in sustaining this ferroptosis-resistant state. Targeted inhibition of ROR1 through both siRNA and small molecules led to suppression of the SLC7A11–GPX4 axis, accumulation of reactive oxygen species (ROS), and restoration of lipid peroxidation, thereby resensitizing cells to ferroptotic death. Furthermore, we identified β-sitosterol, a plant-derived phytosterol, as a potent ferroptosis inducer in radioresistant TNBC cells. Mechanistically, β-sitosterol suppressed the ROR1/YAP/TAZ signaling cascade, depleted GSH levels, and enhanced ROS-mediated lipid peroxidation. When combined with ionizing radiation, β-sitosterol synergistically amplified DNA damage and ferroptosis, significantly reducing clonogenic survival. In a xenograft model, β-sitosterol treatment led to a

pronounced decrease in tumor growth without affecting animal body weight. Tumor tissues from treated mice showed reduced expression of ROR1, YAP, TAZ, SLC7A11, and GPX4, alongside elevated lipid peroxidation markers. Molecular docking studies further suggested that β-sitosterol may directly interact with the extracellular domain of ROR1, potentially disrupting ligand binding. Taken together, our findings uncover the ROR1/YAP/TAZ axis as a critical regulator of ferroptosis resistance in TNBC and propose β-sitosterol as a novel radiosensitizing agent that restores ferroptotic vulnerability through inhibition of this pathway.

B341/P1465

Inhibitory effect of Licochalcone D on atopic dermatitis in HaCaT cells and HR-1 mice

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Atopic dermatitis (AD) is a chronic and recurrent eczematous skin disease characterized by abnormal immune responses and accompanied by severe pruritus. Licochalcone D (LD), a flavonoid isolated from the root of Glycyrrhiza inflata, showed anti-inflammatory effects against lipopolysaccharide (LPS)-induced inflammation in previous studies; however, its role in AD remains unclear. In the present study, we investigated the role of LD and its mechanism related AD on tumor necrosis factor-alpha (TNF-α)/interferon gamma (IFN-γ)-stimulated human keratinocyte and phthalic anhydride (PA)-induced AD mice model. Through in vitro experiment, we found LD reduced pro-inflammatory cytokines and chemokines as well as activation of mitogen-activated protein kinase (MAPK) signaling induced by TNF-α/IFN-γ. In mice model on AD, LD treatment significantly reduced the PA-induced AD clinical score, epidermal thickness of ear, and serum immunoglobulin E (IgE) level. The level of pro-inflammatory cytokines, chemokines and activation of MAPK signaling were decreased in LD-treated group compared to PA-treated group. Therefore, LD alleviated inflammatory response during AD by inhibiting activation of MAPK signaling. These results suggest that LD may be a potential candidate as a treatment for AD.

B321/P1445

Integrative Analysis Reveals Previously Unrecognized Roles for ANKHD1 in Gastric Cancer

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The ankyrin repeat and KH domain-containing 1 (ANKHD1) gene encodes a scaffold protein that mediates protein and nucleic acid interactions in a context- and cell type-specific manner, thereby regulating cancer-related signaling pathways. However, its role in gastric cancer (GC), where ANKHD1 is highly expressed, remains unexplored. Therefore, this study aimed to investigate the biological roles of ANKHD1 and its associated signaling pathways in GC by integrating transcriptomic, proteomic, and functional cellular model approaches. Analysis of The Cancer Genome Atlas (TCGA) GC cohort revealed significantly higher ANKHD1 expression in GC tissues compared to normal gastric samples. Consistently, the metastatic gastric carcinoma cell line HGC-27 exhibited higher ANKHD1 protein levels than AGS cells, derived from a primary gastric adenocarcinoma. Stratification of patients into high- and low-ANKHD1 expression groups identified differentially expressed genes (DEGs), with Gene Ontology enrichment analyses indicating upregulation of cell adhesion and transcription processes in high-ANKHD1 tumors. High ANKHD1 transcript levels also correlated with larger tumor size, advanced TNM stage, and distinct molecular subtypes, supporting its association with

GC aggressiveness. ANKHD1's interactome, mapped via immunoprecipitation (IP) and mass spectrometry, identified 69 potential interacting proteins enriched in biological processes related to cell adhesion, transcription, and translation, corroborating transcriptomic findings. CRISPR/Cas9-mediated ANKHD1 knockout in AGS cells enhanced migratory capacity in Transwell assays and altered chemotherapy responses, with increased resistance to doxorubicin and reduced paclitaxel efficacy after 72 hours of treatment, as shown by MTT viability assays. Co-IP confirmed ANKHD1–ATF6 interaction. Under endoplasmic reticulum (ER) stress induced by 1 mM Dithiothreitol (DTT), ANKHD1-knockout cells displayed earlier ATF6 activation and increased expression of the ATF6 target gene HSPA5 (BiP), indicating that ANKHD1 modulates ATF6-mediated unfolded protein response (UPR). Immunolocalization further revealed ANKHD1 accumulation at cell–cell junctions, the perinuclear region, and within nuclear puncta, suggesting that its subcellular localization is functionally relevant in GC. Taken together, these results demonstrate that ANKHD1 exerts multifaceted functions in gastric cancer, being associated with previously unrecognized processes such as cell adhesion, transcription, and translation. Its loss enhances migration, reshapes chemotherapy sensitivity, and accelerates ATF6-mediated UPR. These findings provide novel mechanistic insights into ANKHD1's contribution to gastric cancer biology.

B329/P1453

Investigating the role of lamin A/C in skeletal muscle using conditional knockout mice

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Mutations in LMNA, which codes for the nuclear lamina proteins lamin A/C, have been linked to different human diseases that affect diverse tissues. Most of these mutations affect straited muscles, causing cardiomyopathy and muscular dystrophies such as Emery-Dreifuss Muscular Dystrophy type 2 and phenotype variants. Recent studies have used conditional knockouts of Lmna in mice to study its role specifically in cardiomyocytes and cardiac fibroblasts, however skeletal muscle has not been as extensively investigated. Here, we characterize the effect of Lmna knockout in mice skeletal muscle using different Cre lines to target specific tissue and cell types. We found that Lmna knockout in embryonic straited muscles led to a dramatic heart dilation phenotype but simultaneously no observable phenotype in the skeletal muscles. When we induced Lmna knockout in skeletal myofibers of adult mice, it triggered their regeneration, presumably by the resident myosatellite cells. This regenerative capacity is essential, as the combined depletion of Lmna from both skeletal myofibers and myosatellite cells was fatal, with the most dramatic degeneration and fibrosis observed in the intrinsic muscles of the tongue. This study lays the foundations for understanding the role of lamin A/C in skeletal muscle maintenance and development.

B337/P1461

Involvement of the glyoxalase pathway in cystic fibrosis

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Cystic fibrosis (CF) is an autosomal multi-organ disease affecting the glandular secretion system and resulting in reduced lung and digestive system function. Chronic inflammation, oxidative stress (OxS) and abnormal

susceptibility to infections are pathogenic traits of CF, causing severe tissue damage. The glyoxalase pathway, which comprises glyoxalase 1 (Glo1) and glyoxalase 2 (Glo2), the latter having a cytosolic (cGlo2) and a mitochondrial (mGlo2) isoform, eliminates the cytotoxic methylglyoxal (MG) in the presence of catalytic amounts of reduced glutathione (GSH). MG, mainly produced as an unavoidable byproduct of glycolysis, can induce both OxS and inflammation (oxinflammation) through the formation of advanced glycation end products (AGEs), which is why its abnormal formation and/or detoxification associates to several human pathologies. We have recently shown that Glo1 expression and activity are defective in murine and human bronchial epithelial cell models of CF upon challenge with Aspergillus fumigatus (ASP), a common pathogen in CF, and this defect is associated with pathogenic inflammation. On the contrary, Glo2 isoforms, which can release GSH and D-lactate either in the cytosol or inside mitochondria, have never been investigated in CF. Here we investigated the glyoxalase pathway in CF as a possible target to treat oxinflammation. Tissue homogenates, as well as mitochondria and cytosolic fractions isolated from lung and liver of CftrF508del mice, a murine model of CF, and control C57BL/6 WT mice, both infected (CF+ASP, WT+ASP) or not with ASP, were analyzed. We found that OxS was higher in CF lungs and livers compared to respective WT, and this difference increased upon ASP infection pointing out the reduced antioxidant capacity of CF tissues. To note, both WT and CF lungs showed higher OxS levels and much lower Glo1 activity than the corresponding livers. Lung Glo1 activity and expression were comparable in CF and WT and increased upon ASP infection in both cases, but the increase was significantly lower in CF+ASP than in WT+ASP. Consistently, the levels of MG-H1, a MG-derived AGE, increased significantly in the formers. Regarding Glo2, CF and WT lungs showed similar activity of both cGlo2 and mGlo2 isoforms, here first analyzed separately. However, upon ASP infection, mGlo2 activity increased significantly only in WT but not in CF lungs, while cGlo2 activity remained unchanged. Similarly to Glo1, mGlo2 activity was much lower in lungs than in livers in all cases. In conclusion, our results suggest that the lower Glo1 and mGlo2 activities, as well as their defective activation in pathogenic infection in CF, may contribute to the higher susceptibility of lungs to tissue damage as compared to liver, and may be pathogenic factors involving a mitochondrial mechanism.

B345/P1469

Maladaptive repair as a catalyst for cystogenesis in ADPKD mouse models

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Autosomal dominant polycystic kidney disease (ADPKD) is a common inherited disorder affecting 1 in 400–1000 individuals and is caused by pathogenic variants in PKD1 or PKD2, which encode the polycystin proteins PC1 and PC2, respectively. Cyst initiation is traditionally explained by a “two-hit” model, but recent evidence suggests that additional insults, such as renal injury, may act as a “third hit” to promote cyst expansion. Following injury, tubule epithelial cells typically attempt to repair and restore their differentiated state; however, in certain cases this process becomes maladaptive, leading to abnormal proliferation, and fibrosis, thereby worsening the impact of PKD mutations. Thus, defining how maladaptive repair intersects with PKD gene mutations will offer new insight into the mechanisms that drive cyst progression and disease severity. To gain mechanistic insight, we examined renal epithelial injury responses, focusing on maladaptive repair processes that may contribute to cyst formation. We hypothesized that proliferative and dedifferentiated epithelial cells create a microenvironment favorable for cystogenesis. To test this, we monitored Sox9, a marker of injured epithelial cells, at multiple time points (0, 3, 7, 28, and 35 days) following cisplatin treatment in both Pkd2 mutants and controls. Sox9+ cells increased post-injury in both groups but with distinct kinetics: in controls, Sox9 expression peaked at 7 days, whereas in Pkd2 mutants it peaked earlier at 3 days and remained significantly higher throughout. Furthermore, Sox9+ cells did not return to baseline by 35 days post-injury,

when repair is typically complete, suggesting persistence of a maladaptive repair state. This defect was more pronounced in Pkd2 mutants, indicating heightened sensitivity to injury and defective repair. To further characterize maladaptive repair, we assessed Wnt signaling and fibrosis-related genes using qRT-PCR and observed increased expression of multiple genes in these pathways in Pkd2 mutants compared to controls. In addition, proliferation analysis using EdU revealed elevated epithelial proliferation during cyst formation, further supporting a link between maladaptive repair and cyst expansion. Altogether, our findings show that markers of maladaptive repair are elevated and remain dysregulated during injury resolution. We propose that Pkd2 dysfunction leads to incomplete epithelial repair, with injured cells entering a maladaptive repair state that act as a catalyst for cyst formation. Understanding the role of maladaptive repair cells will provide new insights into the mechanisms linking renal injury to cystogenesis and may highlight therapeutic strategies to slow ADPKD progression.

B344/P1468

Mechanisms that Implicate Pannexin 1 Channels in the Pathophysiology of Interstitial Cystitis/Bladder Pain Syndrome (IC/BPS)

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Interstitial Cystitis/Bladder Pain Syndrome (IC/BPS) is a chronic condition characterized by bladder-related pelvic pain, pressure, and discomfort, often accompanied by urinary urgency and frequency. Several factors are implicated in IC/BPS pathogenesis, including urothelial barrier dysfunction. A leaky urothelium exposes bladder tissues to high urinary potassium (K+), which induces release of signaling molecules, such as ATP, that contributes to development of IC/BPS symptoms. Pannexin 1 (Panx1) channels are mechanosensitive and participate in bladder sensation by mediating urothelial ATP release in response to bladder distension. Panx1 channels are also activated by elevated extracellular K+, suggesting that in the context of urothelial damage, K+-mediated ATP release and signaling might provide a mechanism that implicates Panx1 channels in IC/BPS pathophysiology. To test this hypothesis, we performed in vitro studies to determine whether urothelial cells respond to elevated K+ and whether these responses are mediated by Panx1 channels. Immortalized mouse urothelial cells were cultured in ibidi µ-slides and exposed to (1) urine-like (10 mM) or normal extracellular (2.5 mM) K+ levels, (2) mechanical stimulation via oscillatory fluid flow (OFF), or (3) combined elevated K+ and OFF. Responses in the presence and absence of the Panx1 channel blocker Brilliant Blue FCF (BB-FCF; 10 µM) were assessed by real-time Ca2+ imaging and quantification of ATP release. Data were compared pairwise using the Mann-Whitney test. Exposure to urine-like K+ levels induced Ca2+ responses in 90% of imaged cells (N=44) and resulted in ATP release that was 4.5-fold higher than controls ($p<0.05$, n=4). Exposure to combined OFF + high K+, as cells would experience during a voiding cycle and breach of urothelial barrier, amplified Ca2+ responses and induced ATP release in amounts 2-fold ($p<0.01$, n=4) and 5-fold ($p<0.001$) higher than OFF and K+ alone, respectively. Notably, all responses were blunted in the presence of the Panx1 channel blocker BB-FCF. Our findings indicate that Panx1 activation by urinary K+ constitutes a mechanism through which these channels can contribute to development of IC/BPS symptoms in the context of urothelial barrier dysfunction. This study supports future preclinical testing of Panx1 channel blockers as potential treatment for IC/BPS. Funding Source: DoD W81XWH-21-1-0465

B318/P1442

Mechanistic Insights into Bifidobacterium bifidum BB1-Mediated Protection of the Intestinal Barrier Against IL-1ß-Induced Permeability

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Background: Defective intestinal tight junction (TJ) barrier function is a key pathogenic factor of inflammatory bowel diseases (IBD). Pro-inflammatory cytokines, including interleukin-1β (IL-1β), cause an increase in intestinal TJ permeability. Bifidobacterium bifidum (BB), a widely studied probiotic, has been shown to produce strain-specific enhancement of the intestinal TJ barrier. A particular BB strain, referred to as BB1, produced a marked enhancement of intestinal TJ barrier, while another BB strain, BB4, had no effect. The major purpose of this study was to determine the mechanism and the microbe-host interaction responsible for the BB1 inhibition of IL-1β-induced intestinal TJ barrier disruption. Methods: Filter-grown Caco-2 monolayers (in-vitro) and recycling intestinal perfusion of live mice (in-vivo) were used to assess intestinal epithelial TJ permeability. Results IL-1β produced a rapid activation of NF-κB, and an increase in expression and activity of myosin light chain kinase (MLCK), a key effector of TJ barrier opening in filter-grown Caco-2 monolayers. BB1 treatment inhibited the IL-1β-induced NF-κB activation, MLCK expression and activity, and an increase in Caco-2 TJ permeability. BB1, but not BB4, caused an increase in apical membrane expression of Toll-like receptors TLR-2 and TLR-6. Moreover, siRNA-induced knockdown of TLR-2 or TLR-6 inhibited the BB1 protective effects against MLCK activation and TJ barrier disruption. The BB1 activation of the TLR-2/TLR-6 complex did not activate the canonical MyD88 pathway, resulted in the activation of the anti-inflammatory adaptor protein TOLLIP. The siRNA-induced knockdown of TOLLIP, but not MyD88, prevented the BB1 protective effect against MLCK activity and TJ barrier disruption. In live mice, BB1 oral gavage inhibited the IL-1β-induced increase in MLCK expression and activity and intestinal permeability to Dextran 10 kDa in wild-type mice, but not in intestinal epithelial-specific TLR-2 knockout (TLR-2^ΔIEC) mice. Conclusion: BB1 protects against IL-1β-induced intestinal TJ barrier disruption by intestinal epithelial TLR-2/TLR-6 receptor-dependent activation of TOLLIP and inhibition of NF-κB and MLCK activity. These results provide important mechanistic insight into how the probiotic bacteria BB1 interacts with the host cells to protects against the cytokine-induced disruption of the intestinal epithelial TJ barrier.

B343/P1467

Medial unicompartmental knee arthroplasty outcomes in full vs. partial cartilage loss: A comparative analysis

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Knee osteoarthritis (KOA) is one of the most common degenerative joint diseases worldwide, is a leading cause of disability and decreased quality of life in older adults. One of the main characteristics of knee osteoarthritis is the deterioration of articular cartilage, which is aggravated by chondrocyte death, loss of matrix integrity, and impaired mechanotransduction. Medial unicompartmental knee arthroplasty (UKA) is increasingly adopted as a minimally invasive and joint-preserving solution. However, it is unclear how much cartilage degradation full thickness cartilage loss (FTCL) vs. partial thickness cartilage loss (PTCL) affects postoperative recovery. This study evaluates functional recovery, joint stability, and complication rates in FTCL vs. PTCL patients undergoing UKA. Based on intraoperative findings, we classified 60 patients receiving UKA for isolated medial compartment OA into two groups: those with full thickness cartilage loss (FTCL, n = 30) and those with

partial thickness cartilage loss (PTCL, n = 30). The distribution of FTCL patients was similar in terms of age and gender, but they were noticeably taller (167.5 ± 6.2 cm vs. 162.8 ± 5.9 cm, p=0.003) and heavier (74.6 ± 8.5 kg vs. 68.9 ± 7.1 kg, p=0.006), which may indicate chronic joint overloading, which is linked to chondrocyte stress and ECM degradation. Range of motion (ROM) was marginally better in FTCL (113.3° vs. 107.6°, p=0.06), and instability patterns were comparable between groups (p=0.45), which could be due to variations in cartilage stiffness and surface wear or adaptive rebuilding. Over a two-year period, postoperative outcomes measured by the Oxford Knee Score (OKS) and Knee Society Score (KSS) improved comparably in both groups. After three months, an early difference in Day 1 KSS (greater in FTCL, p=0.005) vanished by the second year. Regardless of cartilage integrity, neither group experienced any problems or reoperations, suggesting that surgery was effective in both the cases. Crucially, long-term functional recovery was not substantially impacted by the extent of cartilage loss. These results imply that the initial thickness of cartilage loss has no apparent effect on patient outcomes following UKA. Joint mechanics plays a crucial role in preserving tissue-level homeostasis, as evidenced by the research suggesting that even joints with total cartilage erosion can recover functionally when mechanical conditions are restored.

B322/P1446

Molecular Analysis Of Eprenetapopt (APR-246) Activity In A TP53 R248Q-Mutated Acute Myeloid Leukemia Cell Model

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In acute myeloid leukemia (AML), TP53 mutations are strongly associated with poor prognosis. A comprehensive analysis of patient samples from the TCGA and BeatAML cohorts conducted by our group identified 65 distinct TP53 variants, with R248Q being the most frequent. This hotspot mutation displays dominant-negative properties and pathogenic potential. While structural data suggest no major conformational alterations, functional studies confirm impaired p53 activity. Kasumi-1 cells, which harbor the TP53 R248Q mutation, were selected as an optimal model to investigate therapeutic responses. This study evaluated whether eprenetapopt [APR-246, PRIMA-1(Met)] restores mutant p53 function in Kasumi-1 cells and assessed its activity in combination with clinically relevant anti-leukemic agents. Synergy analyses with APR-246 revealed the strongest effect with venetoclax (ZIP score: 18.139), followed by daunorubicin (16.098) and cytarabine (7.474). For molecular analysis, cells were treated with APR-246 (0–10 µM) for 72 h. Combination regimens included: (i) 0 or 6.25 µM APR-246; (ii) 0.5 µM cytarabine ± APR-246; (iii) 0.06 µM daunorubicin ± APR-246; and (iv) 1.25 µM venetoclax ± APR-246. Protein expression was analyzed by Western blot. APR-246 induced a dose-dependent reduction of p53, p62, and LC3B-I/II, accompanied by upregulation of BAX, p21, and cleaved PARP1, indicating modulation of autophagic flux and activation of apoptotic pathways. Drug combinations further enhanced γH2AX accumulation and PARP1 cleavage compared with single agents, suggesting synergistic reinforcement of DNA damage and apoptosis. These findings align with previous evidence supporting the reactivation of mutant p53 by APR-246 in TP53 R248Q-mutated AML. The reduction in p53 protein levels likely reflects functional reactivation followed by degradation through feedback mechanisms. The concomitant increase in apoptotic and cell-cycle inhibitory markers, along with suppression of autophagy proteins, further corroborates pathway restoration. Notably, the synergistic interactions with cytarabine, daunorubicin, and venetoclax highlight APR-246 as a promising adjuvant to standard chemotherapy in TP53-mutated AML. In summary, APR-246 reactivates mutant p53 in Kasumi-1 cells, promoting apoptosis, reducing autophagy, and amplifying DNA damage. When combined with conventional chemotherapeutics, these effects are potentiated, underscoring its therapeutic potential. Future directions

include in silico docking, molecular dynamics, and complementary in vitro studies. Supported by FAPESP, CNPq, and CAPES.

B314/P1438

Novel anti-melanoma compounds targeting splicing regulators hnRNPH1 and H2 induce immunogenicity of melanoma cells and in vivo immune response

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As estimated by the American Cancer Society, there will be about 100,640 new cases in the USA, and the number of deaths will be more than 8,000. Melanoma is one of the most active areas of oncological drug discovery, with more than 300 active clinical trials, but despite this, new therapeutic approaches are urgently needed. We have previously reported the discovery of novel anti-melanoma compounds 2155-14 and 2155-18 that bind and downregulate spliceosomal RNA-binding proteins hnRNP H1 and H2. These compounds are active against BRAFi-sensitive and -resistant A375 cell line-derived xenografts at 25mg/kg and non-toxic to immunocompetent mice at 50mg/kg. The objective of this study was to evaluate the effects of hnRNPH1 and H2 modulation on melanoma cell immunogenicity. In vitro experiments with BRAF, NRAS, and triple-wild type melanoma cell lines demonstrated that treatment by siRNA, 2155-14, and 2155-18 leads to an increase of double-stranded RNA, a known stimulator of interferon signaling. NanoString analysis of gene expression confirmed upregulation of interferon signaling and cellular immune response, which is known to upregulate cellular immunogenicity. Cytokine assays confirmed melanoma cell immune response on the protein level. Finally, treatment of the B16F10 syngeneic melanoma mouse model resulted in increased tumor infiltration of macrophages, dendritic cells, B cells, and CD4+ T cells as determined by flow cytometry analysis. In conclusion, these results suggest a therapeutic potential of targeting spliceosomal proteins hnRNPH1 and H2 and support the potential of 2155-14 and 2155-18 as drug candidates for the treatment of melanoma. Additionally, it demonstrates the potential of spliceosomal inhibition to stimulate immune system response, suggesting the possibility of using such therapies in combination with FDA-approved immunotherapies.

B339/P1463

Pigment epithelium-derived factor overexpressing placental mesenchymal stem cells prohibit neovascularization in streptozotocin-induced diabetic rats

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Diabetic retinopathy (DR) is a major complication of diabetes characterized by microvascular damage in the retina, leading to localized hemorrhage and edema. Neovascularization can cause retinal detachment and subsequent vision loss. Although current therapeutic approaches including anti-VEGF agents and steroid treatment are aimed at suppressing neovascularization and preventing disease progression, it is known that these interventions cannot be a fundamental treatment for patients. Pigment epithelium–derived factor (PEDF) is a multifunctional protein with neuroprotective, anti-angiogenic, anti-tumor, and stem cell–supportive properties. Notably, its potent anti-angiogenic activity makes it a promising candidate for DR therapy. We have previously demonstrated that placenta-derived mesenchymal stem cells (PD-MSCs) exert antioxidant effects in ocular disease models. Based on this, we investigated whether overexpression of PEDF in PD-MSCs (PD-

MSCsPEDF) could enhance their therapeutic efficacy in DR by suppressing pathological neovascularization and promoting retinal regeneration. To establish a DR rat model, streptozotocin (STZ, 60 mg/kg) was intraperitoneally injected into the diabetic group, while the sham control group received only BSS solution. After 8 weeks, naïve PD-MSCs or PD-MSCsPEDF were intravitreally transplanted into the right eye for 4 weeks. Pathological neovascularization drives the progression of DR and subsequent vision loss. The balance between vascular endothelial growth factor (VEGF) and PEDF is critical in regulating angiogenesis. Immunofluorescence staining and mRNA analysis revealed increased VEGF and decreased PEDF expression in retinas of NTx group. These alterations were reversed by PD-MSC transplantation, with the most pronounced effect in the PD-MSCsPEDF group, which showed strong PEDF expression and reduced VEGF levels in the transplanted right retina compared with the naïve group. Interestingly, similar VEGF–PEDF expression patterns were also observed in the non-transplanted contralateral retina of the PD-MSCsPEDF group (R^2 = –0.8965). These findings suggest that PD-MSCsPEDF exert potent anti-angiogenic effects by modulating the balance of pro- and anti-angiogenic factors, thereby promoting vascular repair in STZ-induced diabetic retinopathy as well as they may provide new treatments for ophthalmic diseases. This research was supported by the Industry-Academia-Research Institute (IAR) Collabo R&D Fund Grant (Ministry of SMEs and Startups, Korean government, RS-2025-02313877), the Integrated R&D Fund Grant (National Research Foundation of Korea, RS-2022-00070304), and PLABiologics Co., Ltd.

B320/P1444

Potential antitumor effect of the combination of omega-3 docosahexaenoic acid and a poly(ADP-ribose) polymerase inhibitor in castration-resistant prostate cancer

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Metastatic castration-resistant prostate cancer (mCRPC) represents a major therapeutic challenge, and PARP inhibitors (iP) have improved patient outcomes. The omega-3 docosahexaenoic acid (DHA) has multiple antitumor effects in prostate cancer (PCa). No clinical trial has addressed the specific effects of DHA and its potential interaction with current PCa therapies. This study investigates the effects of DHA alone and in combination with iP on PC3 prostate cells, a cell line representative of mCRPC. PC3 cells were treated with DHA (100 µM), PARP inhibitor AZD2461 (20 µM), or their combinations for 48 h. The counts of DAPI-stained nuclei revealed that the viability of PC3 cells decreased ~13.5% after DHA, ~38.8% after iP and 49% after DHA+iP incubation compared to control, indicating an additive effect of DHA on the cytotoxicity induced by iP. This effect was also confirmed using Trypan blue viability assay and MTS. In the clonogenic assay, colonies were reduced after iP treatment and no colonies were formed in the DHA or DHA+iP groups. Markers of proliferative pathways, nuclear receptors, and programmed cell death were examined using Western blotting. All treatments reduced PI3K expression, notably iP (59%), and pAKT, especially DHA+iP (80.3%). No significant changes occurred in pERK1/2 and GSTP1 among the treatments. The content of ERα was markedly suppressed by DHA+iP (98.3%, 24.9-fold greater than iP alone). GRα decreased in all groups, particularly DHA+iP (48%). DHA and iP isolated induced apoptosis (active caspase-3 fluorescence assay), pyroptosis (cleaved GSDMD) and necroptosis (pMLKL) in PC3 cells, and an additive effect of DHA+iP was observed for apoptosis and necroptosis. DHA and iP treatments also increased autophagy (148.1% and 72.9%, respectively), but no effect was observed with their combination. All treatments significantly reduced PC3 cell migratory capacity within 12 hours, as evaluated using the scratch assay. While control and DHA-treated areas achieved wound closure between 12-24 hours, iP-treated groups showed wound closure between 24-36 hours. Treatments reduced expression of epithelial–mesenchymal transition (EMT) markers linked to invasion/metastasis: N-cadherin, vimentin, and

SNAIL (assessed by Western blotting). N-cadherin decreased by 71.9% (DHA), 86.2% (iP), and 96.3% (DHA+iP). DHA alone was most effective in reducing SNAIL (79.1%), whereas the combination therapy produced the greatest reduction in vimentin (74.6%). In conclusion, DHA enhances the cytotoxic effects of iP in PC3 cells through multiple mechanisms, including suppression of proliferative pathways and induction of apoptosis and necroptosis. These findings reinforce the need for clinical studies and suggest DHA's potential use as an adjuvant in mCRPC treatment.

B319/P1443

Selective cleavage and measurement of mucin with proteases: a diagnostic and treatment

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Airway epithelial mucus functions as a key component of immune response by trapping irritants that are then expelled through mucociliary clearance and coughing. Mucus is composed of secreted proteins mucin 5B (MUC5B) and mucin 5AC (MUC5AC). Healthy mucus is predominantly MUC5B, with less MUC5AC expression. However, asthma and chronic obstructive pulmonary disease are correlated with overexpression of MUC5AC, causing highly viscous airway mucus and interfering with mucus clearance and normal respiration. We developed a MUC5AC-specific protease with the capacity to form a substrate-protease complex after activation, triggering the release of a fluorescent compound and increasing the fluorescence intensity of the sample. This protease interacts with an inhibitor to reduce cleavage activity until the protease localizes to MUC5AC, which is assisted by the inclusion of MUC5AC antibody. This complex may serve as a diagnostic tool for the measurement of MUC5AC within samples through fluorescence intensity, as well as a therapeutic to reduce MUC5AC in unhealthy mucin ratios. To test this, we derived mucus samples from human airway epithelial (HAE) cell lines, including cell lines with MUC5AC or MUC5B knocked out. We developed a plate reader assay to measure the fluorescence intensity of treated and untreated samples across 5-minute intervals to determine the efficacy of the protease-substrate complex. We also use multiple particle tracking microrheology to determine if the protease is effectively cleaving MUC5AC by observing changes to the microviscosity and pore size based on the diffusion of fluorescent nanoparticles in mucus samples. Thus far, the protease complex appears to be selective toward MUC5AC and more effective when paired with MUC5AC antibody. The fluorescence intensity increased significantly in the MUC5AC mucus, while changing minimally in the MUC5B mucus and PBS control. When the mucus samples were incubated with MUC5AC antibody, the fluorescence intensity increased for all samples containing MUC5AC, indicating that the antibody is increasing the localization of the protease. The fluorescence intensity of the protease-substrate complex is more distinct between groups at lower concentrations of mucus. These studies establish proof of concept for the use of the protease-substrate complex as a diagnostic tool and future work will evaluate its potential use as a mucolytic treatment for obstructive lung diseases.

B324/P1448

Simultaneous silencing LDL receptor members CD320 and LRP2 inhibits cancer cell proliferation with no harm to normal cells: New relationships for old receptors

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The low-density-lipoprotein (LDL) receptor family contributes to diverse signaling pathways and ligand importation in both normal and cancer cell biology. We previously reported that the LDL receptor member CD320 facilitates the selective uptake of porphyrins into cancer cells through an endocytic process. We now report that the silencing of CD320 and fellow LDL receptor member LRP2 together by siRNAs kills or severely inhibits the proliferation of cancer cells but leaves normal cells unharmed. Cells were transfected with siRNAs by commercial lipofection reagents. Silencing efficiency was assessed by Western blot. Cell viability was measured by the Cell Titer Glo assay, as well as documented by cell imaging. Cell growth was inhibited by CD320 and LRP2 siRNA silencing compared to a control siRNA in cells derived from brain, lung, breast, prostate, and skin cancers. CD320 and LRP2 silencing did not affect cell proliferation of normal cells derived from lung, breast, and skin tissues. Cancer cells tested were not selected by any specific genetic mutation. Interestingly, in some cancer cells silencing of one receptor increased the protein expression of the other, suggesting a compensatory relationship between CD320 and LRP2. CD320 has only one known natural ligand: Vitamin B12 (cobalamin) complexed to its chaperone TCN2. LRP2 also has Vitamin B12/TCN2 as a ligand among approximately 100 others. We are investigating the mechanism of the selective toxicity of CD320 and LRP2 silencing on cancer cell growth. CD320 and LRP2 cooperate to maintain optimum proliferation of cancer cells, showing a novel function for these LDL receptors.

B333/P1457
TGF-ß1 triggers fibrosis via ROS-mediated inhibition of PHD2 and HIF-1a activation under normoxia
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Idiopathic pulmonary fibrosis is a progressive and deadly disorder with few effective therapies. In this study, we unveil a normoxic mechanism by which TGF-β1 promotes fibrotic progression through oxidative stabilization of HIF-1α. Specifically, TGF-β1 upregulates NOX4 and activates NOX2, leading to excessive generation of reactive oxygen species (ROS). These ROS oxidize the ferrous iron cofactor within prolyl hydroxylase domain protein 2 (PHD2), thereby disrupting HIF-1α hydroxylation and degradation. This redox-driven impairment of HIF-1α turnover creates a state of “oxidative hypoxia”, in which HIF-1α remains aberrantly active despite normoxic conditions. The sustained activation of HIF-1α further amplifies NOX expression, establishing a feed-forward loop that exacerbates fibrogenesis. To therapeutically counter this mechanism, we synthesized ACF-2, a novel small molecule that binds PHD2 and neutralizes ROS. ACF-2 preserved PHD2 function, reduced fibrotic marker expression in vitro, and significantly attenuated lung fibrosis in bleomycin-treated mice—surpassing the efficacy of nintedanib. These findings designate oxidative hypoxia as a key driver of fibrosis under normoxia and support PHD2 redox regulation as a promising therapeutic strategy.

B323/P1447
The role of PAX3-FOXO1 condensates in rhabdomyosarcoma
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Alveolar rhabdomyosarcoma (ARMS) is a highly aggressive childhood cancer driven by PAX3-FOXO1 (P3F), an abnormal protein resulting from the fusion of PAX3 and FOXO1 genes. P3F reprograms cells into a cancerous state by dysregulating the expression of genes involved in cell differentiation, growth, and survival. Emerging research suggests that P3F may cluster into specialized cellular structures called biomolecular condensates,

which lack membranes and are formed through a process known as phase separation. However, the role of these structures in ARMS remains poorly understood. I hypothesize that these condensates could amplify the cancer-promoting activities of P3F by concentrating the machinery that regulates gene expression. I used live-cell imaging and high-throughput sequencing methods to investigate the assembly and disassembly mechanisms of P3F-containing condensates and their pathological role in cancer progression, with the ultimate goal of developing new therapeutic strategies to treat ARMS and improve survival rates for children with ARMS. Our findings will deepen our understanding of P3F-containing condensates and could revolutionize treatment for ARMS by enabling innovative therapies that target the underlying causes of this aggressive cancer, providing hope to affected children and their families.

B317/P1441

The Role of Sonic Hedgehog Signaling in Prostate Cancer Therapy Resistance

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Prostate cancer (PCa) is the most commonly diagnosed cancer amongst American men, and it is primarily driven by androgen receptor (AR) signaling. The subsequent development of AR-targeted therapies, such as enzalutamide, have transformed the clinical landscape of PCa. However, within two years of treatment, many patients develop resistance and progress to lethal stage metastatic castration resistant PCa, which has a 5-year survival rate of only 28%. One key player implicated in conferring this resistance is the Sonic Hedgehog (SHH) signaling pathway. SHH signaling is often tied to the progression of various cancers, particularly through a mechanism called lineage plasticity. Lineage plasticity is the ability of cells to switch between cell states. However, the exact molecular mechanisms behind how this phenomenon occurs in prostate cancer are unclear. Utilizing cloning and lentiviral transduction, PCa cell lines DU145 and LNCaP/AR were engineered to express overexpression (OE) and CRISPR knock-out (KO) vectors of SHH and its principal receptor, PTCH1. Western blotting was used to confirm successful transduction. The resulting cell lines were tested against the parental DU145 and LNCaP/AR lines in competitive cell growth assays, and the results were analyzed with fluorescence-activated cell sorting using fluorescent protein markers in the KO and parental cell lines. The SHH and PTCH1 OE cell lines showcased a significant fitness advantage over the parental cells in both normal and enzalutamide-treated conditions, while SHH and PTCH1 KO cell lines fared significantly worse. To assess how overexpression of SHH and PTCH1 might be conferring resistance, bioinformatic databases were generated by Mu lab. These databases contained RNA-seq based analysis of PCa cell lines and patient tissue, which were used to identify gene markers associated with SHH and PTCH1 in PCa. Both PTCH1 and SHH expression were found to be significantly elevated in progenitor-like cells; this result was further investigated by probing for the expression of several stem cell markers in SHH and PTCH1 OE cell lines using quantitative PCR. Interestingly, SOX2 and HMGA exhibited a twofold and fourfold expression in PTCH1 OE cell lines, respectively. These results indicate that manipulation of the SHH signaling pathway and lineage plasticity could be a prominent mechanism by which PCa develops resistance to AR-targeted therapies. Thereby, uncovering more genetic markers and understanding their correlation with SHH and PTCH1 could hold promise in improving therapeutic avenues and bettering patient lives.

B315/P1439

Visualizing Fucoxanthin Localization in BEAS-2B Cells Using Its Intrinsic Fluorescence

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Fucoxanthin is a bioactive compound derived from seaweeds and microalgae. Although numerous in vitro and in vivostudies have demonstrated its promising biological activities—including suppression of oxidative stress and inflammatory responses—fucoxanthin has not yet been recognized as a therapeutic agent by regulatory authorities. One key limitation is the incomplete understanding of its mechanism of action. Fucoxanthin is known to modulate several molecular pathways, such as NF-κB, Nrf2, and AMPK, yet the specific molecular interactions remain unclear. We hypothesize that fucoxanthin modulates inflammatory responses at the plasma membrane by interfering with the assembly of cytokine receptor complexes. To investigate this hypothesis, we leveraged the intrinsic fluorescence properties of fucoxanthin and conducted non-invasive imaging of its subcellular localization in BEAS-2B, a human bronchial epithelial cell line. This study reports spectral profiling and microscopy-based analyses of fucoxanthin and its major metabolite, fucoxanthinol, focusing on their distinct localization patterns within the cell.

B335/P1459

WKYMVm- functional enhanced placenta-derived MSCs ameliorate liver fibrosis via FPR2 signaling

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Purpose Mesenchymal stem cell (MSC)-based therapies are emerging as potential treatments for liver fibrosis, but their mechanisms remain incompletely understood. WKYMVm is a synthetic peptide that functions as a potent ligand for formyl peptide receptor 2 (FPR2), enabling receptor activation. This study evaluated placenta-derived MSCs engineered with WKYMVm (WKY-PDMSCs) and investigated their therapeutic effects in a thioacetamide (TAA)-induced rat model of liver fibrosis. Methods WKY-PDMSCs were generated by surface modification of PD-MSCs with WKYMVm. Rats with TAA-induced hepatic fibrosis were transplanted with either Naïve PD-MSCs (2x106)or WKY-PDMSCs (2x106) via intravenous injection. Fibrosis severity in liver tissue was assessed by measurement of type I collagen deposition using Sirius red staining Also, markers of oxidative stress and lipid accumulation were examined to analyze regeneration of liver tissues. Their therapeutic mechanism were performed to evaluate autophagy activity and FPR2 signaling pathways. Results WKY-PDMSCs transplantation significantly reduced collagen accumulation and oxidative stress We confirmed that WKY-PDMSC transplantation not only enhanced hepatocyte regeneration but also reduced fibrotic septa in a rat model with TAA-indued hepatic fibrosis Additionally, antioxidant responses in the injured liver tissues were promoted, contributing to reduced hepatic injury. Interestingly, transplantation of WKY-PDMSCs enhanced liver regeneration by increasing autophagic pathway as well as activating FPR2 downstream signaling pathways. Conclusion WKY-PDMSCs display superior antifibrotic effects by reducing fibrosis progression, mitigating oxidative damage, and enhancing liver repair. Their therapeutic efficacy appears to rely on autophagy activation and FPR2-mediated signaling. These findings provide mechanistic insight into how WKYMVm enhances stem cell function and suggest that receptor-targeted MSC engineering represents a promising strategy for liver fibrosis therapy. Supported by National Research Foundation (NRF) grant funded by the Korea Government (RS-2022-00070304) and Tech Incubator Program for startup Korea (TIPS) (RS-2023-00303130).

B332/P1456
Δ40p53a as a Key Modulator of Collagen III Expression in TGFß1-Activated Human Hepatic Stellate Cells
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Chronic liver injury triggers persistent wound-healing responses, leading to excessive extracellular matrix (ECM) deposition and fibrosis. Transforming growth factor-β (TGFβ) is a major profibrogenic driver, yet its crosstalk with specific p53 isoforms remains poorly understood. We investigated the role of the N-terminally truncated p53 variant, Δ40p53α, in ECM regulation in human hepatic stellate cells (LX-2). Analysis of cirrhotic liver tissues by RT-PCR revealed elevated Δ40p53 expression, suggesting clinical relevance. In vitro, antisense-mediated knockdown of Δ40p53 attenuated TGFβ1-induced stellate cell activation and markedly reduced fibrillar collagen III production. Conversely, Δ40p53α overexpression enhanced collagen III expression, acting synergistically with full-length p53 (FLp53). Co-immunoprecipitation assays showed that Δ40p53α forms complexes with FLp53 and phosphorylated Smad3 upon TGFβ1 stimulation. These findings identify Δ40p53α as a critical co-regulator of collagen III transcription in TGFβ1 signaling, implicating it as a potential therapeutic target in liver fibrosis.

Disease Mechanisms and Therapeutics - Rare genetic disorders

B423/P1547
An Unbiased Drug Screen in Drosophila Models of Lamin-Associated Muscular Dystrophy Identifies Calcium Channel Blockers as Potential Treatments
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Mutations in the LMNA gene encoding A-type nuclear lamins cause a collection of human diseases known as laminopathies, including multiple types of muscular dystrophy (LMNA-MD). Lamins are intermediate filaments with three conserved domains (N-terminal head, a coiled-coil central rod, and a C-terminal Ig-like fold). Missense mutations in all three domains can cause LMNA-MD by mechanisms that are not well understood. Currently, there are no treatments available for LMNA-MD beyond symptom management. Our goal is to identify candidate treatments for LMNA-MD through an in vivo drug repurposing screen using Drosophila models of the disease. Expression of the LamC R264Q (orthologous to human LMNA R249Q) substitution in Drosophila muscle causes muscle defects that lead to premature death at the pupal stage. We fed 1,520 FDA/EMA approved drugs to larvae and screened for rescue of lethality. This screen resulted in 68 positive hit compounds that partially restored adult viability. Molecular fingerprinting clustered a subset of these compounds into six structurally related groups, identifying the L-type voltage gated calcium channel blockers clevidipine and amlodipine as top candidates. Secondary testing using independently purchased compound validated these hits in the LamC R264Q model. To broaden our analysis, we next tested whether calcium channel blockers could rescue lethality in additional Drosophila models of the pathogenic LMNA A57P, H222P, R249W, H506P, W514R, and R527P substitutions. Our results indicate that, among the substitutions tested, calcium channel blockers rescue lethality caused by only the H222P, R249Q and R249W rod domain substitutions, potentially identifying a sub-domain of lamin A/C with roles in regulating calcium homeostasis. These findings support L-type voltage gated calcium channel blockers as candidate treatments for LMNA-MD and suggest that altered calcium dynamics is a mechanism of disease pathogenesis.

B421/P1545

Assessing the use of UBC9 inhibitors as chemotherapeutic enhancers for cisplatin and etoposide against non-small cell lung cancer

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Lung cancer including non-small cell lung cancer (NSCLC) is the leading cause of death by cancer in the United States, with a 5-year survival rate of only 21.7%. SUMOylation is a post-translational modification in which a Small Ubiquitin-like Modifier (SUMO) protein is covalently attached to a target protein, thereby regulating protein-protein interactions. SUMOylation regulates multiple cellular processes, including DNA repair, replication, and cell cycle progression. Dysregulation of the SUMOylation pathway is well documented in cancer, where it contributes to carcinogenesis, DNA damage response and proliferation. Central to the SUMOylation pathway is UBC9, the sole E2-conjugating enzyme which is responsible for transferring activated SUMO proteins to their target substrates. UBC9 is upregulated in several cancers, including lung cancer, and therefore it represents a promising therapeutic target. In this study we assessed the specificity and cytotoxicity of a set of newly developed UBC9 inhibitors in A549 cells, a NSCLC model. Immunoblots analyses confirmed that protein expression was significantly decreased by the addition of these inhibitors, highlighting their effectiveness. Treatment with the UBC9 inhibitors reduced cell proliferation and induced cell death. Furthermore, combination treatments with standard chemotherapeutic agents, cisplatin and etoposide, revealed enhanced cytotoxicity and increased apoptosis, suggesting a chemosensitizing effect. Together, these findings suggest that our UBC9 inhibitors disrupt the SUMOylation pathway and have the potential to enhance the effectiveness of current standard chemotherapy. Therefore, our UBC9 targeted inhibitors constitute promising chemosensitizers for the treatment of NSCLC. Future studies will further evaluate the effectiveness of the inhibitors against other cell lines and organoid model systems.

B413/P1537

Calcium as a regulator of myogenesis and differentiation of GNE deficient skeletal muscle cell: pathological relevance to GNE Myopathy

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Muscle myogenesis and differentiation is tightly regulated by calcium mediated co-ordination of regulatory factors in myoblasts and myotubes. Calcium enables actin and myosin crosslinking bridge formation thereby facilitating excitation-contraction coupling process. Altered calcium homeostasis has been observed in various neurodegenerative disorders. One such rare neuromuscular genetic disorder, GNE Myopathy (GNEM), is characterized by muscle weakness and atrophy. GNEM is caused due to mutation in bifunctional sialic acid biosynthetic enzyme GNE. Recent studies demonstrate altered calcium levels in HEK cell-based model for GNEM. Whether disrupted calcium affects muscle cell myogenesis and differentiation of GNE deficient skeletal muscle cell is explored in the present study using L6 skeletal muscle cell GNE heterozygous knock out cell (SKM-GNEHz). Our study showed (~38%) reduction in cytosolic calcium levels as measured by Fura-2AM ratiometric dye. Also 50% reduction was observed in calcium-bound Troponin C in SKM-GNEHz cells. The inhibitory proteins, Troponin I and Tropomyosin, of actin-myosin ternary complex were increased by 35% and 40% in SKM-GNEHz cells suggesting impaired cross-bridging of actin and myosin. Consequently, myosin ATPase activity was 40% reduced in SKM-GNEHz cells as compared to L6 WT. Treatment of SKM-GNEHz cells with

caffeine and calcium ionophore restored cytosolic calcium levels, TnC and inceased myosin ATPase activity by ~0.8 folds and ~2 folds, respectively. In response to reduced cytosolic calcium, the muscle cell differentiation was delayed with ~60% reduced Myosin Heavy chain (MHC) and Myo D levels. These genes are transcriptionally regulated by NFAT, which requires dephosphorylation by calcineurin for activation. We observed 50% reduced calcineurin activity and 40% reduced NFAT translocation to the nucleus that may affect reduced expression of MHC and MYO D. Microtubule Organization was disrupted with reduced number of MTOC origination and length in SKM-GNE Hz cells. The SKM-GNEHz cells were found to be arrested at G1/G0 stage. Treatment with calcium modulators restored the differentiation defect of SKM-GNEHz by 4 days and MHC/Myo D levels by ~2 folds and ~1.5 fold, respectively. Also, MTOC number and cell cycle arrest was rescued after caffeine/Ca2+ ionophore treatment. Our study indicates that regulating calcium homeostasis in GNE deficient can improve muscle motor movement and open a novel area for therapeutic development of rare neuromuscular GNE Myopathy.

B424/P1548

Characterization of Lamin A/C Function in LMNA-Related Congenital Muscular Dystrophy

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Laminopathies are a spectrum of rare human genetic disorders that are caused by mutations in the LMNA gene. Some diseases that fall under this category affect multiple tissue types, such as the accelerated aging disease progeria. Others mainly affect one tissue type, such as cardiomyopathies and muscular dystrophies. One of the more understudied laminopathies is LMNA-related congenital muscular dystrophy (L-CMD). Symptoms of this disease emerge during infancy, and worsen over time. Severe symptoms include weakening and wasting of the skeletal muscles, eventually leading to premature death. L-CMD is a dominant-negative disease caused by different de novo point mutations in the LMNA gene. Here, the focus is on the most prevalent mutation, C745T, which translates to an R249W amino acid mutation. Previous studies suggest irregular nuclear shapes; however, due to the dominant negative nature of mutant proteins, the exact location of these proteins and their impact on nuclear structure and function have not been elucidated, nor does a distinct biomarker exist for therapeutic testing. To identify hallmark phenotypes that could serve as disease biomarkers, an R249W-specific antibody was developed, and patient-derived fibroblasts were analyzed using fluorescence microscopy. Data from this revealed abnormal elongation and asymmetric distribution of Lamin A/C along the nuclear envelope. Transduction of GFP-tagged R249W- Lamin A in fibroblasts and myoblasts reproduced these phenotypes. Furthermore, not only did R249W Lamin A express asymmetric localization, but it also appeared to impair wild-type localization. Additional analysis of patient-derived fibroblasts indicated that the presence of mutant Lamin A/C also disrupted nuclear function during the cell cycle. Analysis showed that patient-derived fibroblasts had an increase in the cell population arrested in G2/M phase. In addition, there was an increased presence of aneuploid cells. These findings provide insight into how mutant Lamin A/C may disrupt nuclear organization and stability, suggesting a potential cellular biomarker for L-CMD that could inform future therapeutic studies.

B412/P1536

Chromatin Dynamics and Mesenchymal Lineage Commitment in Hutchinson-Gilford Progeria Syndrome Using Patient-Derived Fibroblasts

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Hutchinson-Gilford Progeria Syndrome (HGPS) is a rare autosomal dominant disorder caused by LMNA mutations that disrupt genome architecture and nuclear morphology. Patients show mesenchymal lineage defects such as loss of subcutaneous fat, bone abnormalities, and joint stiffness. In vitro studies of these deficits are limited by difficulties in obtaining bone marrow-derived mesenchymal stem cells (MSCs) due to patient comorbidities and the rapid senescence of patient-derived dermal fibroblasts. To address these limitations, we need a robust cellular model to examine mesenchymal lineage differentiation potential and accompanying nuclear abnormalities. In this study, we developed an enrichment approach to increase MSC-like subpopulations from healthy and HGPS fibroblasts. The enriched population displays distinct morphological characteristics and smaller cell size. With scRNA-seq, we find enhanced transcriptional expression of MSC progenitor markers across adipose, neuronal, cartilage, and vascular lineages and reduced myofibroblast signatures in enriched population. We report an increase in adipogenic differentiation potential and commitment in healthy fibroblasts, from 3.6% in basal media to 7% in enrichment media (Mann-Whitney test, $p=0.04$). Chromosome contact analysis (Hi-C) under enriched conditions reveal clusters of genes linked to fibronectin type III domains, SANT chromatin remodelers, and GTPase-activating proteins adopting a euchromatic state. In contrast, the collectins and C-type Lectin proteins associated with the fibroblast markers are characterized by a heterochromatic configuration, suggesting a suppressed fibroblast signature. In the context of HGPS-derived fibroblasts, our enrichment approach can generate enough cells to conduct Hi-C, overcoming a major limitation of the dermal fibroblast model. With enhanced yield and enriched MSCs, we utilize this model to investigate lineage commitment in HGPS patient-derived fibroblasts and to address the inconsistent findings in differentiation potential observed in previous models. Here, we demonstrate rescue of progeria MSCs from growth arrest and defective adipogenic differentiation commitment. We observe increase in the formation of mature adipocytes from 5% in basal media to 8% in enrichment media (Mann-Whitney test, $p<0.0001$). Our findings provide a robust cellular model for investigating genomic architecture changes down the mesenchymal lineage in healthy and HGPS fibroblast models, offering new insights into chromatin dynamics and potential therapeutic strategies.

B409/P1533

COP Subunit Alpha Mutations Disrupt Alveolar Epithelial Homeostasis via Aberrant STING Signaling and ISR Activation

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Type I interferonopathies are a relatively novel class of mendelian autoinflammatory diseases with two genotypes presenting lung pathology, COPA syndrome and STING associated vasculopathy with onset in infancy (SAVI). These diseases lead to the overactivation of cGAS-STING signaling with mortality highly correlated to the development of interstitial lung disease and pulmonary fibrosis (PF). We have shown that ER Stress in alveolar epithelial cells (AEC) activates the ISR and predispose individuals to developing PF. Given the observed lung pathology in these interferonopathies, we hypothesized that overactivation of STING in AECs may promote ER Stress and lead to PF in COPA and SAVI. To explore this hypothesis, we generated murine AEC lines (MLE-12s) with constitutive expression of human COPAD243G (MLED243G), COPAC1013S (MLEC1013S), or COPAWT (MLEWT) allowing us to characterize COPA dependent defects in proteostasis. ER structure, golgi structure, and STING localization each assayed by immunofluorescence (IF), while evaluation of cellular stress, protein glycosylation, and STING activation were evaluated by western blot and qPCR. Protein synthesis and degradation was assayed using puromycin labeling under varying conditions that included cycloheximide,

bafilomycin A1, and bortezomib. MLEWT exhibited a fenestrated ER morphology and a compact Golgi structure, while COPA mutant MLEs displayed fragmented Golgi morphology, and a condensed ER structure. Confirmation of STING activation in COPA mutant cell lines was observed via increased expression of cGAS-STING transcriptional targets and altered STING localization. Western blot analyses revealed altered proteostasis by the accumulation of p62 and altered processing of Heat Shock Protein 70 (BiP). Expression of COPA mutants also resulted in increased expression of ER stress genes, however phosphorylation of EIF2A suggests ISR activation. Decreased glycosylation branching noted via immunoblot analysis suggests defective posttranslational modification in COPA mutants. Puromycin labeling confirmed stalled protein synthesis in MLED243G cells however, this same assay revealed that MLEC1013S cells had greater accumulation of puromycin labeling. Together these results highlight that COPAD243G and COPAC1013S disrupt normal proteostasis leading to abnormal ER architecture and Golgi fragmentation. While qPCR indicated activation of transcriptional ER stress responses, protein analysis suggests that COPA mutant MLEs may manage defective COPI trafficking by decreasing global translation. In addition to promoting STING signaling, we see activation of ISR in our COPA mutant MLEs emphasizing a heretofore, uncharacterized consequence of overactive STING signaling in the alveolar epithelium.

B422/P1546
Effects of isoflavones in combination with chemotherapeutics in breast cancer cells
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Breast cancer is the second most common cancer among women in the United States and ~2.3 million women are diagnosed with breast cancer globally per year. Generally, combination chemotherapy has been shown to be the most effective and results in higher response rates than single-agent chemotherapy. Identifying new effective combinations could lead to potential treatment avenues that could lead to better efficacy, decreased toxicity, or delayed development of drug resistance. Isoflavones are plant-derived estrogen analogs found in many legumes, including soybeans, alfalfa sprouts, and red clover. Isoflavones potentially act in a preventative manner in breast cancer as those with diets high in isoflavones, have reduced risk of breast cancer. Additionally, they have been shown to reduce proliferation in breast and other cancer cells in cell culture. Some work has investigated the role of isoflavones in combination with traditional chemotherapy drugs, particularly in the case of genistein. We hypothesized that other isoflavones, including biochanin A, formononetin, and medicarpin, could increase the effectiveness of traditional chemotherapy drugs in breast cancer. Using ER+, PR+ MCF7 cells and triple-negative MDA-MB-231 cell lines, we tested the effectiveness of isoflavones in combination with each other and in combination with common chemotherapies belonging to the different classes of chemotherapy drugs to determine the effects on cell proliferation, cell death, and metabolism in breast cancer. We identified that isoflavones were able to further enhance the activity of some chemotherapy drugs but not others, when used as a pre-treatment and when used in combination in breast cancer. These results provide insight into potential treatment avenues for breast cancer.

B415/P1539
Efficacy and Mechanism of Plasmon Activated Water on Restenosis Prevention
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Restenosis refers to the re-narrowing of a blood vessel following balloon angioplasty or stent placement. Current strategies to reduce the incidence of restenosis focus mainly on anti-inflammatory approaches, promoting the proliferation and migration of endothelial cells, and inhibiting the proliferation of smooth muscle cells. This study aims to explore the potential of plasmon activated water (PAW) as a candidate for the prevention and treatment of vascular restenosis. We have conducted a series of in vitro and in vivo experiments. The results showed that PAW significantly enhanced the viability of vascular endothelial cells (VECs). Pharmacological experiments using serum from SD rats that consumed PAW revealed that this serum promoted the migration of endothelial cells. In the in vivo experiments, balloon angioplasty was performed on the common carotid artery of SD rats to simulate intimal layer injury. Frozen sections of these carotid arteries were analyzed to observe intimal hyperplasia and assess changes in gene expression levels in carotid artery tissues at the surgical site. The findings indicated that, compared to reverse osmosis water, PAW exhibited a significant inhibitory effect on vascular intimal hyperplasia, downregulating the expression of two genes associated with restenosis (CD44 and IL 18). Moreover, PAW was found to enhance the relative abundance of Limosilactobacillus in the intestine of SD rats, which is closely related to anti-inflammatory effects. In conclusion, PAW significantly prevents restenosis by inhibiting intimal hyperplasia, enhancing endothelial recovery, reducing oxidative stress and inflammation, and beneficially altering gut microbiota, demonstrating its potential for vascular restenosis and inflammatory disease treatment.

B408/P1532

Elucidation of the TRIO GEF1 activation mechanism and its dysregulation in neurodevelopmental disorders

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Objective TRIO is a multidomain scaffold with two GEFs—GEF1 (activates Rac1/RhoG) and GEF2 (activates RhoA)—that translate extracellular guidance-receptor cues into cytoskeletal remodeling that shapes neuronal morphology and plasticity. Large-scale sequencing has identified TRIO as the leading risk gene in neurodevelopmental disorders (NDDs), with pathogenic variants clustering in GEF1 and the adjacent spectrin repeats (SR) 6 through 9. Studies confirmed that SR6–9 acts as an intramolecular blocker that autoinhibits GEF1. We aim to address major gaps in the field: how neuronal receptors engage and relieve this autoinhibition of TRIO-GEF1, and how receptor:TRIO interactions alter Rac1 signaling and its impact on neuronal architecture and function. Methods I selected a curated panel of receptors linked to cell adhesion, neurotransmission, axon guidance, or vesicle trafficking, processes known to be regulated by TRIO. I built a stable Rac1 FRET reporter line for a quantitative screen of receptor-dependent TRIO activity. Identified receptor:TRIO interactions are validated by NanoBiT complementation assay in live cells, followed by biolayer interferometry (BLI) with purified cytoplasmic tails and TRIO fragments. Hits are subjected to interface mapping using AlphaFold-guided fragment scans and crosslinking/mass spectrometry. For receptors with native ligands, I will perform ligand titration in WT TRIO neurons and in TRIO-knockdown neurons rescued with WT TRIO or interaction-site TRIO mutants, and quantify Rac1 activation, dendrite/spine morphology, and synaptic physiology. Results In cells, TRIO GEF1 activity was recorded by a high-dynamic-range Rac1 FRET biosensor: expression of GEF1 significantly increased the FRET ratio compared with cells expressing SR6-GEF1, the autoinhibited GEF1 construct. Constitutive GEF1 activation promoted Rac1-driven cell spreading, whereas autoinhibited constructs or catalytic-dead mutants did not; notably, the SR6-GEF1R1078Q activating mutant increased cell area relative to wild-type fragments. As an initial receptor validation, GST-fused ADAM22/23 cytoplasmic tails bound full-length TRIO and its SR6-GEF1 module, making ADAM22/23 a suitable starting point to map Receptor/TRIO interaction. Conclusion Together, these data define a working platform to connect extracellular cues to TRIO-GEF1 activation and Rac1 signaling. We will use this workflow to map receptor–TRIO contact sites, generate biochemical/structural insights, and perform functional tests in neurons. By elucidating

receptor-driven control of TRIO GEF1 and the impact of disease associated variants, my work will contribute to the understanding of TRIO mediated NDD mechanisms and identify candidate targets for therapeutic intervention.

B414/P1538

Exon-Skipping Morpholino targeting the sentrin-specific protease 1 (SENP1) enhance the chemotherapeutic effects of Cisplatin and Etoposide in a non-small cell lung cancer tissue culture model

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Non-small cell lung cancer (NSCLC) is the second most common cancer and the leading cause of death in the United States, with an estimated 125,070 deaths from lung cancer in 2023. Chemotherapy remains the predominant choice for NSCLC treatment, yet chemoresistance frequently undermines its effectiveness. SUMOylation is a post-translational modification that involves the covalent attachment of SUMO proteins to other proteins. SUMOylation coordinates multiple cellular processes, including DNA repair, transcription, and cell division. SUMO modifications are reversible owing to the isopeptidase activity of the eight sentrin-specific proteases (SENPs). SENPs are involved in tumor growth and survival by regulating the SUMOylation state of various substrates. The inhibition of SENP1 can disrupt the survival, proliferation, invasion, and migration of cancer cells, along with increasing their chemical and radiation sensitivity, establishing it as a promising anti-tumor target. Here, we evaluated the synergistic effects mediated by SENP1IN (a set of Exon Skipping Morpholinos that block SENP1) and cisplatin and etoposide, two chemotherapeutic agents commonly prescribed to treat NSCLC, using A549 cells as a model system. To this end, we evaluated SENP1IN's specificity and chemosensitizing effects in A549 cells. Immunoblot analyses demonstrated that SENP1IN specifically reduces SENP1 protein levels and overall SUMO conjugation, confirming its efficacy and selectivity. A synergistic effect was found between SENP1IN and each of the chemotherapeutic agents in reducing cell migration, thus indicating SENP1IN's potential as an anti-metastatic, a major challenge in cancer therapy. Preliminary data in cytotoxicity and cell cycle analysis indicate that SENP1IN also triggers a synergistic effect characterized by increased apoptosis when combined with either cisplatin or etoposide. Therefore, SENP1IN holds the promise of providing an innovative approach to enhance chemotherapeutic outcomes in NSCLC.

B418/P1542

Identifying novel mechanisms to enhance chemotherapy response in CRC leveraging 3D cultures and anti-ribosomal antibiotics

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Loss of polarity is a hallmark of cancer, and the related epithelial-to-mesenchymal transition (EMT) phenotype also impacts prognosis and therapy outcomes, particularly in colorectal cancer (CRC). However, the mechanisms and drugs that impact these morphological changes are understudied, due to the complete failure of typical live/dead 2D high-throughput screens to capture morphology or the lack of robustness of 3D screens. We designed a high-throughput screen using 3D type I collagen cultures of CRC cells to assess morphological changes in colonies and identified several FDA-approved drugs that re-epithelialize CRC

colonies. Two of these drugs, azithromycin and linezolid, increased colony circularity, enhanced E-cadherin membrane localization, and caused transcriptomic changes consistent with downregulation of epithelial-to-mesenchymal transition. Azithromycin additionally enhanced ZO-1 localization to tight junctions and elevated sensitivity to the chemotherapeutic, irinotecan. A retrospective analysis of patient data demonstrated that the use of azithromycin in patients undergoing treatment for CRC with irinotecan had improved the 5-year survival compared to the chemotherapy alone. Based on their bacterial target, we predicted azithromycin and linezolid inhibited mitochondrial protein translation to drive the observed re-epithelialization. Inhibitors upstream and downstream of mitochondrial translation caused similar morphological changes observed with antibiotics. Linezolid treatment also caused a decrease in the expression of electron transport chain subunits. The effect of antibiotic treatment on mitochondrial function and morphology will be assessed by seahorse assay and microscopy, respectively. In parallel, photo-affinity labeling will be used as an unbiased approach to identify binding targets of both azithromycin and linezolid within the cell.

B417/P1541

Investigating the Molecular Contribution of COL12A1 and FLNB Variants to the Hypermobility and hEDS Phenotype

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COL12A1 encodes collagen type XII, a fibril-associated collagen critical for extracellular matrix organization and tendon/ligament mechanical stability. FLNB encodes filamin B, an actin-binding protein involved in cytoskeletal remodeling and mechanotransduction at the cell-matrix interface. Variants in both genes have been previously linked to skeletal dysplasias and connective tissue disorders, suggesting a possible spectrum of phenotypic consequences. Hypermobility spectrum disorders (HSD) and hypermobile Ehlers–Danlos syndrome (hEDS) are characterized by generalized joint hypermobility, chronic pain, and connective tissue fragility. While the genetic basis of hEDS remains largely unresolved compared to other EDS subtypes, emerging evidence implicates extracellular matrix and cytoskeletal genes in shaping its phenotype. In this study, we explore the potential contribution of COL12A1 and FLNB mutations injoint hypermobility and hEDS pathogenesis using computational modeling to determine the effects of clinically observed mutations in these genes. Using published genomic datasets and literature review, we evaluated reported missense variants, structural alterations, and functional assays relevant to COL12A1 and FLNB. Our analysis highlights that dysregulation of collagen fibrillogenesis (COL12A1) and impaired actin-cytoskeleton crosslinking (FLNB) may converge on pathways governing tissue and joint laxity, tissue elasticity, and mechanosensory feedback. These computational molecular findings support the hypothesis that subsets of hEDS patients may exhibit pathogenic variants or dysregulated expression in these genes, contributing to the observed heterogeneity of clinical presentation. Further studies integrating transcriptomic, proteomic, and biomechanical profiling will be essential to delineate gene-specific contributions to hEDS and advance molecular diagnostics for hypermobility disorders.

B410/P1534

Modeling a rare MOPD II patient mutation in Pericentrin

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The centrosome plays critical roles in many processes including cell division and migration, and its dysfunction has been linked to developmental disorders such as primordial dwarfism and microcephaly. To better understand the mechanisms underlying these conditions, we have developed an animal model to investigate the underlying cell biological causes of a mutation in Pericentrin (PCNT) found in a family with Microcephalic Osteodysplastic Primordial Dwarfism type II (MOPD II). This mutation results from a single amino acid deletion in the conserved PACT domain at the C-terminus of PCNT. We engineered this mutation into the Drosophila PCNT orthlolog, PLP, which we have termed plpΔR, and examined how it affected the fly at the organismal, tissue and cellular level. While the flies carrying these mutations are viable and fertile, behavioral assays revealed defects in gravitaxis and mechanosensation, suggesting impaired sensory cilia function. We also found that this mutation resulted in a reduction in wing area, suggesting issues in the development of epithelial tissues. Examining the wing as it developed, we found effects on mitosis and increased cell death. At the cellular level, we found this mutation resulted in a reduction in the amount of PLP at centrosomes and a reduction in recruitment of pericentriolar material to the centrosome during mitosis. Finally, using yeast two-hybrid and Co-IP experiments we found that this mutation disrupts dimerization of the PACT domain as well at the interaction of this domain with Asterless (Asl), the fly ortholog of another critical centrosome gene CEP152. Overall, these findings suggest that plpΔR is a hypomorphic allele with tissue-specific effects that are consistent with MOPD II's clinical manifestations. This work offers new insights into the failed cellular mechanisms that may underly MOPD II, potentially linking this human disease to the loss of a specific protein-protein interaction, in this case the interaction between PCNT and Cep152.

B425/P1549

Novel mouse models and potential treatments for a rare vision disorder Retinitis Pigmentosa caused by mutations that affect the pre-mRNA splicing factor gene SNRNP200

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Retinitis Pigmentosa (RP) is a progressive retinal degenerative disorder that affects 1:4,000 individuals world-wide. RP is characterized by degeneration of rod photoreceptors followed by loss of cone photoreceptors. Over 300 genes have been implicated in RP, including SNRNP200 encoding an RNA helicase component of the U5 small nuclear ribonucleoprotein complex essential for pre-mRNA splicing. The purpose of this study is to develop pre-clinical models and treatments for SNRNP200-associated RP. We generated mouse models possessing a dominant RP-causing point mutation in Snrnp200 and a rod photoreceptor specific knockout (cKO) mouse using the Cre/lox system. Retinal function in the progeny was assessed via electroretinography (ERG), retinal structure by optical coherence tomography (OCT), and retinal cell integrity by immunohistochemistry. The point mutation is homozygous lethal; heterozygotes show no visual defects. Using fibroblasts from the heterozygous mice we identified an siRNA that has little effect on wild-type Snrnp200 mRNA levels and reduced mutant Snrnp200 mRNA by 61% and protein levels by 75% compared to controls, suggesting a possible treatment. Snrnp200 homozygous cKO mice showed reduced rod ERG amplitudes compared to that of controls while heterozygotes showed no defects. By four weeks of age, rod responses were nearly absent, and cone responses were diminished, while controls amplitudes remained robust. OCT analysis showed mild outer nuclear layer thinning at three weeks, and a dramatic reduction by four weeks. This was confirmed by immunohistochemistry showing decreased photoreceptor markers by four weeks of age, with only minimal staining of cones remaining. Despite this loss, a subset of bipolar cells, interneurons that transmit signals from the photoreceptors to ganglionic cells, remained. The retention of biopolar cells suggest

replacement of photoreceptor precursor cells as a potential therapy. Our findings demonstrate that the Snrnp200 cKO mouse recapitulates key features of human RP, notably the sequential degeneration of rod followed by cone photoreceptors. It is important to note that the cone photoreceptors possess a wild-type genotype, strongly suggesting that their death is a result of rod death. The accelerated retinal degeneration in this new model provides a valuable platform for studying early molecular events underlying photoreceptor death. Moreover, this model enables the testing of time-sensitive therapeutic strategies aimed at preserving cone function, with implications that extend to a broad range of RP caused by mutations in other genes.

B411/P1535

Pathogenic mechanisms of SPTBN1 syndrome variants in human cortical neurons

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βII-spectrin is an abundant cytoskeletal protein highly expressed in cortical neurons, where together with its binding partner αII-spectrin forms a submembrane lattice that organizes micron-scale domains of transmembrane proteins. βII-spectrin also facilitates organelle transport and likely provides structural support and flexibility to neurons. Loss of brain βII-spectrin in mice leads to early postnatal lethality and significant alterations in cortical development and axonal connectivity. In humans, pathogenic heterozygous variants in SPTBN1, the gene encoding βII-spectrin, cause a rare, early-onset neurodevelopmental syndrome characterized by speech and motor delays, intellectual disability, comorbid with autism spectrum disorder, ADHD, epilepsy, dysmorphisms, and cortical deficits, collectively known as SPTBN1 syndrome. Over half of SPTBN1 syndrome variants cluster in βII-spectrin's calponin homology domains (CH-D). Using CRISPR/Cas9, I generated an allelic series of SPTBN1 syndrome iPSC lines harboring CH-D variants and differentiated into cortical neurons to interrogate cellular pathogenic mechanisms that may contribute to the syndrome. Several pathogenic variants cause soma-localized aggregation of βII-spectrin and αII-spectrin in both iPSCs and neurons, accompanied by reduced βII-spectrin protein levels, stability, and solubility, as well as aberrant axon initial segment (AIS) structures. The aggregates cause upregulation and recruitment of proteostasis machinery. Notably, expression of the Hsp104 disaggregase or treatment with an Hsp70 activator cause reduction in βII-spectrin aggregate volume, indicating a potential future therapeutic strategy. Future experiments will assess functional consequences of the observed morphological deficits.

B427/P1551

Pathogenic Mechanisms Underlying CASK-linked Epileptic Encephalopathy

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Background: CASK is an X-linked intellectual disability gene. CASK heterozygous loss causes microcephaly with pontine and cerebellar hypoplasia while CASK hemizygous loss produces early infantile-onset epileptic encephalopathy (EIEE) which is highly devastating and fatal. The mechanisms underlying CASK-linked EIEE remain unclear. Methods: Using Cre-LoxP conditional knockout strategy, we generated a novel mouse model of EIEE harboring neuron-specific CASK deletion (CASKNKO). CASKWildtype, CASKfloxed, and CASKwildtype: SynCre transgenic mice were used as controls. Experiments were performed to determine mice brain electrical

activity using video-EEG analysis, immunohistochemical stainings to examine the expression of CUX1, TBR1 and GFAP proteins, and Western blots to determine the levels of synaptic and mitochondrial proteins. Mitochondrial respiration, morphology and number, and the NAD+/NADH ratio were also examined in the mice brain. Results: CASKNKO mice showed severe growth retardation, early-infantile-onset tonic spasms and myoclonus, and death by P25. At P18, we observed decreased cerebral cortical thickness, increased neuronal density, and CUX1/TBR1 mis-expression in the CASKNKO mice brain. Abnormal brain electrical activity, altered excitatory/inhibitory neurotransmission, and reactive astrogliosis were also observed in the CASKNKO mice. Reduced mitochondrial respiration, number of mitochondria, and NAD+/NADH ratio were observed in the CASKNKO mice brain. Interestingly, we found an increase in DRP1 expression level in the brain of CASKNKO mice. Conclusions: Our results suggests that neuronal CASK loss causes cortical malformation, excitatory/inhibitory synapse imbalance, and mitochondrial dysfunction, which may contribute to the pathogenesis CASK-linked EIEE.

B416/P1540

Phosphoinositide Phosphatase Myotubularin (MTM1) Regulates Myonuclear Architecture and Regional Chromatin Modifications

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X-linked myotubular myopathy (XLMTM) is a severe congenital muscle disease that primarily affects boys and is characterized by ventilator and wheelchair dependence and, in many cases, early death. XLMTM is caused by mutations in the MTM1 gene, which encodes a phosphatase that dephosphorylates second messenger lipids PI3P and PI3,5P2 and has roles in endosomal lipid dynamics and trafficking. Pathologically, the disease is defined by hypotrophic muscle fibers containing disorganized organelles and myonuclei with aberrant appearance and localization. However, despite the fact that XLMTM is partly defined by the appearance of abnormal myonuclei, the contributions of the nuclear alterations to XLMTM pathogenesis are unknown, and the reason(s) for the nuclear aberrations are not well understood. In this study, we provide insight into these longstanding open questions by demonstrating for the first time a role for MTM1 in myonuclear structure and genome architecture, abnormalities in which lead to alterations to the myogenic gene program and muscle development. While previous studies have classified MTM1 as a strictly cytoplasmic protein, we have discovered using high-resolution confocal and immunoelectron microscopy that a subpopulation of MTM1 protein localizes to the nucleus and nuclear envelope. Furthermore, Mtm1 deficiency in vivo gives rise to ultra-structurally abnormal myonuclei ($p<0.0001$), altered genome organization (>30% of the genome), and shifted chromatin accessibility (16% of genomic sequences). Excitingly, introduction of nuclear targeted-MTM1 in the muscle of Mtm1-deficient mice significantly improves the XLMTM phenotype, with increased myofibril size, increased mouse rears (11 vs 6.3, $p<0.05$), and prolonged survival (50 vs 38 days, $p<0.05$), demonstrating an essential role for MTM1 at the nucleus. We are currently investigating the sub-nuclear localization of MTM1 and MTM1 nuclear interactome using high-resolution microscopy and proximity labeling followed by mass-spectrometry. Together, these findings present a paradigm shift in the understanding of MTM1 function during muscle development and provide new insight into the underlying mechanisms of XLMTM pathology.

B426/P1550

Phosphoribosylformylglycinamidine synthase (PFAS) deficiency: Clinical, genetic, and metabolic

characterization of a novel defect in purine de novo synthesis

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Objectives: De novo purine synthesis involves 10 reactions catalyzed by six enzymes, including phosphoribosylformyglycinamidine synthase (PFAS). To date, genetic defects of three of these enzymes, namely ATIC, ADSL and PAICS, have been characterized in humans. Here, we report for the first time two individuals with PFAS deficiency. Results: Probands were identified through metabolic and genetic screening of neurologically impaired individuals. The pathogenicity of the variants was established by structural and functional studies. Probands C1 and C2 presented with prematurity, short stature, recurrent seizures, and mild neurological impairment. C1 had elevated urinary levels of formylglycine amide riboside (FGAr) and bi-allelic PFAS variants encoding the NP_036525.1:p.Arg811Trp substitution and the NP_036525.1:p.Glu228_Ser230 in-frame deletion. C2 is a 20-year-old female with a homozygous NP_036525.1:p.Asn264Lys substitution. These amino acid changes are predicted to affect the structural stability of PFAS. Accordingly, C1 skin fibroblasts showed decreased PFAS content and activity, with impaired purinosome formation that was restored by transfection with pTagBFP_PFAS_wt. The enzymatic activities of the corresponding recombinant mutant PFAS proteins were also reduced, and none of them after transfection corrected the elevated FGAR/r levels in PFAS-deficient HeLa cells. Conclusion: While genetic defects in purine de novo synthesis are typically considered in patients with severe neurological impairment, these disorders, especially PFAS deficiency, should also be considered in milder phenotypes. FUNDING: Grants: NU23-01-00500 from the Ministry of Health of Czech Republic, MULTIOMICS_CZ (Programme Johannes Amos Comenius, Ministry of Education,Youth and Sports of the Czech Republic,//ID Project CZ.02.01.01/00/23_020/0008540) – Co-funded by the European Union. The work presented here have been published previously: Marie Zikanova et al: Phosphoribosylformylglycinamidine Synthase (PFAS) Deficiency: Clinical, Genetic and Metabolic Characterisation of a Novel Defect in Purine de Novo Synthesis. J Inherit Metab Dis. 2025 May;48(3):e70041. doi: 10.1002/jimd.70041. PMID: 40421664; PMCID: PMC12107509.

B420/P1544

Screening for Vaccinia-Related Kinase 1 inhibitors to alleviate pancreatic ductal adenocarcinoma progression as a novel adjuvant

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Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive cancer which remains one of the deadliest cancers with a 5-year survival rate of 12.5%. PDAC is particularly malignant in part due to rapid progression, early metastasis, late diagnosis, and limited response to existing treatments. Many first-line treatments, such as Gemcitabine and FOLFIRINOX, work by inhibiting DNA synthesis to induce cellular death, but have failed to substantially improve even 5-year survival. This is likely because PDAC exists in a cold tumor microenvironment and is resistant to therapies due to an immunosuppressive environment. The Vaughan laboratory is studying how the induction of cellular senescence and the activation of the c-GAS-STING pathway could provide a novel mechanism for targeting PDAC. It has been demonstrated previously that cellular senescence can occur following disruption of nuclear envelope integrity, such as in Progeria disease. Additionally, the Vaughan lab has demonstrated that the interaction between two nuclear envelope proteins, Lamin A and Barrier-to-autointegration factor (BAF), is necessary for nuclear envelope integrity. It is known that Vaccinia-Related Kinase 1 (VRK1) phosphorylates BAF, and phosphorylated BAF interacts with Lamin A. We

have shown that inhibition of VRK1 by known VRK1 inhibitors disrupts the nuclear envelope, but these inhibitors are not ideal drug candidates. The goal of the current project is to find potent, selective inhibitors of VRK1 which inhibit the phosphorylation of BAF and disrupt the nuclear envelope. To this end, we are currently optimizing a kinase assay to measure VRK1-mediated phosphorylation of BAF using the ADP-Glo Kinase Kit. We have optimized substrate and kinase concentrations and have shown the ability of known VRK1 inhibitors to decrease BAF phosphorylation events. We plan to screen 12,000 known pharmacophores from the KINASet Library in collaboration with Dr. Lan Chen at the Chemical Genomics Facility at Purdue University. Following the screen, we will use our established assays to measure the effect of the VRK1 inhibitors on nuclear blebbing, cellular senescence, and immune activation, and cytotoxicity. Overall, this work directly contributes to advancing our scientific understanding of PDAC and the discovery of novel VRK1 inhibitors which could serve as novel adjuvants to enhance the efficacy of PDAC therapies.

B428/P1552

The coordination of voltage-gated sodium channels via ankyrin protein interactions

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Voltage-gated sodium channels (NaVs) are key regulators of neuronal excitability, making the dysfunction of NaVs a serious public health concern. Genetic variants in SCN2A, which encodes the neuronal sodium channel NaV1.2, have been identified in human patient cohorts of autism spectrum disorder (ASD) and developmental and epileptic encephalopathy (DEE). While variants within SCN2A commonly affect the biophysical properties of the channels themselves, in some cases these variants do not impact the channel properties. This suggests that channel dysfunction may contribute to disease through other means such as alterations in localization or binding partners. The proper localization of NaVs is dependent on their interactions with ankyrins, a family of intracellular scaffolding proteins that link membrane-bound proteins to the underlying cytoskeleton. Ankyrin-G, encoded by ANK3, has been established as the master organizer of the axon initial segment (AIS), which is a key site of neuronal excitability. Here, ankyrin-G scaffolds NaV1.2 during early development. More recently, our lab identified a critical interaction between NaV1.2 and ankyrin-B, encoded by ANK2, in mature neocortical pyramidal cell dendrites, where NaV1.2 regulates dendritic excitability. This study aims to investigate the molecular interactions between ankyrins and NaVs, focusing on how SCN2A disease variants impact the ankyrin/NaV binding interface and channel localization. To do so, molecular, biochemical, electrophysiological, and imaging techniques are used. Our preliminary data indicate that several mutations within SCN2A do not affect the electrophysiological properties of the channel, while decreasing the binding affinity between ankyrins and NaV1.2. These findings suggest that a disruption between ankyrin/NaV binding could underly disease phenotype. This work will provide insights into ankyrin/NaV interactions and their role in neurodevelopmental disorders.

B419/P1543

The Phytochemical Diosgenin Modulates the Expression of Chemoresistance- and Metastasis-Associated Genes in EGFR-Mutant Non-Small Cell Lung Carcinoma Cells

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Lung cancer is the leading cause of cancer-related mortality worldwide. Non-small cell lung carcinoma (NSCLC), the most prevalent subtype, presents significant clinical challenges due to acquired resistance to therapy and metastasis. In a subset of patients, this resistance is driven by activating mutations in the Epidermal Growth Factor Receptor (EGFR), which promotes uncontrolled cell proliferation. Phytochemicals are increasingly investigated as sources for novel anticancer agents. Diosgenin, a plant-derived steroidal saponin, has demonstrated anticancer properties in various tumor models; however, its role in EGFR-mutant NSCLC remains poorly understood. This study aimed to evaluate whether Diosgenin modulates the expression of genes involved in chemoresistance and metastasis in the H1975 NSCLC cell line, which harbors EGFR-driver mutations (T790M/L858R). To assess this, H1975 cells were treated with Diosgenin for 24 hours. Total RNA was subsequently extracted, reverse-transcribed to cDNA, and analyzed by RT-qPCR to quantify the expression of markers for chemoresistance (EGFR, ABCB1, NFKB1, STAT3, FOXM1), metastasis (NDRG1, MMP2, MMP9, RAC, PREX1), and immune signaling (PDL1, CD47, IL6, TNF-α). Preliminary findings indicate that Diosgenin downregulated the expression of several genes known to promote chemoresistance (FOXM1, NFKB1, ABCB1), metastasis (MMP2, MMP9), and pro-inflammatory signaling (IL6, TNF-α). Conversely, Diosgenin upregulated the expression of NDRG1, a known metastasis suppressor gene. No significant changes in expression were observed for other key markers, including EGFR, CD47, PDL1 and STAT3. These results suggest that Diosgenin has the potential to modulate key pathways contributing to therapeutic resistance and cancer progression in EGFR-mutant NSCLC. Further research is required to fully elucidate its molecular mechanisms and therapeutic potential.

Genome Biology - Chromatin structure and dynamic

B034/P0618
Annotation of Protein Coding Genes in D. willistoni Contig18
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With the completion of the Drosophila melanogaster genome in 2000, new opportunities emerged for studying heterochromatic regions and exploring the genomes of other Drosophila species. The Genomics Education Partnership (GEP) contributes to this effort by annotating and analyzing Drosophila genomes to investigate specific genomic regions through the study of amino acids, nucleotide, and exon sequences. The primary objective is to improve genome annotations and gain insights into evolutionary patterns across different Drosophila species by identifying conserved elements and unusual genomic features. Such comparative genomic analysis contributes to a broader understanding of chromosome structure, function, and evolutionary conservation. In this project, contig18 a region on chromosomes 3R (E element) of Drosophila willistoni was analyzed. Comparative bioinformatics methods were used to study and annotate this genome, specifically to determine the number of genes present in this contig using D. melanogaster as a reference and utilizing the GEP's genome annotation tools. Initial predictions indicated that there were about 31 genes in this region, our results show that only 7 genes truly exist in contig18 and are homologous to genes found in D. melanogaster. These include Mekk1-PD, Mekk1-PB, ChAT-PA, ChAT-PB, VAChT, CG7714, and CG7715

B035/P0619
Bayesian Inference of cohesin loop extrusion kinetics: searching for a data-driven effective description
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Genome folding, and specifically loop extrusion driven by SMC proteins such as cohesin, are believed to play key roles in regulating time-gated, long-range genomic interactions. Due to such interactions, enhancers can control expression of genes located up to a million of base pairs away along a linear sequence. Mutations of cohesin sub-units rank top among 12 genes significantly mutated across cancers, and also underlie other congenital developmental disorders, “cohesinopathies”. Determination of in vivo loop extrusion speed and its microscopic kinetic rules from live chromatin imaging have been a challenging task. This is due to the polymer connectivity of the chromatin fiber: loop extruders continuously pull the entire fiber, of which the imaging loci are a low dimensional projection. Several claims have been made about extrusion speeds based on these data; however, their mathematical rigor is under debate. Here, we plan to use Bayesian inference to detect cohesin loop extrusion events in vivo using existing microscopy data of mESC. Our Bayesian inference scheme uses the physical model of the chromatin and the kinetic model of the cohesin as priors, takes in the microscopy data as likelihood, and outputs the probability distribution of the inferred cohesin parameters (e.g. loop extrusion speed). We first demonstrate that, for a simple toy model, a bead-spring system undergoing Brownian motion with randomly switching spring constants, we can infer the spring states well from discrete-sampled bead trajectories with sub-unity signal-noise ratio. We plan to further push this to the whole polymer regime, as their mathematical structures are completely identical. Another question we explore concerns a data-driven effective description of cohesin’s microscopic motion. Recent advances in single molecular in vitro experiments allowed the measurement of microscopic motion of cohesin on bare DNA, revealing that besides regular extrusion, cohesin loops can also slip and even shrink. But how differentiable is our current microscopy imaging data to different microscopic models? Bayesian inference provides a rigorous framework to answer it by calculating the ELBO function of a given model prior.

B027/P0611

Characterizing the role of loop extrusion in homologous recombination using chromosome polymer simulations

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Homologous recombination (HR) depends critically on the identification of damaged chromatin's homology site, requiring a search along the sister chromatid. Using the polychrom software, we employ 3D molecular dynamics simulations of chromatin to model the role of loop extrusion in the homology search process. We demonstrate that the recruitment of cohesins to damaged chromatin and associated loop extrusion at double-strand break (DSB) sites is sufficient to explain distinguishing features of experimental Hi-C data. Our simulations further support a model in which DSB ends become tethered after damage in a DNA-PKcs autophosphorylation-dependent manner. Examination of first passage times between the break site and the remainder of the simulated chromatin reveals that loop anchoring properties of the DSB site and the recruitment of cohesins to the damaged chromatid accelerate searches in regions proximal to the break site. Simultaneously, these features spatially constrain the search process by lengthening first passage times to distal regions.

B042/P0626

Chromatin organization regulates plasma membrane tension and cell integrity independently of gene expression

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Primarily studied for its role in gene expression, chromatin organization is emerging as an important regulator of nuclear mechanics. Although the nucleus is in mechanical equilibrium with the cell, we do not know whether and how chromatin reorganization actively regulates the mechanical properties and downstream behaviors of cells. Here, we tested the hypothesis that as a dynamic crosslinked polymer, chromatin directly impacts cell mechanics independently of transcription by studying NETosis: a transcription-independent immune process where chromatin decompacts and the plasma membrane (PM) ruptures. Using high resolution microscopy and ATAC-see, we found that chromatin accessibility progressively increases during NETosis suggesting that chromatin binding proteins (CBPs) dissociate from chromatin during NETosis. To determine the identity and dynamics of these dissociated CBPs, we used fluorescent-recovery after photobleaching to measure the mobility and localization of the linker histone H1, the nucleosomal histone H3 and the heterochromatin binding protein HP1α. We found that the mobile fraction of H1 increases during NETosis while HP1α diffuses faster outside of the nucleus suggesting that it becomes a cytosolic osmolyte. Using optical tweezers, we found that chromatin decompaction during NETosis increases PM tension, independently of the cytoskeleton. In non-NETing U2OS cells, we found a similar PM tension increase upon induced chromatin decompaction and disrupted cytoskeleton, suggesting that chromatin is a global mechanical integrator. We theorized that chromatin might regulate PM tension by generating cellular osmotic pressure. Lattice light sheet microcopy experiments indicate a 10% increase in volume during NETosis, supporting our theory. Our findings reveal a novel non-genetic role of chromatin in cellular biophysics: regulating cell volume, PM tension, and thus, overall cell mechanics. Considering the critical role of cell mechanics in biological processes such as cell migration, proliferation and pathogen killing, our work broadens our understanding of how chromatin regulates cell physiology and pathology.

B036/P0620

Control of non-LB1 genomic assembly and nucleolus formation by NEMP1

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NEMP1 is a transmembrane protein located in the inner membrane of nuclear envelope. Loss of Nemp1 resulted in metazoan infertility but the molecular mechanism is unknown. By using immunofluoresence staining of mouse full grown oocytes, we found that NEMP1 homologous null mutation (Nemp1-/-) lead to global genomic assembly defects, manifesting as multi-nucleoli with perinuclei distributed heterochromatin, a decrease of H3K9me2 modifications and meiotic arrest. Meanwhile, in Nemp1-/- Mouse embryonic fibroblast cells (MEFs), metaphase spindle width is significantly enlarged, suggesting a global genomic assembly defects during chromosomal condensation. MEFs overexpressing NEMP1-GFP fusion proteins were also used for NEMP1 genomic target analysis by using CUT&RUN technology and GFP antibody. The preliminary data analysis results showed that NEMP1 is binding to non-LB1 genomic regions. Intriguingly, among these targets, rDNA clusters are included, suggesting a potential role of NEMP1 in regulating nucleolus formation by directly binding to rDNAs. CUT&RUN results also showed a binding of NEMP1 on subtelomere regions and tandemly repeated gene clusters. Further verification of the binding targets in mouse oocytes, characteristics of DNA modification and histone modifications within the NEMP1 targets are under analysis. In conclusion, NEMP1 has potential role to regulate genomic assembly via binding to non-LB1 genomic region.

B025/P0609

Dbp7 interacts with RNA exosome component Dis3 to mediate CENP-A loading to centromeres

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Centromeres are crucial for proper chromosome segregation during cell division. Centromeres in most eukaryotes are epigenetically specified by the histone 3 (H3) variant, CENP-A. How CENP-A precisely targets to centromeres remains elusive. Centromeres are typically transcribed into non-coding RNAs, which have been implicated in centromere functions. But the exact role of centromeric RNAs in CENP-A loading is still poorly understood. In addition, centromere transcription induces the formation of RNA-DNA hybrids termed R-loops. Excessive R-loops lead to genome instability. Little is known about the mechanism used for safeguarding centromeres against R-loops. Here we show that through a visual genetic screen, we identified a conserved RNA binding protein Dbp7 that plays a critical role in CENP-A loading to centromeres. Deletion of dbp7+ results in CENP-A mislocalization and defects in chromosome segregation and centromere silencing. We found that Dbp7 associates with centromere transcripts, and further showed that Dbp7 prevents accumulation of R-loops at centromeres. Our domain-deletion analysis demonstrated that the DEAD-box helicase domain and the C-terminal extension motif of Dbp7 contribute to its role in centromere function. In addition, Dbp7 interacts with the key exosome subunit Dis3. Importantly, we found that Dis3 promotes CENP-A centromere loading by interacting with the CENP-A chaperone Sim3. Together, this study uncovered a previously unrecognized CENP-A loading mechanism, by which Dbp7 and Dis3 act together to bind to centromeric transcripts and in turn mediate recruitment of CENP-A via the CENP-A chaperone Sim3. Our work also provides mechanistic insight into R-loop resolution at centromeres.

B037/P0621

Decoding Trans-Chromosomal Interactions During Differentiation Using The Oct4 Gene Locus

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Interchromosomal interactions are rare but critical and highly regulated processes in mammals (Maass et al., 2019). One example is the trans-allelic pairing event at the Oct4 locus, which is associated with Oct4 repression during mouse embryonic stem cell differentiation and is concordant with the deposition of H3K9me2 repressive histone marks at the locus (Hogan et al., 2015). Since Oct4 repression is triggered by differentiation signals such as LIF/STAT3 withdrawal and retinoic acid (RA)-induced pathways, this pairing event highlights a direct but not yet fully understood link between upstream signaling networks, gene regulation, and chromatin structure. This event also highlights an important but not fully understood interplay between gene regulation, epigenetic states, and trans-allelic chromatin interactions. To address the interplay between these different players, we use sequential OligoSTORM, a super-resolution imaging technique, utilizing Fluorescent In Situ Hybridization (FISH) with Oligopaints (Beliveau et al., 2012). This approach provides single-cell insights into the chromatin structure of homologs in 3D. Here, we describe how different differentiation pathways lead to different speeds of Oct4 gene repression and can be used to follow the step-by-step process of allelic pairing. These protocols also provide insights into cell-population heterogeneity, highlighting the importance of a single-cell approach. Differentiation of mESCs also results in a decrease in active histone marks at the Oct4 gene locus (for the -LIF/+RA media), a decrease in pluripotent markers, and induces morphological changes.

We reveal 3D chromatin structure of the Oct4 locus using sequential OligoSTORM during this differentiation process and correlate between chromatin macro- and microstructures, gene expression, and epigenetic or pluripotency factor states at the single-cell level. Oligopaint libraries have also been designed to target other genes, including additional pluripotency factors (Nanog, Sox2, Klf4, Myc), to investigate their potential co-localization and co-regulation. Together, these results provide a framework to connect differentiation signaling pathways, such as LIF/STAT3 and RA-driven networks, with changes in chromatin architecture and trans-chromosomal interactions during cell fate transitions.

B038/P0622
Determining the distribution of histone modifications throughout regeneration in Stentor coeruleus
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Studying regeneration in animals informs us about how cells develop and organize themselves in a functional multicellular structure. Similarly, unicellular regeneration requires individual cells to sense and form patterns of intracellular structures, but these processes are less understood. To understand how single cells are able to regenerate, we used an emerging model system, Stentor coeruleus ("Stentor"). Stentor are giant unicellular ciliates, known to regrow both posterior and anterior halves following bisection. Stentor's transcriptionally active macronucleus is arranged in nodes that run along the length of the cell, but how chromatin is arranged among these nodes, including during regeneration, is unknown. We hypothesize that posterior tails growing their anterior half, which contains a necessary structure for survival, will contain more histone modifications that correlate with active transcription than vice versa. Preliminary results show cells in vegetative growth contain H3K4me3 and H3K27ac marks, both of which are correlated with active transcription in ciliates. H3K27ac levels may also increase in posterior nodes 2- and 8-hours post bisection which correspond to the timepoints of the most dramatic nuclear shape change. Understanding how genome organization contributes to unicellular regeneration is fundamental to our knowledge of how cells sense and form patterns of intracellular structures that are necessary for survival.

B029/P0613
Dynamic metabolite signaling to chromatin links the microbiota to gene regulation
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The intestinal microbiota profoundly influences host physiology and metabolites produced by the microbiota are a major way of communication with the host. Yet the mechanisms by which microbial metabolites regulate cellular processes within complex tissue environments remain incompletely understood. Among these metabolites, short-chain fatty acids (SCFAs) are produced by the fermentation of fiber and accumulate to high levels in the intestinal lumen. These small molecules modulate host signaling, metabolism, and chromatin structure. We previously identified microbiota-dependent histone modifications in the mouse intestine that are derived from SCFAs. Several of these posttranslational modifications, including the understudied modifications of histone butyrylation and propionylation, are enriched at active gene regulatory elements in primary intestinal epithelial cells and are linked to the expression of metabolic gene programs. We further hypothesized that SCFA signaling to chromatin regulates gene expression in the human colon through histone modifications. We now show that histone butyrylation is detected in human colon tissues. In colon cancer cell

lines, histone acetylation, butyrylation, and propionylation respond dynamically to SCFA levels and localize to active regulatory regions. Treatment with individual SCFAs or physiologically relevant combinations reveals distinct and overlapping transcriptional responses, with butyrate exerting dominant regulatory effects. Notably, a subset of butyrate-induced gene expression changes are independent of HDAC inhibition and instead require p300/CBP activity, implicating histone butyrylation as a direct mediator of transcriptional regulation. Together, these studies uncover a mechanistic link between microbial metabolites and chromatin-based gene regulation.

B030/P0614

Heterochromatin Protein 1 Dysregulation in Amyotrophic Lateral Sclerosis and Frontotemporal Dementia

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Eukaryotic DNA is compacted into chromatin, a DNA-protein complex. Chromatin can be divided into euchromatin, which is actively transcribed, or heterochromatin, which is transcriptionally silent. Heterochromatin Protein 1 (HP1) is a key heterochromatin protein, which interacts with Histone H3 when it is trimethylated on Lysine 9. Much like histone proteins, which comprise the core of the basic repeating unit of chromatin, several chromatin proteins undergo posttranslational modifications (PTMs). Previous work has uncovered PTMs on HP1. Through Molecular Dynamic simulations, we determine how HP1-H3 interactions can be significantly impacted by PTM alterations. Some of the HP1 post translationally modified residues that significantly impact binding include K24, K39 and S48. We carried out simulations, altering specific PTM residues across trials and running simulations interactions. We then gathered the raw data involving the contacts between residues in each trial and wrote a script to count the total number of contacts. Comparing trials, we observe significant variation across trials based on the PTM's added to specific residues. Specifically, K24 seems to vary greatly in binding affinity with H3 when formylated, monomethylated, or acetylated. Our data demonstrates effects of PTMs on the binding affinity between HP1 and H3. These findings shed light on the effects of a changing HP1 PTM landscape on a molecular scale and expand our understanding of chromatin.

B031/P0615

Histone deacetylation drives apoptotic chromatin compaction to limit DNA shedding from the dying cell

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Chromatin compaction regulates genome accessibility, shaping gene expression and cellular identity throughout the life of a cell. During apoptosis, genome-wide compaction converts a mixture of open and closed interphase chromatin into one coalesced, dense mass. This is thought to suppress immune stimulation by DNA outside the nucleus, however the mechanism and function of apoptotic chromatin compaction remain unclear. A second hallmark of apoptosis is genome fragmentation, exposing DNA to the cytosol. To avoid inflammation, cytosolic DNA-sensing pathways are inactivated in apoptosis. Fragmentation also raises the possibility of DNA escaping the cell in apoptotic bodies (ApoBDs), extracellular vesicles with a crucial role in intercellular communication. Despite mixing of nuclear and cytosolic compartments, ApoBDs exclude DNA,

preventing excessive immune stimulation of surrounding cells during apoptotic clearance. Here, we investigate the pathways underlying apoptotic genome compaction. We find that histone deacetylation is a key feature of apoptosis, necessary for chromatin compaction and exclusion of DNA from ApoBDs. Using synthetically-designed proteins, we probe the role of electrostatic interactions in this process and propose a charge-based model for deacetylation-driven compaction. Our findings provide a mechanistic understanding of apoptotic genome reorganisation, and give further insight into principles governing chromatin organisation in both living and dying cells.

B032/P0616

Insights into Chromatin Packaging From Tissue-specific Chromatin Profiling in C. elegans

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Our lab studies chromosome organization, focusing on meiosis in C. elegans. Chromosomes and nuclei undergo extensive remodeling during meiotic progression. We investigate how this remodeling occurs and how it contributes to meiotic processes such as homolog pairing and double-strand break formation. High-resolution cytology has been instrumental for visualizing meiotic progression. To gain a genome-wide view of meiotic chromatin, we have been developing high-resolution chromatin profiling methods. To circumvent the high input requirements for ChIP-seq, we have implemented and optimized CUT&RUN and CUT&Tag. We find that we can generate high-quality, reproducible data starting from only ~15-50 gonads (each containing ~1,000 cells), which can be dissected in less than an hour, greatly streamlining our experimental workflow and enabling analysis of mutants and other difficult samples. We have recently extended these methods to profile histones in intestinal cells, which yielded new technical and biological insights. Intriguingly, when we compared CUT&RUN and CUT&Tag profiles between germline and intestinal cells, we observed striking differences. In intestines, both methods produced largely concordant H3K4me3 profiles with sharp promoter peaks. In germline nuclei, peaks were considerably broader for both methods. Although H3K4me3 enrichment was still greatest at promoters, it extended further into the gene body, with the promoter-gene body contrast particularly reduced in CUT&RUN profiles. We believe that these observations reflect both biological differences, including higher H3K4me3 levels in gene bodies and greater chromatin compaction in meiotic relative to somatic cells, as well as method-specific effects. While the Tn5 transpososomes used in CUT&Tag can cut DNA only once, the MNase used in CUT&RUN can make multiple cuts wherever it is tethered. The higher compaction of meiotic chromosomes may cause the CUT&RUN signal to spread by bringing more nucleosomes close to the antibody binding sites, an effect that is more subtle in the CUT&Tag data. This spreading effect causes CUT&RUN metagene profiles for three canonical histone modifications – H3K4me3 at active promoters, H3K36me3 along active gene bodies, and H3K27me3 across repressed gene bodies – to deviate from CUT&Tag and ChIP-seq in the germline. Our study demonstrates that CUT&Tag is better suited than CUT&RUN for profiling compact chromatin. Our streamlined protocol not only broadens access to chromatin profiling in challenging contexts but also creates a foundation for investigating how chromatin landscape differs between germline and somatic tissues, and for uncovering principles that govern genome organization across tissue types.

B039/P0623

Investigating Histone Modifications Linked to the Prion [PIN+] in Saccharomyces cerevisiae

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Chromatin, the DNA-histone complex forming chromosomes, is epigenetically regulated by post-translational histone modifications (Histone PTMs). These modifications are done on the N-terminal tails of the histone which can either promote or suppress transcription. In yeast, prions are proteins that adopt heritable alternative conformations which help cells adapt to challenging environments. Based on these heritable traits, yeast prions could be considered another mechanism of epigenetics. The yeast prion, Rnq1 (Rich in Asparagine and Glutamine), in its prion state [PIN+], promotes the formation of the prion [PSI+] and is prevalent in yeast, suggesting that it is beneficial to yeast. Previous findings associate [PIN+] with reduced histone H3 PTMs, indicating a connection to epigenetic regulation. Using western blotting techniques, we examined whether [PIN+] influences histone H4 PTMs but observed no differences compared to the [PIN-] non-prion state in Rnq1. Future studies will explore PTM levels in other histones, such as histone H2B.

B033/P0617
Long-range gene regulation requires both cohesin-mediated loop extrusion and tethering elements in the Drosophila embryo
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Genes regulating developmental programs are often controlled by enhancers located far away from their cognate promoters. Previous studies have demonstrated that protein-oligomerization anchors, known as tethering elements in Drosophila, are required for efficient enhancer-promoter communication in both insects and vertebrates. However, it was unclear if tethering elements were sufficient to establish complex gene regulation programs during the rapid cell cycles in the early Drosophila embryo. Here, we have established an auxin-inducible system to abrogate cohesin-mediated loop extrusion by depleting endogenous Nipped-B protein in Drosophila embryos. Combining this inducible depletion with live transcriptional imaging during Drosophila genome activation, we show that loop extrusion, anchored at an enhancer-proximal CTCF site, plays a role in reducing search time for a long-distance enhancer-promoter interaction of ~240 kb. We propose a two-step model, whereby loop extrusion strongly regulates the timing of gene activation while tethering elements affect levels of expression.

B043/P1807
Measuring Force Propagation and Mechanical Properties of the Chromatin in Live Cells
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In cells, long DNA polymers are highly compacted into a spatially organized, yet dynamic, three-dimensional chromatin structure. Chromatin dynamics underlies core genomic processes such as transcription, replication and DNA repair. The dynamics of chromatin is determined by the response of the DNA polymers and associated factors to a combination of thermal fluctuations and active processes. Chromatin also plays a mechanical role by contributing mechanical stiffness against nucleus deformations from the outside forces. Here, we are seeking to infer mechanical properties of the chromatin in its natural context by measuring how mechanical agitation applied outside of the nucleus propagates through chromatin fibers. To this end, we

have developed a live cell assay that utilizes natural agitation of the centromeres by cyclic, microtubule-dependent motion of the spindle pole body (SPB) in fission yeast S. pombe. Using fluorescent microscopy and high-precision single particle tracking of individual chromosomal loci, we measure the correlation in motion between the agitation point (centromere) and a distant 'probe' locus in a library of strains with varied genomic distances between the centromere and the 'probe' locus. Unexpectedly, we observe correlation in SPB and chromatin locus motion far from the source - at least several hundred kilobases away - much farther than one can expect from the current estimates of chromatin persistence length (250-6,000 bp). Moreover, some correlation is observed even for telomeric regions which are more than 1 Mb away. Our results also suggest that at the shorter scale (~ few hundreds kilobases), the agitation propagates mostly through individual chromatin fiber, while at the longer scale (~1 Mb) the agitation propagates through the chromatin meshwork. We use theory of semi-flexible polymers to estimate persistence length (rigidity) of the chromatin in the nucleoplasmic environment. We also investigate how perturbations of heterochromatin which are known to mechanically soften or stiffen nuclei impact chromatin rigidity and the scale of the force propagation. Our results show that mechanical stress can be directly transmitted to the genome, describing both physical and genomic length scales over which the chromatin is capable of directly responding to the mechanical stress exerted on the nuclear envelope.

B028/P0612

Reproducibility of DNA sequence organisation within mitotic chromosomes

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The mechanism by which rod-shaped mitotic chromosomes are formed from interphase nuclei has yet to be fully understood. Whilst it has been demonstrated through in-vitro loop extrusion studies, in vivo microscopy and Chromosome Conformation Capture (3C) techniques that condensin complexes drive large-scale chromatin folding, whether specific DNA sequences reproducibly occupy distinct positions within mitotic chromosomes has not previously been investigated. Polymer modelling from condensin Hi-C maps has suggested that the local chromatin organisation within mitotic chromosomes can be in different configurations and result in the same global structure, however this has not been tested experimentally. To address the question of whether DNA is reproducibly organised in mitotic chromosomes or not, we applied Genomic Loci Positioning by Sequencing (GPSeq) to simultaneously sequence and visualise DNA positioned on the surface of mitotic chromosomes in cell populations with near-perfect prometaphase synchrony. Using progressive nuclease digestion, we mapped sequences from four biological replicates and six digestion time-points, in order to determine if there is preferential organisation of particular DNA sequences to any regions of separated cohesin-depleted sister chromatids at prometaphase. These datasets showed a degree of reproducibility, in which sequences were consistently identified as more internal or external relative to the rest of the genome. Furthermore, sequences annotated as internal of the mitotic chromosome consistently showed higher G:C-content – and those identified as on the outside of the mitotic chromosome showed higher A:T-content. Integration with unpublished ChIP-Seq data from our lab further revealed that the regions identified as internal and G:C-rich also co-localised with condensin I peaks – which has been shown by fluorescence microscopy to form an axis down the centre of the mitotic chromosome. These results provide the first evidence for reproducibility in DNA sequence organisation in mitotic chromosomes.

B024/P0608

The centrosomal protein Bbp1 regulates telomere-associated LINC complex during budding yeast meiosis

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The Linker of Nucleoskeleton and Cytoskeleton (LINC) complex physically connects the nucleus of the cell to the cellular cytoskeleton facilitating mechanotransduction, chromatin tethering to nuclear envelope, regulation of nuclear shape and position in eukaryotes. The budding yeast LINC complex is composed of the SUN-domain protein Mps3 at the inner nuclear membrane (INM) and the KASH-like protein Mps2 at the outer nuclear membrane (ONM). Bbp1, a spindle pole body (SPB; centrosome equivalent in yeast) associated protein acts as an adapter at the SPB insertion site binding to Mps2 forming the centrosome-associated LINC complex (C-LINC), which is essential for nuclear envelope anchoring and insertion of the SPB. While Bbp1 localizes solely to the SPB during mitosis, we have found that it localizes to the entire nuclear periphery during meiosis and is associated with the telomeres. Using mutant yeast strains and fluorescence microscopy, we show that this nuclear periphery localization is dependent on Mps2 and a coiled-coil domain of Bbp1. While Mps2 and a meiosis-specific protein Csm4 are the currently known ONM components of the telomere-associated LINC complex (t-LINC), the association of Bbp1 to telomeres is unexpected. However, Bbp1 fails to localize to an ectopically produced t-LINC by expressing CSM4 during mitosis suggesting that Bbp1 may be competing with Csm4 to bind to Mps2. Mps2 coimmunoprecipitates with both Bbp1 and Csm4 suggesting a direct binding of the candidate proteins to Mps2. Depletion of Bbp1 during meiosis results in reduced sporulation efficiency. We are currently investigating the role of Bbp1 in homolog pairing and recombination, and the mechanism of Bbp1 recruitment to telomeres during meiosis.

B026/P0610

The inner nuclear membrane protein NEMP1 cooperates with telomeres to maintain genome stability

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Female infertility is a major health concern worldwide, with age-related decline in oocyte quality being a central factor. The nuclear envelope (NE) is critical for nuclear integrity, chromatin organization, and nucleocytoplasmic transport. Nuclear Envelope Membrane Protein 1 (NEMP1), highly expressed in oocytes across metazoans, has emerged as a key regulator of NE stability and developmental competence. Female mice lacking Nemp1 (Nemp1−/−) are sterile, yet the molecular mechanisms underlying NEMP1 function remain poorly defined. Here, we show that NEMP1 localizes to the oocyte NE in discrete condensates and contributes to nuclear stability. Loss of NEMP1 resulted in nuclear envelope deformations, chromatin disorganization, and disrupted telomere organization. In wild-type oocytes, ~12% of telomeres tethered to the NE, and over 90% of these colocalized with NEMP1 condensates. By contrast, Nemp1−/− oocytes exhibited abnormal telomere clustering, increased free telomeres lacking shelterin protection, and accelerated telomere shortening—hallmarks of genomic instability. These findings suggest that NEMP1 transiently associates with telomeres to regulate their tethering to the NE, thereby preserving telomere structure and spatial distribution.Together, our results establish NEMP1 as a critical coordinator of telomere organization and NE integrity in oocytes. Disruption of these processes compromises genomic stability, likely contributing to aneuploidy and infertility. This work highlights the essential role of nuclear envelope proteins in safeguarding female reproductive longevity and suggests new therapeutic avenues for maintaining oocyte quality during aging.

B040/P0624
Topoisomerase 1 inhibition suppresses nuclear blebbing via RNA Pol II stalling and nuclear stiffening
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The function of the nucleus is dependent on both nuclear morphology and compartmentalization which is determined by nuclear mechanical strength and transcriptional activity driving dynamics. Perturbations to chromatin or lamins disrupts nuclear stiffness causing increased nuclear blebbing and rupture, which leads to increased DNA damage and cellular dysfunction. Transcriptional activity is another genomic function that has been shown to be necessary for the formation and stabilization of nuclear blebs and ruptures. The leading mechanism for this finding places heavy importance on the effects of RNA polymerase (Pol) II motor activity on chromatin dynamics and structure. However, it is still unclear whether these findings are due to decreased RNA Pol II motor activity, or due to pleiotropic effects caused by global inhibition of transcription. To address this, I reduced transcriptional activity using the topoisomerase I inhibitor camptothecin (CPT). In perturbed cells with increased nuclear blebbing, camptothecin treatment over 1 hour to a significant decrease in nuclear blebbing. This inhibition of topoisomerase via CPT does not alter RNA Pol II phosphorylation but instead stalls it resulting in significantly decreased levels of RNA via Click-iT RNA imaging. However, inhibition of topoisomerase 1 on DNA also lead to chromatin-chromatin crosslinking that greatly increased chromatin-based nuclear stiffness measured by micromanipulation nucleus force measurements. Thus, one hour treatment of CPT inhibits nuclear blebbing by both increasing nuclear stiffness via chromatin linking and transcriptional activity via stalling RNA Pol II.

B041/P0625
Ultra-high resolution DNA FISH and beyond
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DNA fluorescence in situ hybridization (FISH) is a powerful technique for localizing specific genomic loci within cells and tissues. Traditional DNA FISH methods require high-temperature denaturation of double-stranded DNA for oligo probe hybridization. However, this high temperature treatment disrupts protein molecules, posing challenges for quantitative imaging of proteins along with chromatin. Additionally, global denaturation increases non-specific background noise, preventing imaging at sub-kilobase resolution. To address these issues, we developed GOLDFISH-X, an exonuclease-based genome oligopaint via local denaturation FISH method. This technique uses Cas9 nickase to identify and cleave specific genomic targets, followed by exonuclease digestion to create local single-stranded DNA regions for probe hybridization. Consequently, GOLDFISH-X eliminates heat denaturation, preserves protein integrity, and achieves high signal-to-background ratio for non-repetitive genomic targets. GOLDFISH-X enables detection of genomic segments as short as 150 bps. Combined with very fast CRISPR for opto-controlled double-strand break (DSB) induction, we reveal the dynamics of DNA repair protein recruitment at single DSB sites. In summary, GOLDFISH-X offers a immunofluorescence-compatible platform with sub-kilobase resolution, thereby expanding the scope of high-resolution genomic imaging.

Genome Biology - DNA damage and repair

B074/P0656
A DNA quality control program eliminates defective haploid sperm nuclei
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DNA integrity is fundamental to cellular and organismal health. Survival across generations requires gametes that transmit genetic information faithfully. During spermiogenesis, post-meiotic, haploid germ cells develop over an extended period of time to produce mature sperm for fertilization. Little is known about how post-meiotic gametes ensure genome quality in the absence of any repair templates, such as homologous chromosomes or sister chromatids. In addition, sperm DNA packaging transitions from histone-based to protamine-based packaging during this period, likely precluding any DNA damage signaling. Here, we use irradiation as a model of compromised genome integrity and find that DNA-damaged spermatid nuclei are effectively eliminated via a mechanism involving the histone variant H2Av. We find that haploid spermatid nuclei are capable of sensing DNA damage only prior to histone removal, through phosphorylation of H2Av, a well-known DNA damage response signal. Upon depletion of H2Av, DNA-damaged spermatids are not eliminated during spermiogenesis. Ongoing experiments using H2Av phosphorylation mutants aim to elucidate the mechanism of elimination as well as functional consequences in the early embryos from H2Av-depleted fathers. Overall, we reveal a haploid-specific DNA quality control checkpoint that prevents DNA-damaged sperm nuclei from being passed to the next generation.

B077/P0659
Context-Specific Regulation of Cohesin and Genome Integrity
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Proteins involved in genome regulation were long considered static in function. However, recent work reveals these core cellular processes are more tunable across contexts than previously appreciated. My lab identifies proteins whose requirements or functions differ across cellular contexts—including disease, development, and evolution—and exploits these differences to untangle complex regulatory mechanisms governing genome integrity. I will present an overview of our lab's research, focusing on the context-specific regulation of the cohesin complex. Cohesin is a critical regulator of genome integrity, contributing to DNA organization, gene expression, DNA repair, and chromosome segregation. Its functional diversity is achieved through co-factor proteins that dictate its localization, stability, and function in a context-dependent manner. Our lab seeks to identify these co-factors and exploit them to untangle the diverse, context-specific roles of cohesin in the cell. I will focus on one such regulator we have identified, PRR12, which contributes to cohesin complex stability and DNA repair functions. We show that PRR12 interacts with cohesin, and loss of PRR12 results in significantly reduced nuclear-localized cohesin. This cohesin loss can be rescued by co-depletion of PRR12 with the cohesin-destabilizing protein WAPL, indicating PRR12 promotes cohesin stability by counteracting WAPL-mediated removal. Beyond its broad nuclear localization throughout the cell cycle, PRR12 co-localizes with cohesin at DNA damage sites in a cohesin-dependent manner, and PRR12 removal disrupts this localization and increases DNA lesions, indicating PRR12 contributes to cohesin's DNA repair role. Despite similar localization dynamics and interactions across cell types, different cell lines exhibit significant variation in their sensitivity to PRR12 loss. Unlike mouse NIH-3T3 cells, which are intolerant to PRR12 loss, many human cell lines exhibit more

modest defects. This contrasts with human patient data, where PRR12 mutations cause severe developmental defects, suggesting context-specific roles in cohesin regulation. Our current work builds on these findings, exploring PRR12's role in regulating cohesin at DNA breaks and using this protein to untangle cohesin's contribution to DNA repair. Together, this work exemplifies our approach of leveraging context-specific requirements to identify regulatory proteins and understand how genome maintenance processes are reshaped across different biological contexts.

B075/P0657

Imperfect sequence matching is associated with homology-directed double strand break repair

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Rearrangements are genomic alterations that arise when accurate double-strand break (DSB) repair mechanisms fail, causing deletions, insertions, inversions, and translocations. They affect larger regions of genomes than any other alteration, covering 300,000x more area than SNVs. However, SNVs have been far better studied, as they are easier to detect. Several mechanisms of DSB repair involve perfectly matching bases immediately adjacent to rearrangement breakpoints, termed microhomology (MH). MH's role in DSB repair and rearrangement formation is well-characterized, but the involvement of sequences beyond the perfectly matching bases has not been systematically studied. We hypothesized that imperfect sequence matching (ISM) adjacent to breakpoints may also contribute to DSB repair. We detected and classified ISM near breakpoint junctions via a novel algorithm using Smith-Waterman alignment and a statistical model of background ISM levels. We applied our algorithm to germline and cancer rearrangements in the Cancer Genome Atlas (TCGA) dataset and found more than half of germline rearrangements have significantly more ISM than expected by chance. In contrast, <1% of somatic rearrangements have significant ISM. However, in both contexts, ISM is abundant in rearrangements with >10 bp of MH (and therefore putatively formed by single-strand annealing or homologous recombination). ISM rarely occurs on only one side of a rearrangement - most often it is either present on both sides of the breakpoint or completely absent. Furthermore, ISM sequence features suggest they may assist with heteroduplex formation during repair. Strikingly, ISM is enriched adjacent to germline SVs with breakpoints in programmed meiotic DSB hotspots. Together, our results suggest that ISM plays a role in homology-directed DSB repair. Better understanding of the marked difference in ISM prevalence between germline and cancer genomes will improve our understanding of ISM's contribution to DSB repair and may reveal new therapeutic strategies to treat malignancies, infertility, and other conditions affected by improper DSB repair.

B072/P0654

Topbp1 mediates the response to DNA damage and acentric chromosomes in inviable Xenopus hybrids

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Inviable hybrid embryos generated by fertilizing Xenopus tropicalis eggs with Xenopus laevis sperm lose the long arms of chromosomes 3L and 4L and die at gastrulation, but the molecular mechanisms driving the hybrid-specific genomic instability are poorly understood. Combining nuclei isolated from X. laevis embryos with metaphase-arrested X. tropicalis egg extract revealed that a subset of X. laevis chromosomes lose

centromere foci marked by the H3 centromeric histone variant CENP-A. Rather than a failure of tropicalis centromere assembly factors to properly load new CENP-A onto laevis chromosomes, we have found that CENP-A is actively removed from this subset of laevis chromosomes cycled in tropicalis cytoplasmic extracts. This defect could be rescued by adding the RNA polymerase II inhibitor triptolide to hybrid reactions, suggesting that replication-transcription conflicts at metaphase leads to CENP-A loss from laevis chromosomes 3L and 4L. Despite the consistent loss of CENP-A on a subset of chromosomes in vitro, a low percentage of hybrid embryo cells display micronuclei in vivo. As has been observed in human cells, topoisomerase II binding protein 1 (TOPBP1) localized to the acentric chromosomes in hybrid extract reactions, suggesting a role for TOPBP1 in responding to acentric chromosomes during metaphase. Immunodepletion of TOPBP1 from hybrid extract reactions prevented CENP-A loss, but led to increased DNA damage marked by gamma H2AX, as well as chromosome alignment defects in hybrid reactions. Addition of recombinant TOPBP1 rescued chromosome alignment, yet CENP-A remained localized to chromosomes and elevated levels of gamma H2AX persisted, indicating DNA damage repair factors co-deplete with TOPBP1 and are required for centromere loss. Inhibition of MMEJ-mediated repair enzyme Pol theta also rescued CENP-A loss in hybrid reactions. Altogether, these results are consistent with DNA repair by TOPBP1 and MMEJ leading to gross chromosomal rearrangements at the centromeres of 3L and 4L, and we are currently pursuing a dCas9-based FISH approach to better understand the nature of these rearrangements and determine whether long arm loss is accompanied by isochromosome formation in inviable Xenopus hybrids. Such a phenomenon would likely reconcile the observation that a low percentage of cells form micronuclei in vivo while a subset of chromosomes consistently lack CENP-A in vitro. Ongoing work is further investigating whether this mechanism is hybrid specific, or if non-hybrid embryos utilize similar pathways to maintain proper chromosome alignment and genome integrity when challenged with DNA damage or acentric chromosomes prior to gastrulation, when canonical cell cycle checkpoints are largely inactive.

B078/P1809

Transcription factor Ovo orchestrates an inducible response to episodes of transposon threat in the male germline

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Germ cells are uniquely immortal and require specialized mechanisms to safeguard the heritable genome. Transposable elements (TEs) can threaten genomic integrity by excising and/or inserting themselves throughout the genome; germ cells throughout the Metazoa counter this threat through deployment of the piRNA pathway. This pathway serves as an “immune system” for the genome, preventing further replication and genomic infection by TEs through production of complementary interfering RNAs. Notably, the risk posed by TEs is not constant over developmental or generational time. High-threat scenarios include conditions in which some TE repression is reduced to allow for a so-called "domesticated" TE to be functionally expressed, as well as cases in which cells lack maternally deposited piRNAs against a genomically-encoded TE. Inducible responses to heightened TE risk remain poorly understood. Here, we identify a dynamic, essential role for the transcription factor Ovo in the Drosophila male germline during episodes of TE threat. While ovo mutant testes appear morphologically normal, they lack germline expression of the piRNA factor Piwi, leading to mild global TE derepression under basal low-threat conditions. Under high-threat conditions, however, loss of Ovo results in severe global TE upregulation, extensive double-strand breaks, and largely agemetic and sterile testes. Furthermore, we find Ovo is required for production of de novo piRNAs against genomically-encoded invading elements. Notably, Ovo expression itself is elevated in these high-threat conditions, suggesting its transcriptional autoregulation is responsive to TE challenge. We propose that Ovo functions as an inducible

regulator of the male germline “immune system”: dispensable when increased TE infection risk is low, but rapidly upregulated and essential when TE activity threatens genomic integrity. This work uncovers the first known role for Ovo in the male germline, and reveals how the male germline can harness domesticated TEs while simultaneously preventing invasion by non-domesticated elements.

B073/P0655

Understanding the role of CENP-A in modulating responses to DNA damage and R-loops formation in human cancers

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The human centromere is the primary constriction within mitotic chromosomes made up of repetitive alpha-satellite DNA hierarchically organized in megabase-long arrays of near-identical higher order repeats (HORs). Centromeres are epigenetically specified by the presence of the centromere-specific histone H3 variant, CENP-A, which enables the assembly of the kinetochore for microtubule attachment. Centromere integrity is crucial to ensure the correct chromosome segregation and preserve the stability of intra and inter-generational inheritance of the genetic information. When errors occur at centromere level, the cells undergo aneuploidy, a major contributor to cancer development. Despite being recognized as a hallmark of human cancer, the exact role of aneuploidy as a “driver” is still largely unknown. Here, we describe how replication stress triggers the non-random segregation (NDS) of DNA damage (γH2AX) in hTERT-RPE-1 cells as a mechanism to preserve the genomic information in one of the two daughter cells. Using a Doxycycline inducible RNaseH1 and an Auxin inducible CENP-A degron, we observed an increase in NDS after CENP-A depletion and a reduction after R-loops removal. We also observed how the homologous recombination protein Rad51 manifests the non-random segregation. Using a chemical inhibitor of Rad51 we observed a strong reduction of cells manifesting the NDS of both γH2AX and Rad51, suggesting an active role of the homologous recombination pathway. Here, we propose that the replication stress triggers the homologous recombination pathway to accumulate the damaged newly synthetized strands in only one of the two sister chromatids. We also hypothesize that the accumulation of R-loops affect how the kinetochore proteins bind to the centromere inducing mechanical and/or epigenetic signals that influence the orientation of the sister chromatids on the metaphase plate. This mechanism can be fundamental to maintain the genetic information; however, the impact of the accumulation of DNA damage on the cell fate remains elusive.

B076/P0658

Unveiling a Novel Component of the Homologous Recombination Mediated DNA Repair in Malaria Parasites

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The alarming rise of drug resistance in Plasmodium falciparum, the causative agent of malaria, necessitates the discovery of novel therapeutic targets. DNA double-strand breaks (DSBs) are among the most deleterious forms of DNA damage and P. falciparum encounters a number of frequent DSBs during its asexual life cycle in the human host. As the parasite lacks the Non-Homologous End Joining (NHEJ) pathway, it relies exclusively on Homologous Recombination (HR) for DSB repair. Previous studies highlight that disruption of HR in malaria parasites significantly reduces parasite survival, where PfRad51 serves as the central key recombinase. Although chemical inhibition of PfRad51 using a small molecule inhibitor, B02, impairs parasitic growth, a significantly high sequence identity of 62.7% between PfRad51 and human Rad51 raises toxicity concerns,

emphasizing the need to identify parasite-specific HR components. In order to identify such targets, we employed a Random Walk with Restart (RWR) algorithm on P. falciparum protein–protein interaction network seeded with known DNA repair genes and this analysis identified a novel, previously uncharacterized putative protein, PfNDR (Novel DNA Repair protein). Functional characterization of PfNDR revealed its interaction with PfRad51 and other HR proteins. Expression profiling of PfNDR exhibits that PfNDR is not only maximally expressed at the mitotically active or the DNA damage–prone stages of the parasite but also upregulated under DNA damaging conditions. Immunofluorescence studies show that PfNDR gets predominantly localized from the cytoplasm to the nucleus upon exogenous genotoxic stress, validating its DNA damage–responsive function in the nucleus. Structural modelling identified a critical PfRad51 residue that is crucial for PfNDR interaction. Additionally, DNA repair kinetic studies confirmed that overexpression of PfNDR enhances repair efficiency and also rescues parasites from B02 sensitivity. Collectively, these findings identify PfNDR as a novel HR-associated protein critical for DSB repair in P. falciparum. The apparent lack of an ortholog in humans qualifies PfNDR as a promising and selective candidate for antimalarial drug development.

Genome Biology - DNA replication and recombination

B068/P0650
A CDK-Centered Switch Balances Dormant Origin Activation and Fork Speed to maintain replication rates
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Cells have the capacity to adapt to replication stress to safeguard genome stability, yet the mechanisms underlying cellular plasticity in the face of replication stress are unclear. We reveal that cyclin-dependent kinase (CDK) activity functions as a central regulatory switch that flexibly balances dormant origin firing and fork speed to ensure completion of DNA synthesis. Inhibition of WEE1 kinase elevates CDK2–Cyclin A activity to prevent PCNA-dependent degradation of the origin firing factor TRESLIN, which promotes recruitment of the TRESLIN–MTBP complex to chromatin and drives dormant origin activation. When origin firing is restricted by MTBP loss, cells compensate by accelerating fork speed through a CDK1-dependent pathway. Similarly, ATR inhibition bypasses the requirement for TRESLIN–MTBP via a CDK1-dependent route that suppresses Rif1, thereby uncovering a compensatory fork speed mechanism. Together, these findings define a CDK-centered network that integrates WEE1, ATR, and Rif1 to dynamically balance origin usage and fork progression. By combining multiple replication control pathways into a single regulatory switch, our work has significant implications for maintaining genome stability and for therapeutic kinase targeting.

B069/P0651
CDK and PCNA-CRL4CDT2 regulation of TRESLIN and MTBP reveals novel mechanisms controlling DNA replication initiation and origin selection
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Faithful genome duplication requires precise control of DNA replication initiation, which occurs at thousands of replication origins distributed throughout the genome. Origin firing is regulated by the coordinated recruitment and activation of initiation factors, notably TRESLIN/TICRR and its partner MTBP, which promote the replicative helicase's loading and activation. Although it is known that CDK activity is essential for DNA

replication, the specific mechanisms by which CDK regulates origin firing factor stability and chromatin recruitment have remained unclear. Moreover, the precise chromatin binding dynamics of TRESLIN and MTBP throughout the cell cycle and how these dynamics impact origin selection and replication timing in human cells were not fully defined. To address these gaps, we have uncovered novel mechanisms through which CDK activity stabilizes TRESLIN by preventing its PCNA-dependent degradation, thus enabling robust MTBP recruitment to chromatin and promoting origin activation. Additionally, we discovered distinct cell cycle-dependent binding modes for TRESLIN and MTBP. During the G1 phase, MTBP chromatin association requires TRESLIN but is independent of pre-loaded MCM complexes, suggesting significant divergence from the yeast paradigm of origin selection.

B067/P0649
DNA2 prevents replication stress during Drosophila development
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Replication stress threatens genome integrity during early development, particularly in Drosophila melanogaster, where embryos undergo rapid synchronous divisions. DNA2 is a conserved helicase/nuclease essential for DNA replication and repair. In Drosophila, mus109 encodes DmDNA2; its loss-of-function disrupts replication-associated repair, but its developmental consequences remain unclear. To investigate the role of DmDNA2 in developmental cell maintenance, we analyzed nuclear organization, mitotic synchrony, and DNA damage via immunostaining in DNA2 mutant versus wild-type ovaries and embryos. Wild-type embryos maintained normal nuclear distribution and mitotic progression, while DNA2 mutants exhibited asynchronous mitotic cycles and disrupted development. Morphologically, mutant embryos showed phenotypic variability including short or absent dorsal appendages, translucent chorions, and abnormal “wrinkled” eggshells. Ovary analyses revealed elevated DNA damage in DNA2 mutants in both germline and somatic cell populations. Together, these data support a role for DmDNA2 in preserving genomic stability during both oogenesis and early embryogenesis.

B070/P0652
Essential Roles of BRCA2 in Spermatogenesis and Oogenesis
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BRCA2 (FANCD1) is essential for gamete formation and human alleles have been linked to early menopause. However, its functions in fertility remain poorly defined relative to those in somatic cells. BRCA2 plays a central role in homologous recombination, a chromosome-repair process that is defective in many cancers and is essential for meiosis, the specialized cell division that produces eggs and sperm. The major function of BRCA2 is to load the homologous pairing and DNA strand-exchange protein RAD51 onto the ends of broken DNA molecules. To understand BRCA2 functions in fertility, we have exploited a conditional allele of the Brca2 gene in mouse to inactivate its mediator function at different stages of gametogenesis in spermatogonia (stem cells), spermatocytes, and oocytes. Inactivation in spermatogonia reveals an essential function for BRCA2 in the proliferation and differentiation of germline stem cells. When Brca2 is inactivated early in meiosis, assembly of strand-exchange complexes is defective and the early events of meiotic prophase-I fail, including chromosome pairing and synapsis. These observations are consistent with an essential role for BRCA2 in loading RAD51 and DMC1 (the meiosis-specific RAD51 ortholog) onto DNA ends. Later inactivation of BRCA2 mediator function

reveals an unanticipated function after initial strand exchange and chromosome synapsis have been achieved. At this stage, BRCA2 mediates the de novo loading of RAD51, but not DMC1, to complete the repair of a subset of ~40 residual DNA breaks. Without BRCA2 mediator function, these breaks persist and become excessively resected. Surprisingly, in spermatocytes, these persistent DNA lesions do not impede the progression of meiosis, and repair is eventually completed during diakinesis/metaphase I. Sperm are produced efficiently but fertility is low suggesting that aberrant DNA repair may cause lethal mutations. Consistently, genome sequencing reveals the formation of de novo mutations, including large deletions, in the progeny of Brca2 mutant fathers. By contrast, in females, Brca2 mutant oocytes undergo apoptosis revealing a sexually dimorphic response to BRCA2 dysfunction and DNA damage arising during meiotic prophase I. Unexpectedly, BRCA2 dysfunction also disturbs the patterning of crossovers – the exchanges between homologous chromosomes that are essential for their accurate segregation.We conclude that BRCA2 functions throughout gametogenesis and mediates distinct modes of DNA repair during the early and late stages of meiosis. Our analysis also reveals sexually dimorphic responses to BRCA2 dysfunction with implications for gametogenesis and germline mutation.

B071/P0653

Quantitative Determination of Dielectric Thresholds for DNA Precipitation by Ethanol

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DNA remains the cornerstone of cellular biology; however, current preservation practices rely on empirically determined volumetric rules rather than quantitative theoretical frameworks (Sambrook & Green, 2001). Existing models often overestimate ethanol requirements, limiting reproducibility and preventing efficient protocol scaling. To address this, we derived and validated a dielectric threshold–based model for predicting ethanol volumes in DNA precipitation, establishing a reproducible and scalable framework that reduces reagent waste and environmental impact. We applied Coulomb's Law (Jackson, 1999) and solvent dielectric quantification to define the threshold at which DNA solubility decreases due to reduced electrostatic repulsion and enhanced charge screening (Record et al., 1976). DNA molarity, obtained through Beer–Lambert spectrophotometry (Swinehart, 1962), was used to predict ethanol requirements. Four DNA concentration conditions were prepared with 0.3 M sodium acetate following standard protocols (Sambrook & Green, 2001), with 12 replicates each across an ethanol gradient from 54% to 90% v/v in 2% increments. Using dielectric constants for ethanol ($\varepsilon = 24.5$) and water ($\varepsilon = 80.1$) (University of Washington, n.d.), we calculated the effective dielectric constant of each solution, determining the precise threshold at which precipitation occurred with high reproducibility. Our data confirm that DNA precipitation begins at 58–60% ethanol and reaches a maximum 95% yield by 72%. Across all DNA concentrations tested (2, 5, 8, and 10 ng/µL), replicates produced consistent dielectric thresholds and precipitation profiles, generating a reproducible sigmoidal recovery curve. From these results, we derived $\varepsilon = 40.07$ as the dielectric threshold for optimal DNA precipitation, with yields plateauing consistently within ±3%. Ethanol volumes can therefore be optimized rather than overestimated, as validated by A260/A280 purity ratios. Adopting the dielectric-threshold framework reduces ethanol use by ~3% compared to standard DNA precipitation protocols, while maintaining yield and quality. Standardizing this model will not only improve reproducibility and enable automation but also reduce flammable and biohazardous waste across high-throughput genomic workflows.

Genome Biology - Epigenetics and gene regulation

B048/P0630
A Comparison Of Antisense/Sense Transcription And Histone Modifications At Differential H3k36me3, H3k79me3, And H2bk123ub States In S. Cerevisiae.
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While several studies have examined the relationship between Histone modifications and sense transcription, the connection between histone modifications and antisense transcription remains less clear. By utilizing data that examines S. cerevisiae genome-wide histone modifications at the bottom 20th percentile and top 20th percentile levels of H3K36me3, H3K79me3, and H2BK123ub modifications, which are theorized to increase antisense transcription (Murray, 2015), this study considers the significance of the correlation between various histone modifications and sense and antisense transcription. The analysis examines these groups in relation to sense transcription to elucidate patterns associated with increased histone modifications in cases where these three modifications are either low or high. The results indicate that several histone modifications are significantly correlated with antisense transcription in cases where the aforementioned three modifications are low. Additionally, the study finds that several histone modifications are correlated considerably with sense transcription when the three modifications mentioned above are high. Specifically, H3K36me3, H3K4ac, H3K4me3, H3K9ac, H3K14ac, H3K18ac, H3K23ac, and H3K56ac exhibit increased correlations in situations with greater antisense transcription, whereas they show less correlation with lesser antisense transcription. These results align with various prior studies on the antisense influence on transcription activity, particularly concerning nucleosome depletion at and around transcription start sites. Our findings open the door to further studies that will uncover the mechanism behind antisense transcription.

B050/P0632
Characterizing Histone Post-Translational Modifications In A Yeast Model Of Matrin-3-Associated Neurodegenerative Proteinopathy
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Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative disorder characterized by degeneration of motor neurons. This neuronal loss results in muscle atrophy which can lead to paralysis and ultimately be fatal. RNA dysregulation has been identified as a key mechanism underpinning ALS pathogenesis, and many RNA-binding proteins (RBPs) such as FUS, TDP-43, and MATR3 have been implicated in this disease. Matrin-3 (MATR3) is a highly conserved nuclear matrix protein with diverse functions in RNA stabilization, mRNA export, and transcription. MATR3 has emerged as a target candidate in ALS treatment, as its overexpression induces pathological protein aggregation, disrupts normal cellular processes, and can lead to premature neuronal death. Intriguingly, both the wild-type and mutant forms contribute to pathology, suggesting MATR3 dysfunction is not confined to genetic contexts but part of a broader pathological framework. We are investigating how MATR3 toxicity reshapes the epigenome, which refers to changes in gene expression driven by histone modifications. To explore this, we overexpressed MATR3 in wild-type Saccharomyces cerevisiae to characterize the epigenetic changes associated with this proteinopathy in ALS. This work focuses on post-translational modifications (PTMs) with established roles in chromatin dynamics and gene regulation. Using

western blotting against modification-specific antibodies to quantify PTM levels across control and MATR3-expressing yeast strains, we find H3K14ac levels to be significantly decreased which suggests a loss of transcriptional activity. Ultimately, this work will contribute to our understanding of molecular mechanisms that drive MATR3-related neurodegeneration and identify potential therapeutic strategies for ALS and other neurodegenerative disorders.

B053/P0635
Cohesin-mediated regulation of functional maturation of pancreatic beta-cells
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Both type 1 and type 2 diabetes are characterized by a depletion of functional beta-cell mass, with DNA-damage induced genomic instability being a key driver. But the molecular mechanisms that protect genomic stability in functional beta-cells and their dysregulation during diabetes are poorly understood. Beta-cell mass is primarily established in the early postnatal growth phase via replication, following which beta-cells gradually exit cell-cycle and attain functional maturation. Prior studies in the lab established that beta-cells in the postnatal growth phase are extremely vulnerable to DNA damage, and that the Cohesin complex, an epigenetic regulator of the chromatin 3D architecture, is crucial for genomic stability and beta-cell mass expansion during this vulnerable phase. Moreover, Cohesins have been shown to regulate cell identity and function in other cell types, by coordinating 3D genome organization with cell state-specific transcriptional programs. We therefore asked whether the Cohesin complex also directs the functional maturation of beta-cells. Bulk-RNA seq analysis and immunostaining studies revealed that the beta-cell specific knockout of the Cohesin subunit Smc3 (Smc3BKO) reduced the expression of beta-cell identity and maturity genes (e.g., Mafa, Slc2a2, and Ucn3) at both the transcript and protein level. As mature beta-cell function relies on mitochondrial oxidative phosphorylation, we assessed mitochondrial health using electron micrscopy (EM), quantification of mitochondrial mass (Tomm20 staining), Seahorse Oxygen Consumption rate (OCR) assay, and membrane potential (TMRE-Red) assay. These experiments revealed that beta-cell mitochondrial integrity and function is significantly impaired upon Cohesin Smc3 deficiency. Furthermore, Smc3BKO beta-cells had reduced insulin content and lacked mature insulin granules as determined using insulin ELISA in isolated islets as well as immunostaining and ultrastructure imaging of pancreatic sections from control and Smc3BKO mice using EM. Finally, glucose stimulated insulin secretion studies on islets from 1m control and Smc3BKO mice showed impaired insulin secretion in response to high glucose (16.7mM). Collectively, our data establishes that Cohesins are crucial for the establishment of functional maturity during the transition from neonatal to adult life.

B051/P0633
Environmental exposures in neurodegenerative disease: Effect of copper on post-translational histone modification levels in S. cerevisiae ALS/FTD models
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Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease associated with motor neuron deterioration and loss of voluntary muscle movement. ALS forms a disease continuum with frontotemporal dementia (FTD),

which is connected to the death of neuronal cells in the frontal lobe of the brain. Interestingly, exposure to environmental toxins - including heavy metals like copper - has been linked to an increased risk of ALS. In fact, an elevated level of copper has been observed in neuronal matter of ALS patients, potentially causing copper toxicity connecting to the protein aggregation present in ALS. We and others have revealed that ALS/FTD is associated with certain epigenetic changes, including changes on the post-translational modification profile of the DNA-packaging histone proteins. The addition of chemical groups on histone tails, like methylation and phosphorylation, can impact gene expression. Here, we determine the effects of copper exposure on the genome-wide post-translational histone modification landscape in yeast via Western Blotting. Upon treatment with copper, we find an increase in the levels of H3S10ph and H3K14ac and an overall decrease in the levels of H3K9ac. Meanwhile, the levels of H3K79me3 and H3K27ac remained unchanged. Some of these changes mirror those observed in overexpression models for neurodegenerative disease proteinopathies, such as TDP43, FUS, and c9orf72. For instance, decreases in H3K36me3 are connected to TDP43 toxicity while decreases in H3S10ph are linked to FUS aggregation and increases in H3K9ac are tied to c9orf72 proteinopathy. These changes in the levels of histone modifications can potentially reveal connections between environmental exposures, protein aggregation and cell death associated with ALS/FTD and neurodegeneration.

B049/P0631

Exploring epigenetic targets to mitigate DNA damage-related dipeptide repeat protein toxicity in C9ORF72-based ALS/FTD

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The G4C2 hexanucleotide repeat expansion (HRE) of the C9ORF72 gene is linked to Amyotrophic lateral sclerosis (ALS) and Frontotemporal dementia (FTD). The expansion leads to the production of dipeptide protein repeats (DPR) such as the arginine-rich poly-proline-arginine (poly-PR) DPR. DPRs aggregate in the nucleus, sequester RNA-binding proteins, interfere with nucleocytoplasmic transport, and cause nucleolar stress by binding nucleolar proteins, leading to neuronal death. While genetic causality alone fails to explain these C9-ALS/FTD phenotypes, recent epigenetic investigations highlight histone post-translational modifications (PTMs) as both disease markers and potential contributors to disease onset and progression. Histone PTM marks control gene activity by controlling access to the transcriptional machinery. In addition, histone PTMs are involved in DNA damage repair mechanisms. Histone H2AX phosphorylation (γ-H2AX) is a marker of DNA double-strand breaks (DSBs) that acts to facilitate DNA damage repair. According to current research, C9 cases display elevated γ-H2AX levels, indicating DNA damage and potential DNA repair initiation. However, as DNA damage persists in C9 cases, investigating histone PTMs can provide insights into aberrant DNA repair and consequent neurodegenerative events. Previous work has shown that yeast cells expressing poly-proline-arginine (poly-PR) display increased levels of Histone H4 lysine 16 acetylation (H4K16ac) and H3S10ph. H4K16ac is recognized and bound by bromodomain protein BRD7 in mammals and its homolog Rsc58 in yeast. On the other hand, H3S10ph is installed by Aurora B kinase in mammals and Ipl1 in yeast. Interestingly, H4K16ac participates in repair protein recruitment and facilitates DSB repair. H3S10ph levels are increased in neurodegenerative cases that also harbor increased DNA damage. Here, we further investigate these PTM alterations and their effects in a yeast Rsc58 deletion model expressing (PR)50 and a mammalian C9-ALS/FTD model derived from SH-SY5Y neuroblastomas treated with a FITC-tagged poly-PR peptide (FITC-(PR)20). Thus far, we observe relief of growth suppression in the Rsc58 knock-out yeast expressing poly-PR. While H4K16ac and DNA damage evaluations are currently underway in the FITC-(PR)20 treated SH-SY5Y cells, so far these cells recapitulate increased H3S10ph levels observed in other C9 models. We hypothesize that aberrant H4K16ac levels contribute to C9 disease pathology by furthering DNA damage. Hence, investigating the

connections between histone PTMs involved in DNA damage repair and neurodegenerative context can reveal new druggable targets to ameliorate neurodegeneration.

B046/P0628
Exploring mechanisms of gliding membrane formation in bats
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A central goal of biology is connecting genotype to phenotype, and the field of evolutionary developmental biology does this by studying how genetic changes lead to phenotypic changes throughout evolutionary time. Studying traits that have evolved multiple times in different lineages is a powerful way to dissect this genotype-phenotype relationship and understand how genome evolution instructs changes in cell behavior and tissue morphogenesis. The independent evolution of the lateral gliding membrane, or patagium, in mammals has been instrumental in the novel abilities of flying and gliding. While the patagium has independently evolved up to seven times in mammals, only bats have taken this adaptation beyond gliding to achieve true self-powered flight. Previous research on the molecular basis for the formation of the patagium in marsupial sugar gliders identified several upregulated genes and rapidly evolving regulatory elements active in the developing patagium. However, no research has been conducted in bat tissue to characterize the molecular basis for the formation of the bat's lateral patagium. This project uses field dissected bat tissue to identify which regulatory regions and coding genes are associated with plagiopatagium evolution and development. Using ATAC sequencing and comparative genomics combined with RNA sequencing, I have identified potential enhancers and genes that are upregulated and enriched for nearby rapidly evolving regulatory elements in the patagium, such as the transcription factor Creb5. In the future, I will functionally test these elements using luciferase assays and visualize gene expression using HCR RNA-FISH to characterize noncoding and coding genomic elements that are associated with plagiopatagium outgrowth in bats.

B054/P0636
Expression of suboptimal ribosomal RNA variants is a 'genomic fuse' to trigger germline ribosomal DNA copy number expansion
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Ribosomal DNA (rDNA) loci contain the hundreds of tandemly duplicated copies of ribosomal RNA (rRNA) genes needed to transcribe sufficient rRNA for ribosome biogenesis. The repetitive nature of rDNA loci makes them susceptible to copy number instability, yet the consistent inheritance of functionally sufficient rDNA copies at each generation indicates that the germline monitors and maintains rDNA copy number. Germline rDNA maintenance mechanisms are most well understood in the Drosophila male germline, where rapid expansion of rDNA copies in germline stem cells (GSCs) restores lost rDNA. This activity is tightly regulated to only function when rDNA copy number is reduced, yet it has remained unclear how genomic rDNA copy number is sensed to control this process. Here we find that ribosome biogenesis serves as a cellular sensor to monitor rDNA copy number status within GSCs and regulate rDNA expansion activity. Combined transcriptomic and cellular analyses reveal that ribosome biogenesis is disrupted in low rDNA GSCs, and we find that perturbing ribosome biogenesis in normal rDNA conditions induces rDNA expansion. Intriguingly, we do not find any difference in rRNA transcription in the GSCs of low rDNA animals, despite having limited rDNA copies. Instead, we find that ribosome biogenesis in low rDNA GSCs is disrupted by the aberrant expression of

normally silent rRNA variants that are erroneously processed. Strikingly, reduced rDNA conditions that do not express this suboptimal rRNA variant do not induce rDNA expansion, and conversely, normal rDNA conditions forced to express this rRNA variant ectopically induce rDNA expansion. Together, these findings reveal that Drosophila rDNA copy number is not directly monitored, but that it is the disruption of rDNA function that triggers rDNA expansion. We propose that genomes retain suboptimal rRNA variants whose consequential expression upon rDNA reduction acts as a 'fuse' to disrupt ribosome biogenesis prior to rDNA copy number being reduced below functionally insufficient – and potentially unrecoverable – levels. We are now working to uncover why these rRNA variants are defectively processed and to understand why these effects are limited to GSCs.

B055/P0637
Gapmer Mediated Knockdown of HSATII RNA in U2OS Cells
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Human Satellite II DNA (HSATII DNA) is a relatively unexplored region of the human genome, consisting of repetitive and interspersed repeat sequences. HSATII RNA is usually not expressed in healthy cells, however is highly expressed in certain cancerous cells where it accumulates in the nucleus. Based on preliminary data, we believe that the accumulation of HSATII RNA into nuclear foci recruits and sequesters regulatory proteins such as TDP-43 to form colocalizing accumulations (Hall et al., 2017). My project aims to knockdown HSATII expression, and to quantify any changes in HSATII distribution and TDP-43 distribution. I achieved this through the use of LNA gapmers, which are single stranded antisense oligonucleotides that can attach to a target RNA sequence and initiate degradation through the recruitment of RNaseH. My results demonstrate a significant reduction of HSATII RNA expression and focal accumulations for trials involving a sequential gapmer knockdown procedure. We have also quantified a significant redistribution and possible knockdown of TDP-43, further suggesting a potential interacting mechanism between HSATII RNA and TDP-43. These studies will give us insight into whether LNA gapmers can serve as an effective method to reduce HSATII RNA expression and its role in cancer progression, and subsequently affect the distribution of TDP-43 across the nuclei.

B056/P0638
Genetic Insights into Autism Spectrum Disorder: The Role of De Novo Variants in Drosophila
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Autism Spectrum Disorder (ASD) is a complex, highly polygenic neurodevelopmental condition characterized by deficits in social communication and repetitive behaviors. Although over 200 genes have been associated with ASD, the cellular and functional roles of many candidate genes remain unclear. Understanding these mechanisms is essential not only for advancing molecular neuroscience but also for informing the broader public health challenge of improving early diagnosis and intervention for ASD. This study investigates five novel human ASD candidate genes: FBXO11, ITSN1, GABA-RP-3, PHRF1, and Ralgapb using Drosophila melanogaster as a genetic model. FMR1, a well-established ASD-related gene, served as a positive control due to its conserved role in synaptic development and regulation. To evaluate the potential involvement of these genes in ASD-related pathways, we combined morphological and behavioral analyses. Larval neuromuscular junction (NMJ) morphology was examined to assess synaptic structure and bouton count, while adult flies underwent social space assays (to quantify sociability) and activity assays (to evaluate locomotion and motor behavior).

Preliminary findings from the social space assay revealed that FBXO11 knockdown flies displayed social spacing patterns comparable to controls, suggesting that this gene alone may not significantly impact social behavior in Drosophila. Moreover, NMJ morphology analysis confirmed that FMR1 knockdown larvae exhibit synaptic overgrowth and excess bouton formation. Ongoing work will extend NMJ and behavioral characterization to the remaining candidate genes to identify shared or divergent cellular mechanisms that may contribute to ASD-relevant phenotypes. By integrating cellular neurobiology with behavioral outcomes, this study leverages Drosophila as a powerful model to uncover conserved molecular pathways underlying ASD ultimately informing translational efforts to better understand, predict, and address ASD at both the biological and population health levels.

B047/P0629

Histone Post Translational Modifications and Chromatin Changes are Linked to C9orf72 FTD/ALS

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Frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) are fatal and incurable neurodegenerative diseases that form a disease continuum. Hexanucleotide repeat expansions (HREs) in chromosome 9 open reading frame 72 (C9orf72) are one of the most common causes of FTD/ALS. HREs lead to the formation of toxic dipeptide repeat proteins that are connected to distinct epigenetic signatures. Alterations in various epigenetic mechanisms such as histone post-translational modifications and changes to chromatin structure have been implicated in FTD/ALS. A Saccharomyces cerevisiae model over-expressing the dipeptide repeat protein Poly-Proline-Arginine display significant increase in a transcriptionally active mark, H3S10ph. H3S10ph changes are also present in patient-derived fibroblasts and induced pluripotent stem cells (iPSCs) from patients bearing C9orf72 expansions. Fibroblasts also reveal changes in a histone PTM linked to constitutive heterochromatin, H3K9me3, as well as its protein reader, Heterochromatin Protein 1 alpha (HP1α). These findings suggest that defects in transcriptionally silent heterochromatin are connected to C9orf72 expansions and proteinopathy. Altogether, this study highlights the role of epigenetics in neurodegenerative disease mechanisms and illuminates novel therapeutic targets.

B044/P1808

Systematic assessment of gene regulatory network inference methods with single-cell multiome data

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Gene regulatory networks (GRNs) are essential models for understanding the molecular mechanisms that govern gene expression, cell differentiation, and cellular function. Constructing accurate GRNs requires integrating diverse layers of genomic information beyond gene expression alone. Existing GRN resources primarily focus on transcriptomic data and often overlook the epigenetic mechanisms of gene regulation, such as chromatin accessibility, and three-dimensional genome organization. This limitation presents a significant barrier to evaluating and improving computational methods for GRN inference. To address this challenge, we developed SC-MO–GRN-DB (https://scmogrndb.psu.edu), a curated, publicly accessible database that houses experimentally validated GRNs alongside harmonized single-cell multiomic datasets. The database includes ground-truth GRNs comprising over 10 million regulatory edges, constructed from high-confidence

experimental sources. In addition, it hosts single-cell multiome datasets totaling more than 2 million cells across six molecular modalities: scRNA-seq, scATAC-seq, scChIP-seq, scDNA-Met, scHi-C, and scCRISPR-seq. All datasets are rigorously processed and standardized to enable consistent analysis across different tissues and modalities. Another gap in the single-cell multiomic GRN research field is the absence of a robust and standardized benchmarking framework. To address this gap, we developed BEAR-GRN, a comprehensive benchmarking framework that provides standardized input data, matched ground truth networks, and evaluation metrics to assess GRN inference methods in an unbiased and reproducible manner. Using BEAR-GRN, we benchmarked ten widely used GRN inference tools across seven single-cell multiomic samples from macrophages, K562 and mouse embryonic stem cells. Performance was assessed using both rank-dependent (AUROC, AUPRC) and rank-independent (precision, recall, F1) metrics, along with stability and scalability. As a result, we found that approaches integrating prior regulatory knowledge to constrain and guide model inference achieved more accurate and robust GRN reconstruction. In particular, approaches employing regularized multi-modal integration were less susceptible to noise. Overall, BEAR-GRN enables unbiased evaluation of GRN tools and supports future method development in the field of single-cell multiomics.

B045/P0627
Telomeres Rewired: A New Perspective from Regenerative Muscle Biology
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All eukaryotic cells have telomeres, specialized nucleoprotein structures that cap the ends of our chromosomes and safeguards them from DNA damage. Disruption of telomere function has been linked to pathological conditions such as cancer and aging. While most telomere research has been performed on isolated cells in vitro and/or in relation to cancer, the in vivo roles of telomere-associated proteins, particularly in non-cancerous tissues, remain underexplored. Our group investigates these proteins in the context of development and regeneration in skeletal muscles that exhibit low carcinogenic potential. We have discovered that muscles utilize telomere-associated proteins in ways that deviate from their canonical roles in telomere maintenance, especially when compared to tissues with higher cancer susceptibility. Stem cell-mediated regeneration is a highly organized process which is essential to maintain tissue integrity. In skeletal muscle, the ability of muscle stem cells (MuSCs) to make precise fate decisions depends on adequate transition between stem cell states. However, the molecular signatures guiding the shift from quiescence to myogenesis remain incompletely understood. Here, we reveal that MuSCs engage TRF2, a telomere-associated protein, through a mechanism distinct from its canonical telomeric roles. During regeneration, TRF2 serves as a molecular switch that governs stemness, affects progression towards reparative myogenesis and sustains stem cell self-renewal. MuSC-specific TRF2 disruption exacerbates muscular dystrophy pathology in mice, closely resembling key features of the human disease. Mechanistically, TRF2 binds to DNA G-quadruplexes located in the regulatory regions of lineage-specific stem cell genes, sustaining expression and supporting regenerative capacity. These findings uncover a previously unrecognized, extra-telomeric role for TRF2 in adult stem cell function and position it as a pivotal regulator of tissue-specific regenerative responses. These findings challenge conventional models of telomere biology and provide fresh insights into the molecular basis of muscle diseases.

B057/P0639
The Effect of Barasertib on Histone H3 Phosphorylation in Yeast ALS/FTD Models

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Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD) are closely related neurodegenerative disorders that share genetic and pathological features. Both have been linked to a hexanucleotide repeat expansion (HRE) in the C9ORF72 gene. This expansion leads to the production of dipeptide repeat proteins (DPRs), which aggregate in cells and contribute to neuronal death. We investigated the effect of Barasertib, an Aurora B kinase (AURKB) inhibitor, on histone H3 serine 10 phosphorylation (H3S10ph) in PR50 yeast models of ALS/FTD. Barasertib treatment reduced H3S10ph levels with increasing effect at higher concentrations relative to untreated and vehicle controls. These findings suggest that AURKB inhibition can regulate histone phosphorylation in DPR-expressing yeast and support further exploration of kinase-targeted strategies as a potential therapeutic approach in ALS/FTD.

Genome Biology - Transcription

B062/P0644
Cellular localization predictions of a folic acid metabolism gene in C. elegans
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One-carbon metabolism is essential for all life. Serine hydroxymethyltransferase (SHMT) is an enzyme which utilizes folate to catalyze essential metabolic steps in two distinct cellular compartments: the cytoplasm and mitochondria. Many animals, including humans, contain two SHMT paralogs that are differently targeted to these cellular locations. However, the genome of Caenorhabditis elegans has a single SHMT gene, mel-32. We investigated the hypothesis that this single gene produces two different isoforms, one that is targeted to the mitochondria, thus contains a mitochondrial signal sequence, and one targeted to the cytoplasm. Our sequencing data suggest that the trans-splicing mechanism, which is common in nematodes, creates unique transcripts from the mel-32 gene, including a transcript that contains a putative mitochondrial signal sequence. These data suggest C. elegans utilizes a unique mechanism for regulating the cellular compartmentalization of folate metabolism, which could provide insights into the evolutionary history of this essential pathway.

B058/P0640
Decoding Enhancer-Mediated Control of Transcription Dynamics through Live-Cell Super-Resolution Imaging
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Eukaryotic transcription is a highly stochastic, discontinuous process occurring in intermittent ‘bursts’. Conventional RNA sequencing provides only static snapshots of gene expression, missing this dynamic behavior. Here, we used the MS2-MCP system in live mouse embryonic stem cells to visualize nascent transcription of the Klf4 pluripotency gene. A distal super-enhancer ~70 kb downstream of the Klf4 promoter is essential for its transcription, as we observed that deletion of a Sox2 binding site within this enhancer region reduces gene expression by ~85%. To investigate such long-range enhancer-mediated transcription dynamics, we combined the photostable mStaygold protein with gentle super-resolution structured illumination microscopy (SR-SIM) to track Klf4 transcriptional bursting for over 12 hours. Hidden Markov modeling of transcription-site intensity trajectories revealed two bursting states, active (“On”) and inactive (“Off”), and enabled quantification of burst duration, frequency, and amplitude. To probe bursting regulators, we used

CRISPR genome editing to introduce a dTAG degron and acutely (<2 h) deplete four classes of factors: Brd4 (co-activator), cohesin (structural), Med14 (Mediator), and Sox2 (transcription factor). Burst frequency was significantly reduced by depletion of all regulators (Brd4: 63%, Med14: 67%, cohesin: 56% and Sox2: 47%) while the impact on burst duration was relatively mild with only Brd4 and Sox2 depletion resulting in a 30-35% reduction. Except cohesin (6%), depletion of other regulators led to a significant (~35-60%) reduction in burst amplitude. The reduction in bursting parameters was in good agreement with our TT-seq (Transient Transcriptome Sequencing) results, which showed strong reduction (>50%) in nascent transcription for Brd4, Med14, and Sox2, but only a modest effect for cohesin depletion (~20%). To probe enhancer regulation, we fluorescently labeled the Klf4 enhancer (E) and promoter (P) using the tetO/tetR system and performed fast dual-color SR imaging, revealing transient (~5–30 s) E–P contacts with long periods of separation. Cohesin depletion increased average E–P separation by ~40%, while other factors had only minor effects (<15%). Using three color volumetric SR imaging, we observe that during gene bursting, the enhancer is stably associated (>10 min) with a well-defined proximal Brd4 condensate (~270 nm away, ~400 nm in size), which gradually dissociates after the end of the burst. Taken altogether, our multi-color labeling and SR imaging of regulatory DNA elements, proteins, and nascent RNA reveal dynamic mechanisms involving long range enhancer-promoter communication and condensate association toward regulation of transcription bursting.

B064/P0646

Differential contribution of TFE3 isoforms to cell motility and invasion

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TFE3 is a basic helix–loop–helix leucine zipper transcription factor that orchestrates cellular responses to a variety of stress conditions, promoting restoration of cellular homeostasis and cell survival. Here we report the presence of two different TFE3 isoforms generated by the use of alternative promoters. The long isoform (TFE3-L) was previously shown to undergo continuous proteolytic degradation due to the presence of a phospho-degron in its N-terminal region and only accumulates under specific stress conditions. In contrast, we show that the short isoform (TFE3-S) lacks the first 105 residues containing the phospho-degron and is constitutively expressed at high levels in most cell types. Both isoforms share the same Rags/mTORC1-dependent mechanism of regulation, being retained in the cytosol in the absence of stress and display comparable capacity of inducing expression of lysosomal and autophagic genes upon activation. Likewise, both isoforms significantly reduce cell proliferation and disrupt cell-cell interaction when over-expressed. However, TFE3-L is considerably more efficient than TFE3-S promoting cell migration and invasion, likely through its ability to modulate a unique set of migratory target genes. Accordingly, specific TFE3-L depletion in a cellular model for tuberous sclerosis causes a significant reduction in cell motility and invasiveness. Our data reveal that the two TFE3 isoforms exhibit partial redundancy and that the appearance of TFE3-L following prolonged stress correlates with metastatic behaviors that may have an important relevance in cancer.

B065/P0647

Investigating the Role of Free Histone H3 in Cell Cycle Control and Zygotic Genome Activation

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The Drosophila embryo undergoes 13 fast, synchronous nuclear divisions that lack checkpoints and gap phases before it slows down at the mid-blastula transition (MBT). Previous work in the lab indicates that free histone H3 competitively inhibits Chk1 to prevent cell cycle slowing prior to the MBT. Embryos that overexpress the first 47 amino acids of histone H3 (the H3-tail), which cannot be incorporated into chromatin, exhibit faster cell cycles leading up to the MBT than controls. The MBT coincides with other key developmental events, including large-scale changes in chromatin structure, differentiation, and the zygotic genome activation (ZGA). During ZGA, the embryo transitions from relying on a maternally loaded pool of RNA transcripts and proteins to initiating its own transcription. Free histone proteins are among these maternally loaded products that allow the embryo to progress through the initial fast cycles. Given the role of H3-tail in timing the MBT, this suggests a possible role for free histones in regulating transcriptional activation. This project aims to investigate if and how the H3-tail affects transcription, and how transcriptional regulation and cell cycle control are intertwined. To examine the effect of excess H3-tail on ZGA, we will conduct time course RNA-seq on H3-tail:GFP and control GFP embryos that will be carefully staged using fluorescence microscopy. Preliminary data from embryos in the last fast cycle before the MBT (NC13) indicate that excess H3-tail delays ZGA for a subset of transcripts. We identified 212 differentially expressed genes with mean log fold change greater than 2. Of these, 183 were downregulated upon H3-tail overexpression. The majority of these genes overlapped with the early zygotic genes from other datasets. To further evaluate the molecular mechanism behind these effects upon H3-tail overexpression, we plan to identify zygotic genes that experience the greatest delay in transcription as a result of the free H3-tail. These results will shed light on the targets and timing of H3 tail regulation as the cell cycle slows in ZGA.

B061/P0643

Live imaging and bioinformatic analysis reveal the molecular mechanism of direct nuclear-to-cytoplasmic ratio sensing in early Drosophila development

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The first steps of embryonic development are conserved across different eukaryotes, with maternally deposited mRNAs and proteins initiating embryonic development upon fertilization. Later, the zygotic genome activates and takes over the control of subsequent development. While the timing of zygotic genome activation (ZGA) is crucial for proper embryonic development, the mechanisms by how this timing is well-regulated have not been fully understood. Over the past few decades, studies have suggested that the increase in nuclear-to-cytoplasmic (N/C) ratio following each cell division serves as a timer for the ZGA in Drosophila. However, since the increased N/C ratio is tightly linked to lengthened cell cycle duration, identifying direct N/C ratio-responsive genes and investigating the underlying mechanism is challenging without detangling the two factors. In this study, we curated a list of 280 N/C ratio-sensitive candidate genes based on RNA-seq data from early Drosophila embryos with arrested cell cycles. Utilizing in vivo live imaging on wild-type (diploid and normal cell cycle), sesame mutant (haploid and short cell cycle), and checkpoint mutant (diploid and short cell cycle) embryos, we verified several N/C ratio-sensitive genes by separating the effect of N/C ratio manipulation from cell cycle length. We found that the probability of activation in frühstart (frs) and rhomboid directly responds to the N/C ratio. We further confirmed that the frs 1kb and 300bp regions upstream from the transcription start site retain complete response to N/C ratio, identifying the minimal regulatory region that confers N/C ratio sensitivity. Through motif enrichment analysis, we identified potential transcription factors (TFs), such as Cropped, that may convey N/C ratio measurement to gene activation decisions. Our study provides the first quantitative characterization of the role of N/C ratio in transcriptional regulation in Drosophila embryos. In addition, our identification of potential N/C ratio-sensing TFs proposed a novel mechanism of N/C ratio-mediated ZGA regulation during early embryonic development.

B066/P0648

The Role of Repetitive Extragenic Palindromes (REPs) in the E. coli Genome

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While the E. coli genome is remarkably gene-dense and efficient, the most abundant noncoding genomic elements, repetitive extragenic palindromes (REPs), do not have a clearly established function. The 355 REPs in the E. coli genome have one of two conserved sequence motifs and a well-conserved structure. Since palindromes can form DNA cruciform structures and RNA hairpins, previous studies have suggested that REPs could be chromosomal protein binding sites, transcription termination sites, or mRNA degradation barriers. We combine molecular biology approaches to study model REP, REP325, with bioinformatic approaches to interrogate the possible biological role of REPs. Here we show the primary role of REPs is regulating gene expression. We did not find a significant role of REP325 in chromosome organization, although we recapitulate a very small effect of REP325 deletion on nucleoid expansion. When we further probe the interactions between bacterial chromosomal protein HU, REP RNA, and cruciform DNA, we do not observe the HU-RNA-DNA complex in vitro that was previously proposed to organize the chromosome. Additionally, we do not see evidence of REP-REP contacts in Hi-C data. Instead, we find that REP325 affects the expression of the genes within its operon, yjdM-REP325-yjdN. The operon contains two nonessential 'y-genes' of unknown function. We find that REP325 both stabilizes the upstream yjdM transcript and decreases transcription of yjdN in RNA-Seq data and in Northern blots probing different operon permutations. Upon direct probing of REP325 expression, we find that the 12-hairpin construct is expressed from a plasmid as a mixture of products corresponding well to different numbers of hairpins. The longer hairpin products increase upon inhibition of rho-termination, suggesting that REP325 acts as a rho-dependent terminator in vivo. We find evidence in publicly available sequencing data that some REPs, particularly between convergent gene pairs, could act as previously unannotated terminators. We find that genes with terminal REPs are slightly enriched for primary metabolism genes by GO term analysis, but we do not find any clear signature of stress response genes or a unified regulatory pathway. Our analyses suggest that REPs affect expression of the surrounding genes, primarily through regulating transcription termination and mRNA stability.

B059/P0641

The yeast checkpoint kinase Mec1p functions in transcription termination by facilitating recruitment of Pcf11p and regulating the torpedo exonuclease Rat1p

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Transcription cycle of RNA polymerase II (RNAPII) features three stages: initiation, elongation, and termination. Termination, closely linked with pre-mRNA 3' processing, dissociates RNAPII from DNA and releases the nascent RNA transcript. Efficient termination is required for maintaining a pool of RNAPII that is available for re-entry into new transcription cycle. Previous results showed that inactivation of checkpoint kinase Mec1p in the absence of exogenous genotoxic stress downregulates the efficiency of transcription termination and reduces the efficiency of pre-mRNA cleavage at the polyadenylation (pA) sites. We report here that Mec1p impacts transcription termination at two distinct steps. Mec1p promotes recruitment of Pcf11p, a subunit of the cleavage factor IA (CF IA), to 3' ends of genes, and regulates the activity of torpedo exonuclease Rat1p. Deletion of Mec1p or mutations that prevent activation of Mec1p partially suppress both transcription

termination defects as well as rRNA and snoRNA processing defects in rat1-1 cells. These results suggest that Mec1p regulates features of Rat1p function that are shared by termination of RNAPII transcription and rRNA and snoRNA processing and imply that the kinase activity of Mec1p downregulates Rat1p function. Cumulatively, our results reveal a new role for checkpoint kinase Mec1p in transcription termination and regulation of the torpedo exonuclease Rat1p.

B060/P0642
Transcriptional regulation in response to cell size during Drosophila zygotic genome activation
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Cell size changes drastically during development. In early Drosophila embryogenesis, several rounds of nuclear divisions occur without changing the embryo's size, increasing the nuclear-to-cytoplasmic ratio (N/C ratio). The changing N/C ratio determines the timing of key developmental processes, including zygotic genome activation (ZGA) and the slowing of the cell cycle during the mid-blastula transition (MBT). The transcriptional activation of many zygotic genes relies largely on the slowing of the cell cycle, which lengthens the window in which transcription can occur. However, previous work uncovered a handful of genes that respond directly to the N/C ratio, including the mitotic inhibitor fruhstart. Transcription of other cell cycle regulators is known to respond to cell size in yeast, suggesting a potential feedback mechanism between the N/C ratio and cell cycle control. The molecular mechanisms that govern a gene's transcriptional sensitivity to the N/C ratio remain unknown. Using the MS2-MCP live imaging system to quantify transcriptional dynamics at high temporal resolution, we have identified a 300bp portion of fruhstart's cis-regulatory region that drives N/C ratio-sensitive expression of a transgenic reporter, indicating that cis-regulatory interactions play a role in this sensitivity. To identify the specific cis-regulatory components that give rise to N/C ratio sensitivity, we re-analyzed published RNA-seq data to identify candidate genes that are activated at a specific N/C ratio. These include genes regulating cell cycle, DNA replication, and chromatin remodeling. We hypothesize that a common regulatory mechanism allows such genes to sense the changing N/C ratio. To uncover this mechanism, we performed DNA motif analysis on the regulatory regions of N/C ratio-sensitive genes to identify factors whose binding is enriched near these genes and could modulate their transcription. Preliminary results show an enrichment of binding motifs for the Myc-pathway transcription factor cropped (AP-4 in mammals), as well as insulator-associated proteins BEAF-32 and Dref near N/C ratio-sensitive genes. Published ChIP-seq data also shows an enrichment of origins of replication near N/C ratio-sensitive genes, indicating that DNA replication might also contribute to N/C ratio sensitivity. Further investigation is ongoing to determine if and how these pathways may contribute to the cell size-sensitive regulation of transcription.

Immune Cell Biology - Adaptive immunity and cellular memory

B310/P1434
Induce the generation of high-quality stem cell-like CD8/CD4 double-positive T cells and their application in anti-tumor immunity
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Tumor immunotherapy has made breakthroughs in cancer treatment in recent years, but the success rate is still very low due to T cell function defects. We recently found that knockout of the small G protein Arf1 in mouse cancer stem cells caused an organelle senescence cascade and released cytokines and tumor-specific

antigens through exosomes and MDEV to remodel and activate the tumor immune microenvironment, enhance T cell activation and effector function, prevent T cell exhaustion, and at the same time upregulate TCF1 in T cells through dendritic (DC) cells to increase T cell stemness, and also release sphingolipid S1P to bind to S1PR1 on the surface of T cells to promote T cell conversion to CD8/CD4 double-positive T cells (DPTs), resulting in a significant increase in DPTs with stem cell properties. This new T cell subset brings together several superior properties of T cells and has extremely strong tumor killing ability. To further improve the abundance of this cell subset, we performed Crisp-Cas9 screening and found that knockout of the Smarca2 gene or treatment with its inhibitor Camibirstat increased the abundance of this DPT to 8-10%. In a variety of mouse tumor models, Arf1 inhibitors combined with Camibirstat can achieve complete tumor shrinkage. We expect this DPT to be a powerful weapon for tumor immunotherapy in the future.

B312/P1436

Mechanism of lactate-mediated immunosuppression in the tumor microenvironment

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Tumors alter tissue environments to promote their own growth and limit immune responses. Metabolic changes in the tumor microenvironment (TME) impair T cell immunity, limiting the efficacy of both native lymphocytes and adoptive cell therapies. Many different tumor types exhibit elevated lactate levels which correlate to tumor grade and worsened patient survival. Additionally, cancers deplete amino acids such as serine from their environment. Our primary data in human CD4 and CD8 T cells, and published data in mouse T cells, show the dual metabolic stresses of serine deprivation and high lactate synergize to impair T cell proliferation. However, the biological mechanism by which lactate potently suppresses T cells is unknown. A number of hypotheses for immunosuppression have been proposed. Lactate may accentuate stress responses or metabolically rewire T cells, for example through action of lactate dehydrogenase (LDH), which depletes NAD+ equivalents in high lactate conditions. We find that supplementation of electron acceptors such as alpha-ketobutyrate can partially restore cell proliferation in support of this hypothesis. Unexpectedly, we find that increasing NAD+ through precursor supplementation does not improve proliferation, arguing against the LDH hypothesis. Interestingly, we find that serine starvation in combination with high lactate reduces mTORC1 signaling beyond serine starvation alone. These data suggest that the anti-proliferative effect of high lactate and serine starvation is more complex than redox stress alone. Understanding how these dual metabolic stressors impair T cell function in the tumor microenvironment will provide new insights for generating more durable cell-based drugs, and improving the metabolic robustness of T cells may be a broadly useful strategy to increase the fidelity of cancer immunotherapies.

B311/P1435

Oral Lysophosphatidylserine Nanoparticle Administration Mediated Immune Tolerance via cDC1 Signaling

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Protein-based therapies are used to treat a wide range of medical conditions. However, their effectiveness are often reduced when patients develop an immune response to the therapy. To overcome these issues, a novel nanoparticle-based immunotherapy has been designed to induce tolerance towards unmodified proteins in an antigen-specific manner. The nanoparticles contain phosphatidylserine (PS) to mediate uptake and signaling

mechanisms similar to those of apoptotic cells. Further, we have found nanoparticles containing lysophosphatidylserine (LysoPS), a derivative of PS with only one acyl chain, induce tolerance in mice comparable to PS when introduced subcutaneously, and preferentially to PS when introduced orally. However, the cellular mechanism involving LysoPS nanoparticle-induced oral tolerance is not well understood. Recently, we utilized single-cell RNA sequencing on mice treated with LysoPS nanoparticles to investigate the interactions and responses of immune cells that generate tolerance. We found LysoPS promotes tolerance through multiple immune cells (T cells, B cells, and NK cells) via TGFb signaling. While we have gained a better understanding of how tolerance is developed, we still lack a mechanistic understanding of the initiation of tolerance, specifically the role of antigen-presenting cells (APCs). This study examined APCs in the single-cell LysoPS tolerance database to identify the cells responsible for initiating tolerance. Our findings indicate that during LysoPS oral administration, APCs increased the expression of genes involved in proteasome function, antigen processing, and antigen presentation. Specifically, we found that APCs upregulate a nonclassical MHC-I receptor, H2-Q7. We further confirmed that the expression of this receptor increased during LysoPS treatment in vitro. Single-cell analysis reveals that the cDC1 cell cluster was the second-highest signaling cluster during LysoPS treatment, as determined by CellChat analysis, with upregulated signaling pathways that facilitate interactions with T cells and NK cells. Many signaling interactions upregulated in the LysoPS cDC1 cluster were related to immune suppression (PD-L1 and OX40) and T cell activation (CD200). Overall, the APCs involved in the oral administration of antigen-loaded LysoPS nanoparticles indicate that cDC1 is a key signaling contributor among all APC clusters, and this cell cluster communicates with both T cells and NK cells. This implies that cDC1 cells assist with tolerance induction by interacting with T cells and NK cells, and may be important in the development of regulatory T cells. This study highlights a potential role for cDC1-mediated oral tolerance during treatment with LysoPS nanoparticles.

B313/P1437

Tropomyosin and integrin engagement cooperate to promote actomyosin arc-dependent force generation at the T cell immune synapse required for efficient target cell killing

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Efficient target cell killing by CD8+ T cells requires the exertion of force on the target cell. This force may be generated by a contractile actomyosin arc network that comprises the medial, pSMAC portion of the T cell's immune synapse. The pSMAC is also where the T cell's integrin LFA-1 binds to ICAM-1 on the target cell to drive T cell: target cell adhesion. Here we show that ligating LFA-1 with ICAM-1 promotes the formation of the actomyosin arc network, and that a tropomyosin (Tpm3.1/3.2) decorates this network. Abrogating Tpm3.1/3.2 function using either a small molecule inhibitor or CRISPR-mediated gene deletion disrupts the concentric organization of the arc network, reduces its content of myosin 2A, reduces T cell traction force, reduces T cell: target cell adhesion, and reduces the ability of effector CD8+ T cells to kill antigen-pulsed target cells without attenuating signaling. These and other results argue that Tpm3.1 and LFA-1 ligation cooperate to promote the generation of actomyosin arc-dependent force at the T cell immune synapse required for efficient target cell killing, and they add to a growing appreciation for the role that positive feedback between adhesion and myosin force plays in promoting T and B cell effector functions.

Immune Cell Biology - Immune cell migration

B306/P1430
Biomechanics of JAM-C-mediated neutrophil reverse transendothelial migration
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Neutrophil reverse transendothelial migration (rTEM) is a critical mechanism in inflammation resolution, enabling neutrophils to reenter the circulation after exiting inflamed tissues. However, compared to direct transendothelial migration, the biomechanical regulation of rTEM is understudied and remains poorly understood. Here, we investigated how Junctional Adhesion Molecule-C (JAM-C) coordinates endothelial biomechanics and neutrophil trafficking. Using siRNA-mediated JAM-C knockdown in vascular endothelial cells (VECs), combined with live-cell imaging, three-dimensional traction force microscopy (3D-TFM), monolayer stress microscopy (MSM), and micropatterned endothelial islands, we quantified neutrophil migration and rTEM events. Micropatterned endothelial islands provided a controlled platform to examine the mechanical interplay between neutrophils and VECs by inducing a reproducible increase in monolayer tension from island edge to island center. JAM-C depletion disrupted VEC F-actin organization, reduced monolayer tension, and elevated Intercellular Adhesion Molecule-1 (ICAM-1) expression, resulting in significantly increased frequency of rTEM events. Under these conditions, neutrophils exhibited slower uropod detachment consistent with increased ICAM-1 mediated neutrophil-VEC adhesion. Quantitative analysis of neutrophil trajectories on micropatterned endothelial islands revealed that, under control conditions, neutrophils preferentially migrated toward regions of higher monolayer tension, whereas JAM-C depletion abolished this tension-guided directional migration. Results from 3D-TFM revealed that VECs imposed strong compressive forces on neutrophils under the monolayer, which were released when monolayer gaps widened during rTEM. These findings indicate that loss of JAM-C weakens endothelial contractility, creating mechanically permissive junctions, while increased ICAM-1 provides additional anchorage points that strengthen neutrophil–endothelial adhesion and enable traction-driven reentry. Collectively, our results identify JAM-C is a key regulator of rTEM through dual control of endothelial tension and adhesion dynamics, underscoring rTEM as a mechanobiological pathway with therapeutic relevance in chronic inflammation.

B305/P1429
Differential adhesion and contractility govern the spatial organization of polarized macrophages
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Macrophages are tissue-resident immune cells that accumulate in many pathologies, including cancer, infection, and injury. The spatial organization of macrophages within tissues likely contributes to their diverse functions but remains understudied. As a reductionist approach to understanding macrophage organization, we cultured various macrophage subtypes on low-adhesion surfaces that are often used for spheroid and organoid culture. Macrophages polarized to M1 states with interferon-γ (IFNγ) or lipopolysaccharide (LPS) exhibited rapid clustering into cohesive aggregates, whereas macrophages polarized to an M2 state with interleukin-4 (IL4) dispersed relative to control macrophages. To determine the molecular mechanisms underlying these behaviors, we analyzed gene expression in M1 and M2 BMDMs. We found that Itgal encoding integrin αL and Icam1 encoding ICAM-1 are both strongly upregulated in M1 macrophages with kinetic profiles

similar to inflammatory genes based on unsupervised clustering. Genes associated with actomyosin contractility are also coregulated based on unsupervised clustering but exhibit more complex kinetics marked by modest early upregulation followed by significant downregulation. Knockout of any αLβ2−ICAM-1 component partially abrogates macrophage clustering mediated by IFNγ whereas pharmacological inhibition of contractility enhances clustering. Importantly, the low-contractility, clustering M1 phenotype observed in our reductionist model recapitulates key features of recently described antitumor macrophage subtypes in human cancers, suggesting they might be relevant to immunotherapy strategies targeting macrophages.

B308/P1432

Mechanical Regulation of Cytoskeletal Dynamics and Function in Cytotoxic T Lymphocytes

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Cytotoxic T lymphocytes (CTLs) are crucial components of the adaptive immune response, responsible for recognizing and eliminating infected or cancerous cells. CTL activation requires recognition of antigenic peptides bound to major histocompatibility complex (MHC) molecules on target cells through the T cell receptor (TCR). This interaction triggers intracellular signaling cascades that drive immune synapse (IS) formation. The IS serves as a dynamic interface where cytoskeletal reorganization facilitates force exertion, targeted lytic granule delivery, and thus, drives the killing of infected or cancerous cells. Recent studies suggest that CTL function is mechanosensitive, with cytotoxic efficiency being influenced by the stiffness of the target cell. However, the biophysical mechanisms underlying this mechanosensitivity remain poorly understood. We used high-resolution spinning disk confocal microscopy to study immune synapse formation and CTL function during activation on polyacrylamide hydrogels of tunable rigidity. We quantified actin reorganization, myosin light chain phosphorylation (pMLC), polarization of the lytic granule machinery, and lytic granule release across different substrate stiffnesses. CTL spreading, cytoskeletal reorganization, and pMLC were all stiffness-dependent, with increased substrate stiffness enhancing contractility and force generation, as measured by traction force microscopy (TFM). Additionally, lytic granule polarization and release correlated with substrate stiffness. To investigate the cytoskeletal mechanisms important in stiffness-dependent responses, we treated CTLs with cytoskeleton-targeting small-molecule inhibitors during activation on gels of differing stiffness. We found that inhibition of Arp2/3 selectively reduced centrosome polarization in cells activated on soft gels, while formin inhibition reduced centrosome polarization regardless of stiffness. We also performed high-resolution imaging assays using confocal and lattice light-sheet microscopy to assess killing efficiency and polarization of the cytolytic machinery during interaction with targets plated on gels of differing stiffness. Our studies revealed that target cell stiffness modulates CTL-mediated cytotoxicity. These findings suggest that CTL mechanosensing directly impacts synapse architecture and cytotoxic efficiency, providing insight into how immune cells adapt to mechanically heterogeneous environments, such as solid tumors.

B309/P1433

Optimizing the mpx promoter for neutrophil-specific gene expression in zebrafish

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A current impediment to the study of neutrophils within zebrafish (Danio rerio) is the inefficient expression of transgenes in only this cell type. The current standard promoter-holding sequence used for neutrophil-specific expression includes 8353 bp upstream of the mpx gene, encoding the myeloperoxidase enzyme. Due to the large size of this promoter region, construction of both expression plasmids and transgenic fish lines are challenging. To mitigate this inefficiency, we wanted to determine the minimal mpx promoter sequence that retains neutrophil-specific expression in zebrafish. We first identified potential transcription factor binding sites within the mpx promoter region, and generated a truncated (3132 bp) mpx promoter retaining the majority of putative binding sites with close proximity to the transcriptional start site. To visualize protein expression from our truncated promoter, we used it to drive expression of a single-pass transmembrane protein, TMEM154, conjugated to the red fluorescent protein mCherry. To assess its performance in zebrafish, we used the fully transparent casper line and performed microinjections at the 1-2 cell stage. The injected embryos were raised until 6-8 days post-fertilization, and then imaged using spinning disk confocal microscopy to determine whether the truncated promoter retained neutrophil-specific expression. Preliminary data suggest that the truncated mpx promoter sequence retains neutrophil targeting, but shows variable mislabeling of other cell types that may be due to the loss of unknown regulatory sequences present in the untruncated promoter sequence.

B307/P1431
X-Linked Moesin Associated Immunodeficiency (X-MAID) leads to T cell dysfunction through altered proliferation kinetics and protein interactions
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X-Linked Moesin Associated Immunodeficiency (X-MAID) is a rare genetic disorder caused by a single point mutation (R171W) in the FERM domain of moesin. Patients suffer from multiple symptoms including lymphopenia and frequent opportunistic infections. Moesin is a protein that links the cortical actin cytoskeleton to the plasma membrane and is critical to T cell functions such as migration and mitosis. However, the precise cellular mechanisms underlying X-MAID remain largely unknown. To study this disease, we use Jurkat T cells as a model system expressing either wild-type (WT) or mutant (X-MAID) moesin. The objective of this study was to investigate how the R171W mutation affects the binding partners of moesin and the subsequent impact on T cell proliferation. To identify differences in moesin-associated protein interactions, we immunoprecipitated moesin and conducted western blotting and mass spectrometry. T cell proliferation was evaluated by flow cytometry using CellTrace Violet dye to track cell divisions. The results demonstrate a distinct difference in protein binding profiles between WT and mutant moesin. Cells overexpressing X-MAID moesin show reduced proliferation compared to WT controls. In conclusion, these findings suggest that the R171W mutation disrupts critical moesin interactions, impairing T cell proliferation and contributing to the immunodeficiency observed in X-MAID. This study provides important mechanistic insights into disease pathogenesis and may inform future therapeutic strategies.

Immune Cell Biology - Immune cell signaling and activation

B300/P1424
A PGE2-GPCR signaling axis suppresses virus-triggered type I interferon response via elimination of damaged mitochondria
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Innate immune cells like macrophages and dendritic cells respond to stimuli like infection or inflammation by rapidly producing key mediators, such as Prostaglandin E2 (PGE2). PGE2 acts as a central, pleiotropic lipid mediator that signals primarily through different G protein–coupled receptors (GPCRs) to trigger diverse immune responses. While immunosuppressive effects of PGE2 on adaptive immunity are well established, much less is known about how it shapes innate immune responses. To address this gap, my project investigates how PGE2 signaling regulates type I interferon response, a key component of the innate immunity that restricts viral infection as well as promotes antitumor activity. Our results suggested that PGE2 serves as a negative autocrine feedback loop of type I interferon and hence suppresses antiviral responses. We propose a model in which PGE2, through GPCR signaling, induces mitophagy to eliminate damaged mitochondria. By doing so, it reduces cytosolic mitochondrial DNA release, suppresses interferon signaling, and ultimately enhances viral replication. Given that type I interferon response also plays a critical role in shaping antitumor immunity within the tumor microenvironment, our study provides a framework to explore how PGE2-driven pathways contribute to immune suppression in cancer and how such biology may restrict the efficacy of existing therapies. These mechanistic insights open an exciting opportunity to leverage existing GPCR-targeted drugs to modulate innate immune responses in the tumor microenvironment. Such approaches have the potential to enhance the efficacy of immunotherapies and other therapeutic modalities, with the ultimate goal to improve patient outcomes.

B302/P1426

A time limit for phagocytosis: FcRIIB triggers delayed inhibition of phagocytic cup extension

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Whole cell engulfment, or phagocytosis, is regulated by activating and inhibitory signals. Fc Receptors on the surface of innate immune cells like macrophages bind the Fc region of IgG on pathogens or diseased cells to trigger phagocytosis. While Fc Receptor activity is critical for pathogen clearance, overactive Fc Receptor signaling is associated with autoimmune conditions. The Fc Receptor family contains several activating receptors and one inhibitory receptor: FcRIIB. All Fc Receptors respond to the same ligand of IgG. From a systems-level perspective, FcRIIB is puzzling – why express a dedicated inhibitory receptor paired to activating receptor engagement, when macrophages could more simply modulate activating FcR expression? To isolate FcRIIB signaling, I designed a synthetic DNA receptor system that allows selective stimulation of FcRIIB and activating Fc Receptors. Using this system, I examined how FcRIIB activation affected the kinetics of phagocytosis. I found that FcRIIB activation did not affect the early stages of phagocytosis, but caused phagocytosis to abruptly fail near the end of the process, when the macrophage membrane was covering >75% of the target. We confirmed that this effect required the recruitment of SHIP1, an inositol phosphatase known to complex with FcRIIB's intracellular signaling motif in B cells. SHIP-1 dephosphorylates the key signaling molecule PIP3, which is required for phagocytosis. Interestingly, I found that blocking PIP3 synthesis reduced initiation of phagocytosis instead of affecting completion of phagocytosis like FcRIIB. I then developed an in silico model of phospholipid conversion during concurrent activating and inhibitory FcR signaling over the course of a single phagocytic event. This model suggests that the rapid generation of PIP3 at the phagocytic cup by the activating Fc Receptors and the slow depletion of PIP3 by FcRIIB generates an incoherent feedforward loop that limits the duration of activating Fc Receptor signaling. To test this model, I challenged macrophages with targets of various sizes. I found that with increasing target size, the percentage of the target’s surface engulfed at failure decreased, while the total surface area engulfed and the duration of the phagocytic attempt were consistent between sizes. Collectively, this work establishes FcRIIB signaling as a

molecular timer that limits phagocytic persistence. We postulate that this molecular fail-safe could prevent long, unproductive interactions with a target.

B304/P1428
An automated imaging platform for quantitative antibody-dependent cellular phagocytosis
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Targeted therapies, such as monoclonal antibodies (mAbs) that bind to tumour-associated antigens have become a staple of many cancer treatment regiments. Although several therapeutic mechanisms have been proposed, compelling evidence supports the notion that treatment efficacy involves antibody-dependent cellular phagocytosis (ADCP) by macrophages. Phagocytosis is a specialized process reserved to “professional phagocytes” such as macrophages, whereby such cells ingest large particles (≥0.5 µm), aiding in tissue development, remodelling, and for immunity to infection. Combined with fluorescent markers, microscopy and flow cytometry have become hallmark techniques to visualize spatiotemporal signalling and quantify rates of phagocytosis. However, these methods have inherent limitations – microscopy experiments require lengthy data processing and are prone to observer bias, while flow cytometry does not reliably distinguish between fully engulfed target cells and mere cell-cell contacts with macrophages. To overcome these challenges, I developed an automated imaging platform on an open-source framework that uses a machine-learning-based segmentation model to quantify ADCP through long-term time-lapse imaging. This high-throughput, unbiased acquisition-to-analysis pipeline not only enables robust quantification of total phagocytic efficiency but also provides temporal insights into engulfment speed and phagocytosis rates over several hours. Furthermore, the platform can produce visual annotations of quantified data directly on imaging acquisitions for better visual interpretation. As such, this robust and quantitative image-based assay accelerates data analysis relative to manual quantification methods, providing a powerful tool to investigate how various signalling cascades, mutations and treatment conditions influence ADCP.

B301/P1425
Bacterial sensing caspases cleave RNA-binding proteins
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Inflammasomes are signaling complexes that sense pathogens and elicit an inflammatory cell death program, whereby intracellular contents and mature cytokines are released into the extracellular milieu. Inflammasome activation recruits and activates immune cells to eliminate the threat. Sterile activation of inflammasomes plays a causal role in autoimmunity, while overactivation during infection can cause sepsis. The inflammatory caspases 1/4/5 (CASP1/4/5) execute inflammatory cell death by cleaving and activating the pore-forming capabilities of gasdermin D, and pro-inflammatory cytokines. However, the complete repertoire of substrates that inflammatory caspases cleave remains to be explored. For example, many RNA binding proteins contain cleavage motifs for inflammatory caspases. We empirically demonstrated that the RNA binding protein heterogeneous nuclear ribonucleoprotein (hnRNPK) is cleaved by all inflammatory caspases. Furthermore, hnRNPK is cleaved in response to bacterial infections in both mouse and human infection models, suggesting that inflammasome-mediated regulation of RNA metabolism may be a feature of intracellular innate immunity.

B297/P1421

Chimeric Antigen Receptor T Cells generate antigen-specific mechanical forces

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Chimeric Antigen Receptors (CAR) are engineered to bind to cancer-associated antigens, with this interaction leading to cancer cytotoxicity. Recently, the T cell field has come to recognize the role of small, piconewton level forces in establishing specificity and cytotoxicity in naïve T cells with the αβ TCR, raising the possibility that these forces could be present in CAR T cells despite the structural differences between CARs and TCRs. We developed single-receptor cancer-antigen DNA tension probes and utilized them to reveal the presence of F>18 pN mechanical forces in healthy, active anti-CD19 CAR T Cells directed against target protein CD19. Remarkably, these forces occur at levels similar to the known mechanoreceptor CD3ε in human T cells. We found that CAR forces are spatially and temporally dynamic and appear more disordered compared to that of the TCR-pMHC forces likely reflecting the structural differences between the CAR and TCR synapse. We further find that CD33 CARs also transmit pN forces to CD33, indicating that is a general feature of CARs. The magnitude of CAR forces display donor-to-donor variability reflecting the heterogenous activity of human T cells. These forces are highly dependent on the CAR T cell's ability to restructure the local cytoskeletal network and recruitment of the actin cytoskeleton, and can be inhibited with pharmacological inhibitors. We show that factors that modulate the cytotoxicity of CAR T cells such as exhaustion and immunomodulatory agents like dasatinib also modulate the mechanical forces exerted by CAR T cells on CD19 antigens, showing that these forces are strongly correlated with their cytotoxicity. Taken together, our work identifies a previously ignored aspect of CAR signaling and suggests potential applications in screening and as novel biophysical marker of CAR T cell health and efficacy.

B299/P1423

Deciphering VISTA binding mechanisms for cancer immunotherapy

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VISTA (V-domain Immunoglobulin suppressor of T cell activation) is an immune checkpoint receptor expressed on T cells and myeloid cells where it represses immune activation through unclear mechanisms. Also, VISTA is found in malignant cells of triple-negative breast cancer, acute myeloid leukemia and others, where it modulates tumor immunity and cancer cell proliferation. However, one major question entails the nature of VISTA binding partners, which have been reported to include VSIG3, VSIG8, PSGL1, LRIG1 and VISTA itself. Moreover, VISTA binding to these receptors is pH-dependent. To clarify mechanisms by which VISTA can engage different receptors, we characterized cell surface molecules recognized by VISTA. Protein sequence alignments and 3D protein modeling suggested that VISTA could bind glycans. Screening of glycan microarrays identified pH-selective binding to different glycan species including sialic acid. Since glycans are heavily enriched on cell surfaces we tested for recombinant VISTA binding to cell surfaces of various cell lines including K562, THP1, HL-60, Jurkat, and HCC-1806 at high and low pH. Recombinant VISTA-Fc strongly bound to all cell lines at pH 6.0, but not at pH 7.4. VISTA also bound primary CD4+ T cells, which increased after T cell receptor stimulation. VISTA binding was diminished by deletion of the SLC35A1 gene, which encodes a CMP-sialic acid transporter that is critical for sialic acid incorporation into glycans, suggesting that sialic acid partly contributed

to recognition by VISTA. Other studies have also shown that VISTA binds to heparan sulfate (HS), biophysical studies showed that VISTA binds HS with a Kd of 14 μM. Heparinase and heparanase enzyme treatment strongly diminished VISTA binding to cells. These studies suggest that glycan recognition by VISTA may be critical for repressing tumor immunity and could be blocked by clinical-grade VISTA-targeted antibodies. These findings should advance cancer immunotherapies by defining mechanisms by which VISTA engages disparate ligands. This could lead to new ways to disrupt the VISTA pathway or allow for better selection of patients for VISTA-targeted therapies.

B303/P1427

Glycosylation inhibition enhances natural killer cell cytotoxicity through nanoscale reorganization of CD16a signaling and effector domains

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Natural killer (NK) cells mediate cytotoxicity through antibody-dependent cellular cytotoxicity (ADCC). NK cells express the Fc receptor CD16a (FcγRIIIa), which binds to antibodies' Fc regions to facilitate ADCC. Engagement of CD16a at the immune synapse triggers phosphorylation of CD3ζ and Syk, leading to target cell lysis. Post-translational glycosylation modulates ADCC and inhibiting glycan processing enhances NK cell cytotoxicity. We investigated the effects of kifunensine (kif.), a Golgi α-mannosidase I inhibitor, on NK cell killing kinetics and CD16a activation. We previously showed that detection of phospho-CD3ζ (pCD3ζ) via confocal microscopy serves as a proximal marker for CD16a activation and that following kif. treatment there is a significant increase in the size and number of pCD3ζ-containing foci. In parallel, downstream signaling is enhanced, as reflected by elevated pSyk levels. This led us to the hypothesis that inhibiting the formation of branched glycans enables the formation of larger signaling clusters. To determine how inhibiting glycan processing translates into functional gains, we performed quantitative live-cell imaging which revealed that kif. treated NK cells lyse target cells in approximately half the time and display increased serial killing efficiency compared to their untreated counterparts. To uncover the structural basis of this enhancement, we employed high resolution imaging. SIM-TIRF microscopy showed the formation of discrete actin foci at the NK cell-target interface that consistently colocalized with pCD3ζ, indicating cytoskeleton-mediated reorganization of signaling machinery during Fc receptor engagement. Single-molecule imaging further revealed that pCD3ζ assembles into tightly organized microclusters in response to Fc receptor binding to ligand, highlighting a nanoscale architecture that underpins signal amplification. Super-resolution imaging confirmed that CD16, like pCD3ζ, formed larger and more numerous microclusters under these conditions. Together, these data underscore a coordinated reorganization of signaling and effector domains and suggest that inhibiting complex glycan formation permits more effective microcluster formation. In summary, kif. treated NK cells exhibit faster and more efficient ADCC, driven by enhanced proximal signaling and nanoscale reorganization at the immune synapse.

B298/P1422

Hemagglutinin receptor-binding site alterations modulate pathogenicity of Influenza A Virus

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Objective Influenza A virus (IAV) remains a major global health threat due to its seasonal epidemics and occasional pandemics. A critical factor underlying its rapid adaptability is the high mutation rate of the surface

glycoprotein hemagglutinin (HA), which enables host adaptation and immune evasion. Mutations occurring in the HA receptor-binding site (RBS) are particularly important, as they determine viral tropism and transmissibility. However, most previous studies have been limited to examining single-point mutations, which do not fully capture the complex and combinatorial nature of HA RBS variation underlying viral adaptation and pathogenicity. Method To investigate the functional consequences of HA RBS mutations, we generated a mutant library through multi-site saturation mutagenesis of the HA gene from an H3N2 strain and incorporated these variants into recombinant IAVs. Serial passaging was performed in cell culture and viral replication efficiency was assessed under different conditions. Viral replication and infectivity were evaluated using plaque assays and qPCR in human lung epithelial A549 cells and canine kidney MDCK cells. Result We observed that several HA RBS mutations modulated viral replication, with a variable infectivity in both A549 and MDCK. These findings suggest that alterations to the RBS within the HA head domain can directly affect viral fitness in cells. Furthermore, adaptive mutations were enriched during serial passaging, demonstrating their role in promoting sustained viral replication and host cell adaptation. Conclusion Our study demonstrates that mutations in the HA RBS play a critical role in determining the infectivity of IAV through their impact on receptor-binding properties. These results highlight the significance of HA binding changes in viral adaptation and pathogenicity, providing insights relevant to antiviral strategy development and pandemic preparedness.
Key Words: Influenza A viruses, Hemagglutinin, Receptor-binding site, Pandemics, H3N2, Viral adaptation

B296/P1420

Loss of AIRE results in increased signaling of JAK-STAT pathway in human keratinocytes

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The autoimmune regulator (AIRE) is known for its canonical role as a transcriptional regulator for the establishment of central tolerance in the thymus. Patients with pathogenic AIRE variants are at increased risk of multi-organ autoimmunity with a 15-fold increased risk of developing alopecia areata (AA), an immune mediated form of hair loss that affects approximately 160 million people worldwide with no cure. We recently reported spontaneous AA development in Aire deficient (Aire-/-) mice, as well as the expression of AIRE in skin keratinocytes. To better define the mechanistic role of AIRE in keratinocytes associated with AA pathogenesis, we generated CRISPR/Cas9-engineered AIRE -/- keratinocyte cell lines. After IFNg stimulation (50 ng/mL, 30 min), immunoblotting revealed increased activation of JAK1 (21% increase) and JAK2 (60% increase) in AIRE deficient keratinocytes (n=3 cell lines) compared to wild type controls. Furthermore, AIRE-/- keratinocytes exhibited downregulation of PIAS1 (43% reduction), a STAT1 inhibitor and AIRE binding partner, and SOCS1 (63% reduction), a JAK1 inhibitor, compared to wild-type controls(n=3). We confirmed our results with immunofluorescence microscopy which also showed a 43% decrease of nuclear PIAS1 expression in AIRE-/- keratinocytes compared to wild-type ($p < 0.0001$). We also engineered AIRE-/- keratinocytes to re-express AIRE fused to mCherry. Strikingly, we observed PIAS1 to localize in nuclear foci adjacent to mCherry-AIRE nuclear foci. Altogether, our data support a model whereby AIRE recruits PIAS1 to nuclear foci for the suppression of STAT1 activity, which is then lost in AIRE deficiency syndromes such as AA and APECED that exhibit unchecked STAT1 activation. These findings establish AIRE as a novel immunoregulatory factor in keratinocytes through endogenous suppression of JAK-STAT, with its deficiency playing a role in pathogenesis of AA.

B291/P1415

Phagocytosis via mannose receptors analysed using chemically functionalized lipid particles

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Phagocytosis is a cellular process that enables uptake and digestion of particles larger than 0.5 micrometer, crucial in innate immunity and tissue homeostasis. Phagocytic cells, like macrophages, have various surface receptors for specific target recognition and internalisation. These receptors undergo lateral reorganisation, triggering signalling, actin remodelling, membrane deformation, and phagosome formation. While opsonic receptor function is well-studied, here we focus on the mannose receptor (MR), part of the C-type lectin family. It binds glycoconjugates with terminal mannose, fucose, or N-Acetylglucosamine on microbial walls, but its role in phagocytosis is not fully understood. Despite the relevant role for bacteria and fungi uptake, the potential mannose receptors clustering in the plasma membrane and the signals elicited need to be further explored, taking into account that the cytoplasmic tail of the receptor lacks any intracellular signalling motifs. To model the uptake of pathogens, we use deformable lipid droplets functionalised with fluorescent glycoplipids coupled to mannose. We have shown that the recognition of these coated particles by human primary macrophages is specific, using preincubation with mannan and naked droplets as controls. We have monitored in live confocal microscopy their interaction with living cells to identify phagocytic receptor clustering, and revealed dynamic actin remodelling at the site of phagosome formation. The signalling pathway and actin nucleators involved have been investigated. In addition, we show that integrins contribute to the efficient uptake of mannolipid-coated droplets. In conclusion, we report a novel approach leading to new insights regarding mannose-mediated phagocytosis, from receptor clustering to intracellular signalling to actin.

B293/P1417

Resolving cholesterol metabolism and primary cilia interaction and its implications on neuroimmune signaling

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Genes like TREM2 and APOE are established markers of neuroinflammation, but the link between their role in cholesterol metabolism and neuroimmunity remains vague. Cholesterol is a crucial element in primary cilia, a sensory organelle that has received increased interest as an immune modulator in a variety of human tissues. Our own molecular profiling suggests a mechanism for the immunomodulatory cytokine pleiotrophin (PTN) in the regulation of cilia in myeloid cells and that this fundamental sensory structure is more involved in microglia function than previously thought. We used HMC3 cells as a model of microglia immune reaction, with a panel of immune stimulants (LPS, PIC, PMA), with and without PTN. We harvested cells for bulk RNA data from each condition, analyzed using differential expression for classical markers and Weighted Gene Correlation Analysis (WGCNA) to discover gene modules associated with immune stimulation and PTN modulation. To study cilia morphological changes, we performed immunocytochemistry (ICC) on HMC3 cells using the ciliary marker, Arl13B visualized by fluorescence microscopy. Images were analyzed using ImageJ plugin CiliaQ to quantify cilia presence and cilia length. Differential expression analysis of microglia showed classical markers of myeloid activation across all stimulations, with upregulated cholesterol synthesis 24 hrs after PTN exposure. WGCNA revealed a novel module in samples treated with LPS enriched in genes of primary cilia assembly. ICC showed reduction in cilia length with LPS stimulation and a significant increase in length after PTN treatment. To test

the hypothesis that PTN stimulated cholesterol biosynthesis is causal to cilia formation in HMC3 cells, we treated cells with atorvastatin, an inhibitor of cholesterol metabolism. Atorvastatin treatment reduced both the number of cilia and the length of those remaining. Our results suggest a mechanism of immunomodulation by PTN, and a microglia specific function of primary cilia supported by cholesterol metabolism. Further work will perturb cholesterol regulators like TREM2 to probe the role of cilia in neuroinflammation and connect cilia to the signaling pathways identified by our panel. Cilia are understudied in immune cells, however understanding the role of this fundamental organelle in neuroimmune biology offers a novel perspective on neuroinflammatory disease.

B295/P1419

Ruxolitinib is a P-glycoprotein modulator, a delayer of T-cell activation, and a reducer of CCL19 chemokine-directed migration in human cytotoxic T lymphocytes

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Cytotoxic T lymphocytes (CTLs) rely on tightly regulated activation and trafficking to fulfill their adaptive immune responses, which are enforced by immune memory. Modulation of these pathways can profoundly affect T cell differentiation and function. Earlier, we recognized that long-lived small human $CD8^+$ memory pools maintain a higher level of P-glycoprotein (Pgp, MDR1, ABCB1) expression (Biwott et al., Biomed Pharmacother. 2025 Jan:182:117780). Here, we investigated how ruxolitinib, a clinically approved JAK1/2 inhibitor, affects Pgp expression and function, T cell activation markers, and chemokine-driven transmigration in human $CD8^+$ T cells. Ruxolitinib modulates Pgp activity at multiple levels. It induced and stabilized ABCB1 mRNA during acute TCR activation, directly inhibited Pgp efflux function, and stimulated basal ATPase activity in a manner similar to the known Pgp substrate verapamil. Functional assays confirmed ruxolitinib as a Pgp modulator agent and its inducer. In addition, treatment with ruxolitinib suppressed TCR-induced upregulation of PD-1 and CD8 surface markers, suggesting interference with activation-associated differentiation pathways. Finally, ruxolitinib significantly impaired CCL19-driven transmigration of CTLs across human umbilical vein endothelial cell (HUVEC) monolayers at clinically relevant concentrations, highlighting its inhibitory effect on lymphoid homing mechanisms. Our findings reveal that ruxolitinib profoundly reshapes CTL function by altering efflux mechanisms, activation dynamics, and migratory behavior. These insights have significant implications for optimizing JAK-targeted therapies in immune modulation, vaccination, transplantation, and memory T-cell-based immunotherapies (Biwott et al., Int J Mol Sci. 2025 Jun 26;26(13):6123).

B294/P1418

Tumor-associated CD47 regulates macrophage phagolysosome acidification and antigen presentation by suppressing the phagocyte NADPH oxidase during tumor immunotherapy.

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Antibody dependent cellular phagocytosis (ADCP) is an important effector mechanism contributing to the depletion of cancer cells. Expression of CD47 by tumors suppresses ADCP by engagement of SIRPα on macrophages. Through genome-wide CRISPR screens, we have found that tumor-expressed CD47 suppresses activation of the phagocyte oxidase (NOX2), promoting rapid phagolysosome acidification and antigen

degradation. In vitro analysis revealed that target-associated antibody promoted NOX2 activation leading to slowed phagosome acidification, which was antagonized by target-associated CD47. Importantly, NOX2 deficient macrophages had no defects in phagocytosis or in their ability to kill B16F10 tumoroids. To determine if this effect was relevant in tumor immunotherapy, we challenged mice with syngeneic B16F10 melanoma bearing a CRISPR gene disruption in CD47. Disruption of CD47 rendered subcutaneous melanoma tumors susceptible to treatment with anti-Tyrp1 antibodies, leading to slowed tumor growth and ~40% of mice attaining complete cure. 50% of the mice that attained cure rejected a second tumor challenge 80 days later suggesting strong engagement of the adaptive immune system. In contrast, anti-Tyrp1 provided no benefit to animals inoculated with wild-type B16F10. The therapeutic effect against the CD47-deficient B16F10 required interferon-γ and CD8 T cells, but not CD4 T cells, indicating a key role for antigen cross-presentation. All therapeutic benefits of anti-Tyrp1 were lost in NOX2-deficient mice, suggesting a loss of antigen cross-presentation. Together these findings indicate that CD47 regulation of phagolysosome function via NOX2 critically shapes antigen cross-presentation and immunotherapy responses to antibodies targeting tumor surface antigens.

Immune Cell Biology - Innate immunity and inflammation

B287/P1411
Assessing Impact of Atenolol on Oyster Hemocytes
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The Eastern oyster Crassostrea virginica is a filter feeder that is exposed to a variety of compounds in the seawater within its natural habitat. Hemocytes, the main immune effectors of C. virginica, are potentially exposed to various bioaccumulated compounds. Contaminants of emerging concern, including pharmaceuticals such as the beta-blocker atenolol, are increasingly detected in estuaries and waterways. Although there are growing number of studies on the toxicity of contaminants of emerging concern, its impact on marine invertebrates and specifically on immune cells such as hemocytes is less well studied. The purpose of this study was to assess the impact of atenolol on oyster hemocyte function and evaluate the use of hemocytes as a model for cell toxicity assays. Hemolymph, the fluid hemocytes are circulating within, was collected from live C. virginica and hemocytes were exposed in-vitro to atenolol (0-1 uM) over a 24-hour period. Impact of atenolol on hemocytes was evaluated using a colorimetric cell viability assay and flow cytometry (cell viability, apoptosis, ROS, phagocytosis, acidic organelle abundance). Results suggest that in-vitro exposure of hemocytes to environmentally relevant concentrations of atenolol was non-lethal. However, differential response was observed for hemocyte subpopulations. C. virginica hemocytes can potentially be utilized as a model for preliminary assessment of the impact of environmental contaminants on marine invertebrates with consideration taken for differences in sub-populations. Further investigations comparing differences in response of subpopulations of hemocytes to other contaminants are needed.

B271/P1395
Breaking stereotypes: investigating the role of cell cycle regulators in macrophage inflammatory response to infection
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Mitochondria are traditionally known for their role as major cellular energetic hubs through oxidative phosphorylation, yet recent work has highlighted their role in cellular signaling, metabolism, and the immune response. Our lab and others have identified an increase in macrophage mitochondrial abundance in primary macrophages during methicillin-resistant Staphylococcus aureus (MRSA) infection. Intriguingly, this occurs despite a well-established decrease in respiration, leading us to investigate how mitochondrial remodeling regulates macrophage activation. A chemogenomic screen identified that inhibitors of cyclin-dependent kinases (CDKs) inhibited this mitochondrial remodeling during infection, revealing a potential pathway that has remained relatively unexplored. Notably, no increase in macrophage proliferation or DNA content is observed during infection. This data raises the critical questions: which cell cycle regulators are responsible for macrophage activation during infection and what are their underlying mechanisms? Analysis of transcript levels reveals elevated expression of G1-associated genes Cdk6, Ccnd2 (cyclin D2), and E2f5 in MRSA-infected primary macrophages, with coincident downregulation of Cdk2, which is involved in S-phase entry. We also identified elevated transcript levels of Cdkn1a (p21), a CDK inhibitor, during infection. These findings support a working model in which MRSA infection induces Cyclin D2 to bind and activate CDK6, leading to phosphorylation of the retinoblastoma protein (pRB) and de-repression of the transcription factor E2F5, thus regulating the macrophage inflammatory program. Downregulation of CDK2 and upregulation of the inhibitor p21 during infection supports the idea of an active block in proliferation and DNA replication by inhibiting the transition from G1 to S phase of the cell cycle. However, the protein levels of CDK6 and CDK2 do not fully reflect the changes in transcript levels. We are currently testing whether cells need a secondary signal to change the abundance of the cell cycle proteins during infection. We will also test the necessity of cell cycle regulators for this mitochondrial remodeling program by genetic perturbation using a CRISPR-based approach. Further experiments will focus on measuring changes in the interactions and post-translational modifications of CDKs and cell cycle-related proteins and perturbing them to determine their contribution to the macrophage inflammatory response to infection.

B286/P1410

Circadian Control of Innate Immune Functions: Investigating the Rhythmic Expression of Colonic RelmB (retnlB)

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Most life forms on Earth are synchronized to the 24-hour light–dark cycle created by the planet's rotation, exhibiting daily rhythms in physiology and behavior. The circadian clock is the biological system that coordinates these rhythms. Recent studies have shown that the circadian clock influences multiple aspects of immunity, yet the mechanisms by which it drives specific immune rhythms remain poorly defined. Antimicrobial peptides (AMPs) are critical innate immune effectors that protect mucosal surfaces throughout the body. In the mammalian intestine, AMPs must strike a balance between preventing pathogen invasion and maintaining symbiosis with the microbiome. Here, we identify resistin-like molecule β (RelmB), an AMP produced exclusively by specialized epithelial goblet cells, as exhibiting rhythmic expression in mice. Using mouse genetics and germ-free mouse models, our investigations reveal that both the microbiome and the transcription factor STAT6 are required for generating these rhythms, in concert with the circadian clock. Current work seeks to address how these signals converge to promote rhythmic expression of a key AMP. Beyond defining the mechanisms underlying rhythmicity, we have developed in vitro and in vivo tools that uncouple RelmB expression from circadian regulation, enabling us to address a central question: why are these antimicrobial rhythms generated in the first place?

B283/P1407

Clustering of CAR Macrophages and synergy with cancer-opsonizing antibodies against 3D tumoroid growth

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Introduction: Solid tumors sometimes show macrophage clusters, which are associated with survival as well as with M1 polarization of the macrophages. Such polarization can be induced by various means including expression of chimeric antigen receptors in macrophages (CAR-Ms), but clustering of CAR-Ms is understudied. Solid tumors in the clinic are also genetically diverse compared to liquid cancers, and CAR-Ms can in principle engage various tumor-opsonizing antibodies to other targets via Fc-Receptors although synergy remains unclear to date. Results: We find that the combination of a CAR and a distinct mAb can suppress 3D tumoroid growth over days even though no synergy is evident in conventional 2D phagocytosis assays (where macrophages are dispersed on a dish). CAR-Ms do surpass parental mAbs in driving 2D phagocytosis, which is consistent with a pro-phagocytic M1 polarization that promotes the macrophage clustering and a cooperative engulfment that increases potency in 3D. Tuning of CAR signaling strength with phospho-mutants is also uncovering differential effects on polarization and cooperative phagocytosis. The findings illustrate how synergy with antibodies and CAR re-design can be leveraged to enhance macrophage-mediated anti-tumor activity, offering an alternative to CAR-T cells that have proven ineffective against solid tumors in the clinic.

B272/P1396

Cyclin-dependent kinase inhibitors alter infection-induced mitochondrial biogenesis and inflammatory output

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Inflammation and the subsequent resolution from inflammation are vital and complex processes. Macrophages are early responders to the sites of infection and coordinate the inflammatory response. In response to infection, macrophages undergo significant remodeling of subcellular architecture and metabolism. We recently identified an increase in mitochondrial network abundance in macrophages during infection with Methicillin-resistant Staphylococcus aureus (MRSA), despite a concomitant decrease in mitochondrial respiration. We hypothesized that this infection-induced mitochondrial expansion is part of a specialized program, which we have termed inflammatory biogenesis. A chemogenomics screen for compounds that could perturb inflammatory biogenesis identified inhibitors of cyclin-dependent kinases (CDKs) as prominent hits. While CDKs are well characterized in the context of cell proliferation, we found no evidence of proliferation following infection. As CDK inhibitors perturb inflammatory biogenesis, we hypothesize that CDKs contribute to inflammatory biogenesis. However, CDKs regulate broad cellular programs, including regulating mitochondrial mass through post-translational modification of nuclear respiratory factor 1 (NRF1). To identify downstream mediators of the inflammatory biogenesis program, we investigated expression of peroxisome proliferator-activated receptor gamma coactivator-1 (PGC1) family members, known regulators of mitochondrial biogenesis. We determined that peroxisome proliferator-activated receptor gamma coactivator-related protein 1 (PPRC1) expression patterns following infection might position this regulator as a mediator of inflammatory biogenesis. We hypothesize that inflammatory biogenesis is mediated by PPRC1 and its binding partners - including NRF1 - to preferentially upregulate select proteins or pathways to support macrophage function following infection. To identify proteins which define the inflammatory biogenesis program, we have taken an

organelle-specific proteomics approach. Here we present our work which supports a model where cell cycle regulators are engaged during MRSA infection, in turn engaging PGC1 family member PPRC1 to mediate an expansion of the mitochondrial network. Our work suggests novel roles for cell cycle regulators and PPRC1 in coordinating macrophage mitochondrial expansion following infection with MRSA.

B279/P1403
DGKe depletion suppresses LPS-mediated NF-kB activation and reduces NO-mediated DNA damage in the liver.
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Diacylglycerol kinase (DGK) phosphorylates diacylglycerol (DG) to produce phosphatidic acid (PA). DG activates protein kinase C (PKC), RasGRP and transient receptor potential channels. PA also activates atypical PKC and hypoxia inducible factor 1α (HIF-1α). Thus, DGK regulates lipid-mediated intracellular signaling machinery. Of the DGK family, DGKε shows the simplest molecular structure (64kDa) and is unique in terms of substrate specificity toward arachidonates-containing DG. Previously, we have reported DGKε is the only isozyme which responds to the stimulation by lipopolysaccharide (LPS) in human T cells. However, it remains to be unclear how DGKε deletion affects the process of LPS-mediated NF-κB activation mechanism. The present study examined how DGKε depletion affects LPS-mediated inflammatory responses at the cellular and organismal levels. DGKε-deficient mouse embryonic fibroblasts (MEFs) showed reduced phosphorylation levels of NF-κB p65 subunit in the early phase. Immunofluorescent and nuclear fractionation studies revealed delayed nuclear translocation of p65 subunit in DGKε-KO MEFs. In addition, phosphorylation levels of Akt was reduced in DGKε-KO MEFs. In the animal model of LPS-injection, DGKε-deficient mice showed full survival (100%) 24 h after administration, whereas the survival rate of wild type mice was 53%. In this setting, , The NF-κB-inducible inflammatory genes such as, tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) were downregulated in DGKε-deficient liver. Furthermore, NO-mediated cytotoxicity as evaluated by 8-OHdG staining was significantly reduced in DGKε-deficient liver. Taken all together, present study suggests that DGKε depletion leads to suppressed NF-κB pathway, thereby conferring resistance to LPS-induced endotoxin shock in mice.

B273/P1397
Distinct Effects of Licochalcone B: Anti-inflammatory Activity and High-Dose Cytotoxicity in HaCaT Keratinocytes
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Atopic dermatitis (AD) is a disease that causes chronic skin inflammation due to overactivation of the Th2 cell-related inflammatory response. Currently, topical steroid hormones are widely used as a treatment for AD, but they are limited by various side effects when used for a long time. Since AD patients mainly require long-term treatment, alternative medicine based on natural compounds has been actively researched in recent years. This study aimed to evaluate the anti-inflammatory effects and cytotoxicity of Licochalcone B (LicB), a flavonoid derived from licorice (Glycyrrhiza spp.), and to explore its potential as a natural product-based therapeutic agent for AD. HaCaT keratinocytes treated with Tumor Necrosis Factor-alpha (TNF-α) and Interferon-gamma (IFN-γ) were used to mimic conditions similar to AD, while normal keratinocytes were used to assess the toxic effects of LicB on cell viability. Low concentrations of LicB significantly inhibited mRNA expression of

inflammatory cytokines and chemokines such as Interleukin-6 (IL-6), Thymus and Activation-Regulated Chemokine (TARC), Macrophage-Derived Chemokine (MDC), and Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES). This suggests that it may contribute to alleviating inflammation by suppressing the recruitment of related immune cells. On the other hand, at high concentrations, TUNEL-positive cells increased, and activation of the apoptosis pathway was observed as cell viability decreased. Moreover, an increase in the ratio of cleaved PARP, cleaved caspase-3, -8, and Bax/Bcl-2 suggests that both intrinsic and extrinsic apoptosis pathways were stimulated. This study evaluated the concentration-dependent effects of LicB, provided basic data for appropriate dose setting and toxicity management of licorice-derived ingredients, and assessed the potential of LicB in the development of AD treatments.

B288/P1412
Effects of TirC in Dictyostelium edited by CRISPR-Cas9
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Dictyostelium discoideum, a model amoebozoan that lacks the canonical Toll-like receptors (TLRs) found in animals, possesses several proteins containing Toll/interleukin-1 receptor (TIR) domains. These domains, first identified in TLRs, are now known to mediate a broad range of cellular signaling functions across diverse organisms, including bacteria. One such D. discoideum TIR domain-containing protein, TirC, is transcriptionally upregulated during phagocytosis, yet its role remains uncharacterized. To investigate its function, we generated tirC mutant strains of D. discoideum using CRISPR-Cas9 genome editing. Two independent mutants were obtained, one containing a premature stop codon and another with a frameshift mutation at AA162, within the TIR domain. Preliminary data suggest that disruption of tirC does not affect growth in axenic media but may alter multicellular development. Ongoing work is examining how loss of TirC impacts bacterial phagocytosis. Together, these studies aim to elucidate the role of TIR domain–containing proteins in D. discoideum and contribute to understanding how TIR-domain signaling may have evolved across eukaryotes and prokaryotes.

B285/P1409
Human Non-canonical Inflammasomes Activate CASP3/7 To Limit Intracellular Salmonella Replication In Macrophages.
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Inflammasomes are multiprotein signaling platforms that activate inflammatory caspases to induce pyroptosis. In humans, canonical inflammasomes activate CASP1, which cleaves the pore-forming protein gasdermin D (GSDMD) and the cytokines IL-1β and IL-18. In contrast, the non-canonical inflammasome detects bacterial lipopolysaccharide (LPS) through CASP4/5, which cleave GSDMD to drive pyroptosis. While CASP1 substrates are well characterized, CASP4/5 substrates remain less defined. Furthermore, though CASP4/5 are central to antibacterial defense, the underlying mechanism has not been fully-defined. Here, we show that in response to LPS and bacterial infection, CASP4/5 also cleave and activate the executioner caspases CASP3/7. CASP3 in turn cleaves gasdermin E (GSDME) to trigger pyroptosis. Surprisingly, CASP3 was essential, but GSDME dispensable, for restricting intracellular Salmonella replication, suggesting that CASP4/5-induced apoptosis

contributes to host defense. We further demonstrate that most GSDMD cleavage during non-canonical inflammasome activation is mediated by CASP1, and that both GSDMD and GSDME contribute to CASP4/5-dependent pyroptosis. Finally, we confirm that CASP4/5 activate CASP3/7 and GSDME in human primary macrophages. These findings establish CASP4/5 as dual apoptotic initiator and inflammatory caspases and reveal a central role for the apoptotic signaling cascade in non-canonical inflammasome-mediated immunity.

B281/P1405
IL-10 Induction by MAB_0676c Impairs Autophagic Flux through the MTOR/RUBCN Pathway
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Mycobacterium abscessus subsp. abscessus (M.abs) is a nontuberculous mycobacterium that can infect human lung macrophages, which poses a public health concern. Understanding its mechanism is crucial for developing strategies to combat M.abs infections. M.abs survives within host cells by inhibiting autophagy, a defense mechanism used against intracellular pathogens; therefore, we investigated the mechanism underlying autophagy inhibition and human lung macrophage infection by M.abs. This study focuses on the M.abs UC22 strain, which exhibits stronger inhibition of autophagic flux compared to the M.abs ATCC 19977 strain. Central to this study is MAB_0676c, a protein secreted by M.abs UC22, and its effects on autophagic flux and the innate immune response, particularly its role in enhancing IL-10 production, a known autophagy regulator. Experiments showed that MAB_0676c expression stabilizes autophagy-related proteins while reducing LC3-LAMP2 co-localization in macrophages, thereby inhibiting autophagy and promoting bacterial growth. Furthermore, blocking IL-10 reduced both autophagy-related protein levels and the intracellular growth of MAB_0676c-expressing bacteria. Therefore, M.abs UC22 mediates intracellular survival by inhibiting autophagy through IL-10 production. Our study reveals bacterial immune-evasion tactics and identifies a potential therapeutic target for treating infectious diseases caused by nontuberculous mycobacteria.

B290/P1414
Investigating Suppression of C. elegans Intracellular Pathogen Response by Viral Proteins
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Intracellular pathogens have evolved to evade an organism’s immune response and cause an infection. How viruses elude a host’s immune system is not entirely understood. C. elegans are a useful model organism to study host-pathogen interactions due to its dependence on innate immunity, eliminating complex interactions in adaptive immunity. The intracellular pathogen response (IPR) is an innate immune response that protects C. elegans against certain stresses and pathogens. The only known virus to naturally infect C. elegans is the Orsay virus. It is a positive-sense single-stranded RNA virus that contains four viral proteins: RDRP (RNA-dependent RNA polymerase), capsid, alpha-delta, and delta proteins. The goal of this study is to investigate the effects of capsid and alpha-delta proteins on the IPR in C. elegans. Preliminary research suggested that the Orsay virus suppresses the IPR. We hypothesized that one or more of the viral proteins of the Orsay virus are responsible for the suppression. In order to study the effects of viral proteins individually, we used transgenic strains that contain a heat shock promoter driving either Orsay capsid or Orsay alpha-delta proteins. These strains were treated with bortezomib, a known IPR activator, to compare the IPR activation levels with and without heat shock-induced viral protein expression. Pals-5, a gene that is upregulated when the IPR is activated, was

measured using qPCR to quantify IPR levels. Our data suggests that the Orsay capsid protein is contributing in the suppression of the IPR. From this project, we will get closer to understanding how the Orsay virus suppresses the innate immune system of C. elegans.

B277/P1401

Neutrophil secretory proteins inhibit calcium oxalate crystallization and crystal growth, but promote crystal aggregation

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Neutrophil granule proteins are frequently found in calcium oxalate (CaOx) kidney stone matrix, suggesting the involvement of neutrophil secretory proteins in stone pathogenesis, but with unclear mechanisms. We therefore investigated the effects of secretome (a set of secretory proteins) from CaOx monohydrate (COM)-exposed vs. control neutrophils on crystallization, growth, aggregation and tissue invasion of COM crystals. Quantitative proteomics was also performed to determine significantly altered secretory proteins, followed by analyses of their physicochemical properties and biological relevance. The data demonstrated that both COM-treated and control secretomes inhibited crystallization and crystal growth, but the inhibitory effects from the COM-treated secretome were slightly weaker. By contrast, both of them promoted crystal aggregation, with the more potent effect from the COM-treated secretome. However, neither of them had a modulatory effect on crystal invasion. Quantitative proteomics revealed 20 decreased and 9 increased proteins in the COM-treated secretome compared with the control. Analyses of physicochemical properties showed that the increased secretory proteins tended to have lower instability index and number of oxalate-binding motifs/protein. Main molecular functions of the increased group were catalytic, hydrolase and transporter activities, whereas those of the decreased group included RNA binding, molecular adaptor activity and catalytic activity. These data indicate that neutrophil secretome inhibits COM crystallization and growth, but promotes crystal aggregation. The COM-treated secretome exerts weaker inhibitory effects on crystallization and growth but has a stronger promoting effect on crystal aggregation. These findings enhance our understanding of the roles of neutrophils in kidney stone pathogenesis.

B274/P1398

Pinpointing D. discoideum Genes Targeted by Uropathogenic E. coli Virulence Factor

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The over prescription of antibiotics is causing a surge of bacteria that are resistant to antibiotics, leading to increasing rates of hospitalization and an increase of hospital spending. Characterization of bacterial virulence factors can offer development of alternative methods of treatment towards microbial infections. Uropathogenic Escherichia coli (UPEC) virulence factor, TcpC, is a Toll/interleukin-1 receptor (TIR) domain containing protein that disrupts innate immune signaling cascades. TcpC disrupts signaling in mammalian macrophages by association with Toll-Like receptors and MyD88, disrupting interactions and priming the molecules for the ubiquitin proteosome system. The model host organism, Dictyostelium discoideum, is a soil-dwelling amoeba that consumes bacteria in a similar fashion to mammalian macrophages. Previous experiments in our laboratory using D. discoideum as a model host organism indicate that TcpC has an inhibitory effect on D. discoideum grazing responses. We use lawn assays to identify potential targets of TcpC,

reasoning that if a D. discoideum strain is deficient for a gene target of TcpC, then there should be no difference in its ability to graze bacteria with and without TcpC. Our initial results suggest that the actin-binding protein, AbpC, might be a target of TcpC, while the glycoprotein Gp130, known to play a role in bacterial phagocytosis, does not appear to be a target. Through qRT-PCR analysis, results suggest that the expression fold change in AbpC determines it may not be targeted by TcpC at the transcriptional level. We are currently examining the downstream processes in the AbpC signaling pathways to characterize the effects that TcpC might have on this protein's function. These studies may provide insight into novel pathways targeted by TcpC that could also be targeted in mammalian macrophages.

B289/P1413

TFEB and TFE3 regulate STING1-dependent immune responses by controlling type I interferon signaling

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STING1 is an essential component of the innate immune defense against a wide variety of pathogens. Whereas induction of type I interferon (IFN) responses is one of the best-defined functions of STING1, our transcriptomic analysis revealed IFN-independent activities of STING1 in macrophages, including transcriptional upregulation of numerous lysosomal and autophagic genes. This upregulation was mediated by the STING1-induced activation of the transcription factors TFEB and TFE3, and led to increased autophagy, lysosomal biogenesis, and lysosomal acidification. TFEB and TFE3 also modulated IFN-dependent STING1 signaling by controlling IRF3 activation. IFN production and cell death were increased in TFEB- and TFE3-depleted iBMDMs. Conversely, TFEB overexpression led to reduced IRF3 activation and an almost complete inhibition of IFN synthesis and secretion, resulting in decreased CASP3 activation and increased cell survival. Our study reveals a key role of TFEB and TFE3 as regulators of STING1-mediated innate antiviral immunity.

B278/P1402

The Convergent Impact of Sex, Immunity, and Metabolism on Infection Susceptibility Revealed by snRNA-seq in Drosophila

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Individuals in a population often show phenotypic variation in susceptibility to bacterial pathogenic infection. Using Drosophila melanogaster and Pseudomonas entomophila - a lethal entomopathogenic bacterium - as a host-pathogen model, our lab's previous work highlighted that variation in infection susceptibility within populations is driven by energetic trade-offs facilitated by immuno-metabolic signaling networks. Prior work revealed two phenotypic cohorts after infection: high-resistance flies, showing low levels of innate immune activation but efficiently cleared bacteria through defecation driven by lipid mobilization; and low-resistance flies, which had elevated immune signaling that limited lipid turnover and defecation, having higher infection susceptibility. Infection responses are often sexually dimorphic, although it remains unclear if and how biological sex and reproduction influence immuno-metabolism to shape infection outcomes. We are now leveraging innovative RNA-sequencing techniques to further investigate sex-specific determinants of energy

metabolism that affect infection responses. Single-nuclear RNA-sequencing of whole, adult Drosophila has revealed an intimate integration of innate immunity and metabolism by providing holistic, comparative analysis of organism-wide gene, cell, and tissue interactions. Analysis of males and females has further emphasized that biological sex and female-specific reproductive traits critically define energetic trade-offs and immuno-metabolic responses to infection that contribute to population variation in pathogen susceptibility. Our preliminary data highlight that female reproductive tissues, part of a multi-tissue signaling network, influence immuno-metabolic responses and lipid-dependent energy resource allocation after bacterial infection. Innovative transcriptomic techniques coupled with molecular analyses suggest that sex-specific and mating-dependent changes in reproduction potentially represent an evolutionary strategy to enhance population defenses against infection.

B284/P1408

The Lysosomal Solute Carrier SLC29A3 Controls Phagosomal Dynamics and Promotes Autophagy in Murine Dendritic Cells

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Phagocytes like dendritic cells (DCs) capture bacteria into newly formed phagosomes. Phagosomes then mature by fusing with the endolysosomal system, thereby acquiring toll-like receptors (TLRs), degradative enzymes, and solute carriers (SLCs) like SLC29A3. SLC29A3 exports nucleosides out of phagolysosomes to maintain solute and pH homeostasis, as the accumulation of nucleobases may increase phagosomal pH. SLC29A3 deficiency causes the human inflammatory disorder H-syndrome, which resembles lysosomal storage diseases and is characterized by hyperinflammation and immune dysregulation. However, the mechanisms driving hyperinflammation in H-syndrome are incompletely understood. Notably, the role played by DCs, the main link between innate and adaptive immunity, remains unknown. We examined the role of SLC29A3 in DC-mediated inflammation using murine bone marrow-derived (BM) and tissue-resident splenic DCs from wild-type (WT) and SLC29A3-/- mice as model systems. Using fluorophore-coated beads in a flow-cytometric assay, we found that phagosomes in SLC29A3-/- splenic DCs are more alkaline than their WT counterparts. Phagosome alkalinization may impair TLR processing and the generation of microbial patterns from phagocytosed bacteria. Accordingly, SLC29A3-/- BMDCs secrete lower amounts of cytokines and chemokines upon phagocytosing Salmonella typhimurium (STm) compared to WT BMDCs. In addition, the activation of transcription factor EB (TFEB), the master regulator of autophagy (a major catabolic and anti-inflammatory mechanism), is impaired in SLC29A3-/- BMDCs in response to STm capture. In consequence, autophagy function in SLC29A3-/- DCs is reduced, as seen by immunoblotting of the autophagy-associated proteins LC3 and p62. We found that SLC29A3 promotes TFEB activation by modulating the activity of mTORC1 and the lysosomal Ca2+ channel TRPML-1, which requires an acidic pH for activity. SLC29A3 inhibits mTORC1 after phagocytosis, as suggested by the increased phosphorylation of mTORC1 and its downstream substrates in SLC29A3-/- BMDCs. By using a genetically-encoded Ca2+ indicator fused to TRPML-1, we visualized that the number of phagosomes actively exporting Ca2+ was significantly reduced in SLC29A3-/- BMDCs compared to WT cells after STm phagocytosis. This suggests that SLC29A3 promotes TRPML-1 activity, leading to Ca2+-dependent TFEB activation after phagocytosis. Together, our data suggest that the export of phagosomal nucleosides by SLC29A3 is critical for phagosome-associated pro-inflammatory signaling and its regulation by autophagy in response to bacteria phagocytosis in DCs. Our data also suggest that defects in TLR signaling and autophagy may contribute to immunodeficiency and hyperinflammation in H syndrome.

B282/P1406

The Role of Host Stress Granules in Promoting Mycobacterium tuberculosis Pathogenesis

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Stress granules (SGs) have been identified as cytoplasmic condensates that regulate mRNA translation and signaling during periods of stress. While SGs have been demonstrated to regulate antiviral responses, their function during bacterial infections remains to be elucidated. In this study, we demonstrate that SG-mediated translational control contributes to the pathogenesis of Mycobacterium tuberculosis (Mtb) by suppressing mitochondrial energy metabolism and host immune defenses. Mtb-induced SGs have been shown to inhibit mTORC1 activity and cap-dependent mRNA translation, resulting in a reduction in the expression of mitochondrial and immune-related proteins in macrophages. Disruption of SG formation has been shown to restore mTORC1 signaling, enhance mitochondrial respiration, and boost antimicrobial mediators such as ROS, NO, and pro-inflammatory cytokines. This, in turn, restricts Mtb survival. Intracellular ATP depletion has been demonstrated to induce SG assembly, suggesting that energy stress during Mtb infection favors SG formation and immune suppression. The present findings suggest that Mtb utilizes the host's stress granules (SGs) to regulate translation and energy metabolism, thereby facilitating its ability to evade the immune system and persist within host cells. These observations underscore the potential of SGs as a target for host-directed therapies aimed at restoring mitochondrial function and immune responses to regulate Mtb infection.

B276/P1400

The Role of TcpC's NADase activity on mammalian macrophages' immune response

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The innate immune system defends against pathogens using Toll-like Receptors (TLRs) that identify bacterial molecular patterns and trigger immune responses by initiating signaling cascades through Toll/interleukin-1 receptor (TIR) proteins. Uropathogenic E. coli produces TcpC, a TIR protein that binds to host TIR proteins, blocking immune response. TcpC also exhibits NADase activity. We hypothesize that TcpC’s NADase activity contributes to its immune response inhibition. To test this, we compared the effects of a TcpC mutant EA without NADase activity and wild-type TcpC on macrophage response. TcpC was synthesized in-vitro and successfully isolated by adding streptavidin beads and elution with biotin. A MyD88 pull-down assay shows that E244A and WT TcpC bind to MyD88 at similar rate, indicating that NADase activity is not related to the MyD88 binding ability of TcpC. As a strategy to produce a higher yield of TcpC TIR protein for further functional assays, we used site-directed mutagenesis to create an E244A mutant plasmid for recombinant protein expression, and purified WT and E244A TcpC proteins using a Streptactin column followed by size exclusion chromatography. Using size exclusion chromatography, we have isolated single monomer TcpC.. We are currently treating macrophages with single monomer WT and E244A TcpC to test the effects of NADase activity on cytokine production in response to treatment with LPS. Comparison of macrophage response in the presence of TcpC WT or E244A would give insight into the potential importance of NADase activity to the TLR inhibition function of bacterial TIR proteins.

B280/P1404

The roles of caspases-8 and -10 in the TLR4-induced innate immune response in human macrophages

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In response to infection, a host cell engages innate immune defense mechanisms, including the upregulation and secretion of pro-inflammatory cytokines and the induction of regulated cell death. In murine macrophages, the cysteine protease caspase-8 is a key mediator of multiple innate immune signaling pathways, including apoptosis, necroptosis, and pro-inflammatory cytokine expression, in response to microbial infection. However, in contrast to mice, which only encode caspase-8, humans express caspase-8 as well as caspase-10, a homologous protein whose function remains poorly defined. We thus aimed to elucidate the roles of caspases-8 and -10 in human macrophage responses to bacterial stimuli. We found that the dependencies upon caspase-8 for pro-inflammatory gene expression, cytokine secretion, and cell death observed in murine macrophages were not recapitulated in human macrophages. At the transcriptional and protein levels, pro-inflammatory cytokine production in human CASP8-/- and CASP10-/- macrophages was equivalent to that in wild-type macrophages, and Salmonella-induced cell death was also independent of caspases-8 and -10. These findings suggest that caspases-8 and -10 have divergent roles in human and murine macrophages, highlighting species-specific differences in innate immune regulation.

B275/P1399

Therapeutic Potential of Galangin in Inflammatory Diseases

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Cardiovascular disease remains one of the leading causes of mortality in the United States. A primary contributor is atherosclerosis, which underlies conditions such as coronary artery disease and ischemic stroke. This process is characterized by the buildup of lipids within blood vessels, leading to vascular narrowing, secretion of inflammatory cytokines, and leukocyte adhesion. The resulting plaque formation can progress to thrombosis, vessel rupture, and cellular damage. Galangin, a naturally occurring flavonoid found in honey, has demonstrated promising anti-inflammatory activity in previous studies. To evaluate the anti-inflammatory effects of galangin in vitro, Bovine Aortic Endothelial Cells (BAOECs) and THP-1 cells served as model cell lines. The anti-inflammatory and wound healing properties of galangin were assessed using BAOECs, while THP-1 cells were used to examine galangin’s ability to reduce cellular adhesion to commonly used biomaterials. Initial viability assays showed that galangin is non-cytotoxic to both cell lines. Endothelial cell studies revealed enhanced cellular migration in wound-healing assays along with a reduction in actin stress fiber formation in cells exposed to TNF-α. THP-1 adhesion assays did not yield significant differences, suggesting that while galangin is effective as a mitigator of inflammation relevant to the prevention of heart disease, it does not prevent immune cell recognition of implantable materials. These results suggest that galangin should be studied further for potential therapeutic benefit in certain inflammatory diseases.

Membrane Biology and Trafficking - Endocytosis and recycling mechanisms

B235/P0813
Alpha-arrestin regulation of nutrient transporter endocytosis in response to glycolytic inhibition
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Our research defines the cell signaling, trafficking, and metabolic response to 2-deoxyglucose (2DG), a toxic analog of glucose and promising therapeutic in metabolic diseases and cancers, using the yeast Saccharomyces cerevisiae and human cell lines. Like glucose, 2DG enters cells through glucose transporters. Unlike glucose, which is converted to energy via glycolysis, phosphorylated 2DG (2DG6P) accumulates in cells and inhibits glycolysis. 2DG and other glycolytic inhibitors are often used in anti-cancer and other therapeutic trials because many diseased cells undergo a metabolic shift, --known as the Warburg effect, where they become reliant on glycolysis. Unfortunately, spontaneous 2DG-resistant mutations arise at a high frequency, and a clear mechanistic understanding of 2DG toxicity remains elusive, even after >50 years of study. Without a deeper mechanistic understanding of how cells acquire 2DG resistance, we cannot fully capitalize on 2DG's therapeutic potential. Our studies provide new fundamental insights into how metabolism, signaling, and trafficking are altered in response to 2DG. Like many cancerous cells, yeast ferment glucose and are sensitive to 2DG but rapidly acquire resistance. Our work shows that 2DG resistance is associated with activating mutations in AMP-activated protein kinase (AMPK), a central energy sensor and signaling regulator in cells. We discovered that 2DG-resistant cells impair 2DG-induced endocytosis of nutrient transporters, and we have comprehensively mapped the scope of this endocytic response. Importantly, this 2DG-induced endocytosis is controlled by a suite of alpha-arrestins, whose activity is regulated by AMPK. We present the first evidence for a massive cellular rewiring in response to 2DG that stimulates alpha-arrestin-mediated endocytosis of a wide-array of membrane proteins and show that this is important for 2DG-induced toxicity. Based on our studies, we propose that internalization of key nutrient transporters, including glucose and amino acid transporters, by the alpha-arrestins results in cellular starvation and death. Thus, we propose a novel mechanism for 2DG's toxicity that is reliant on a-arrestin-directed endocytosis. All of the factors described in our studies are conserved from yeast to humans and there is good evidence that this 2DG-responsive endocytic remodeling is conserved in humans. We are currently defining the role of a-arrestins in controlling disease progression in glioblastoma models, as a pro-drug of 2DG was recently approved as a therapeutic intervention in this cancer. These findings will be help design future combinatorial therapies with 2DG, identifying the key resistance pathways that must inhibited if 2DG is to become an effective treatment.

B238/P0816
Avl9 contributes to recycling and sorting of endosomal cargo
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Trafficking of cargos between cellular compartments requires numerous proteins including Rabs and their activating regulators, guanine nucleotide exchange factors (GEFs). Biochemically, GEF activity may be encoded in numerous domains, including the Differentially Expressed in Normal and Neoplastic tissue (DENN) domain. In yeast and humans, the conserved DENN-like protein Avl9 has a poorly characterized role in trafficking and secretion. Here, we show that Avl9 localizes to punctae at the cell periphery that are consistent with secretory

vesicles. Moreover, we show that Avl9 shows minimal coincidence with the trans-Golgi network (TGN) marker Sec7, Golgi/endosomal marker Tlg1 or with the endosomal marker Ist1. We next demonstrate that avl9Δ results in defects in recycling of the endosomal cargo Snc1 but does not alter secretion of an endocytosis-defective Snc1 mutant, suggesting that Avl9 is not directly involved in secretory traffic from the TGN to the plasma membrane. The avl9Δ recycling defect is exacerbated by the additional loss of RCY1or SNX4, but not VPS35. Each of these three genes contributes to a distinct endosomal recycling pathway, indicating that Avl9 acts in conjunction with multiple pathways.

B244/P0822

Bridging protein TTR-53 mediates phosphatidylinositol phosphate signaling for cell corpse clearance

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Phagocytic cargos expose cytofacial lipids like phosphatidylserine (PtdSer) to signal for engulfment, which is important for tissue homeostasis and appropriate immune responses. The secreted transthyretin-like protein TTR-52 recognizes PtdSer for receptor-mediated phagocytosis but is not required for the engulfment of all dying cells in Caenorhabditis elegans. Therefore, we asked whether a closely related secreted protein, TTR-53, was required for phagocytosis. We discovered that endogenous TTR-53 tagged with fluorescent proteins localizes to the surface of cargos prior to their phagocytosis in early embryos, including polar bodies born during meiosis and midbody remnants formed during cell division. Using RNAi and deletion mutants, we found that TTR-53 promotes the phagocytosis of dying polar bodies, but phagocytosis of midbody remnants is not delayed after deletion of ttr-53. This indicates that an individual cell can use both TTR-53-dependent and TTR-53-independent pathways to carry out phagocytosis of distinct cargos. To establish whether TTR-53 binds to PtdSer similar to TTR-52, we induced PtdSer exposure by disrupting the PtdSer flippase TAT-1. TTR-53 localization did not change after tat-1 RNAi treatment or in tat-1 mutants, revealing that TTR-53 is unlikely to interact with PtdSer. We purified recombinant Flag-tagged TTR-53 and discovered that it binds to phosphatidylinositol 3,4,5-trisphosphate (PIP3) and phosphatidylinositol bisphosphate (PIP2) lipids in a calcium-dependent manner. This supports recent findings that PIP2 and PIP3 exposure can serve as an eat-me signal for apoptotic, ferroptotic, and necroptotic cell clearance in cultured mammalian cells (OH Kim et al., Cell Death Differ 2022). Therefore, we propose that TTR-53 acts as a bridging molecule between PIPs and engulfment receptors to promote phagocytosis of dying cells. To determine whether PtdSer and PIP exposure form the basis of redundant phagocytic signaling pathways, we asked whether preventing synthesis of PtdSer could enhance phagocytic defects when TTR-53 is absent. We found that knocking down the PtdSer synthase pssy-1 enhanced internalization defects in ttr-53 mutants, but pssy-1 knockdown did not disrupt polar body internalization when TTR-53 was present. These data indicate animal cells use interchangeable and conserved lipid signals to recognize dying cells, emphasizing the importance of clearing cell corpses for normal physiology.

B240/P0818

CCDC32 collaborates with the membrane to assemble the AP-2 clathrin adaptor complex

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Cells have evolved a variety of assembly chaperones to aid in the difficult process of forming macromolecular complexes in a crowded cytoplasm. Assembly of adaptor protein complex 2 (AP-2), the primary cargo adaptor in clathrin-mediated endocytosis, is regulated by the chaperones AAGAB and CCDC32, whose deletion causes loss of all AP-2 subunits in vivo. AAGAB and CCDC32 are thought to act sequentially to assemble the AP-2 tetramer from its constituent heterodimers. However, the molecular requirements and structural consequences of CCDC32 interaction with AP-2 are not yet understood. Here, using in vitro reconstitution and integrative structural analysis, we describe the molecular mechanism of CCDC32-mediated AP-2 assembly. First, CCDC32 interacts with the appendage domain of the AP-2 α subunit, using the same binding site as canonical endocytic regulators in addition to a novel, yet highly conserved pocket on α. CCDC32 contains cargo sorting motifs normally found in trans-membrane cargo and binds to AP-2 heterodimers using canonical cargo-binding sites. Additionally, two amphipathic helices in CCDC32 bind to the α/σ2 heterodimer. Surprisingly, in solution, we find that CCDC32 prevents complex assembly and actively disassembles AP-2 tetramers. Inhibition requires the amphipathic helices of CCDC32, which also mediate binding to PIP2-containing membranes. The presence of PIP2-containing membrane stabilizes the final stages of assembly. We propose that the membrane acts as a molecular switch to release inhibitory interactions, allowing for full complex assembly to proceed. Using cryo-EM, we visualize an assembly intermediate that mimics the conformation of AP-2 found in vesicles, with CCDC32 bound at both cargo binding sites and both membrane-binding sites, suggesting that assembly leads to deposition of active complexes on the plasma membrane.

B236/P0814

CREG1 regulates endocytosis in visceral endoderm

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Endocytosis in the visceral endoderm is crucial for fetal development, supporting histotrophic nutrition and cellular signaling during early embryogenesis. During the late fetal period, visceral yolk sac endoderm-mediated endocytosis is critical for maternal-fetal exchange and immune regulation. CREG1, an evolutionarily conserved glycoprotein, is highly expressed in the visceral endoderm, but its function remains unclear. Using immunofluorescence microscopy in mouse embryos, we detected strong CREG1 expression at E7.5. It colocalized with early and late endosomal markers on the apical side of the visceral endoderm. At E16.5, CREG1 colocalized with cubilin, disabled-2, and internalized immunoglobulin in the subapical region, and partially overlapped with the lysosomal marker LAMP-2 basally. Targeted deletion of Creg1 markedly reduced immunoglobulin uptake in the visceral yolk sac endoderm, a finding confirmed ex vivo by impaired endocytosis of FITC-labeled dextran in CREG1-deficient endoderm. Under stress conditions, such as intraperitoneal administration of tamoxifen, Creg1 knockout embryos exhibited growth retardation. Altogether, these findings identify CREG1 as a key regulator of endoderm endocytosis and embryonic development, particularly under stress.

B248/P0826

Defects in N-linked glycosylation cause delays in Rab5-mediated endosomal transport.

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The post-Golgi transport pathways involve the vacuolar protein sorting (VPS) pathway, which sends cargos to lysosomes/vacuoles, and the secretory pathway, which sends cargos to the cell surface, via transport vesicles from the trans-Golgi network (TGN). Recent studies have demonstrated that the TGN can be divided into sub-domains that produce specific carriers for efficient cargo sorting. Recently, we have shown that endocytic sorting compartment distinct from the secretory compartment exists within the TGN and Rab5-mediated endosomal transport is regulated by the post-Golgi vesicle trafficking in budding yeast. Many yeast proteins transported from the TGN are known to be modified with the high mannose-type N-glycans and these proteins are referred to as mannoproteins. Mannoproteins are important for the cell wall integrity and control the cell wall porosity. Disruption of the mannoprotein network by deletion of mannoproteins or alteration of protein glycosylation modification damages the cell wall integrity and increase the cell wall porosity, which often leads to increased protein secretion. Since secretion and endocytosis are closely related and both pathways involve transport via TGN, we examined whether defects of the N-linked glycosylation affect the endocytic pathway. We first examined the effect of deletion of gycosyltransferase family genes on the endocytic pathway and found that disruption of M-Pol I and Pol II subunits, including Mnn9 and Mnn10, causes defects of endocytic cargo trafficking. Interestingly, while no apparent defect was observed in endocytic vesicle internalization, marked delay in Rab5-mediated endosomal transport was observed in these mutants. Similar to these mutants, we also found that deletion of β-1,3-glucanosyltransferase Gas1p caused delay in defects in endosomal transport. Previous study reported that the inactivation of Gas1p led to the cell wall defect and increased the secretion of insulin-like growth factor 1 in mammalian cell. Thus, these results suggest that N-glycan modification of cell surface proteins is involved in both transport from the TGN to the plasma membrane and to endosomes.

B246/P0824

Development of chemical and genetic tools to inhibit a Clathrin and Dynamin independent (CLIC/GEEC), CG endocytic pathway.

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Cells actively take in material from their extracellular milieu via multiple endocytic mechanisms . The most well-studied of these mechanisms is the Clathrin-mediated endocytic pathway (CME) where clathrin coated vesicles are scissioned by a large GTPase dynamin. However, some endocytic pathways function independent of clathrin and dynamin. One such endocytic pathway was observed to takes in bulk extracellular fluid and Glycosyl-phsophatidylinositol (GPI)-anchored proteins into Clathrin Independent Carriers (CLIC). These CLICs fuse to form distinct GPI Enriched Early Endosomal Compartments (GEECs), hence is referred to as CLIC/GEEC or CG pathway. The CG pathway plays a crucial role in many cellular functions such as nutrient uptake, membrane tension homeostasis and in pathogenic events like cancer metastasis and viral infection, thus necessitating the need to develop chemical and genetic CG modulators . Activation of the small GTPase Arf1 by its GEF, GBF1 initiates the formation of a nascent CG endocytic pit. Inhibiting the catalytic activity of GBF1 by a chemical compound named LG186 or expression of a catalytically inactive GBF1 mutant, blocks CG endocytosis. However a few drawbacks of these inhibitory strategies called for development of better tools to specifically modulate this pathway. LG186 was observed to have cellular toxicities or loose activity upon short storage times. LG186 structure consists of an aromatic phenolic ring which is connected via a hydrazine linkage to the rest of the heterocyclic structure. The aromatic phenol undergoes hydrolysis during storage and the hydrazine linkage gives rise to inactive isomers during synthesis. Hence we designed a series of LG186 analogs

and characterized their Structure Activity Relationship with CME, CG inhibition and effect on Golgi morphology as GBF1 maintains Golgi structure and protein secretion . Modifying the phenolic ring or the hydrazine linkage alone lead to non-specific, unstable or inactive compounds. Therefore a combination strategy to modify both these components was adopted, which led to stable and specific GBF1 inhibitors. GBF1 protein consists of N-terminal DCB domain, followed by HUS domain, the catalytic sec7 domain responsible for Arf1 activation and lipid binding domains. A point mutation (E794K) in the sec7 domain leads to catalytically inactive GBF1 that inhibits CG endocytosis but also leads to disruption in Golgi function. Therefor various mutations in the fly homolog of GBF1; Garz were first screened for effects on CG endocytosis and secretion. Deletion of the DCB domain in Garz and GBF1, results in a genetically encoded CG inhibitor that does not affect golgi structure. Thus, we have developed chemical and genetic tools to inhibit the CG endocytic pathway.

B252/P0830

Elucidating Autophagic Mechanisms of GPCR Desensitization in S. cerevisiae

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G-protein coupled receptors (GPCRs) are a crucial class of extracellular sensor proteins. Mechanisms that desensitize GPCRs to their ligands are essential, as prolonged receptor activation is deleterious to a cell's ability to respond appropriately to its environment. In brewers yeast, Saccharomyces cerevisiae, several desensitization mechanisms attenuate the signaling of Ste2, a GPCR that promotes mating competency in response to mating pheromone. One mechanism of Ste2 signal regulation is receptor endocytosis. Ligand binding stimulates clathrin-mediated endocytosis and eventual delivery of the receptor to the vacuole (lysosome). Our data suggest that autophagy, a set of stress-responsive pathways that promote cell survival, may also drive Ste2 desensitization. Specifically, our data show that deletion of Atg8, a core autophagosome component that facilitates bulk and selective autophagy leads to an increase in transcription of Ste2 specific mating genes. Furthermore, Atg8Δ Ste2-GFP cells exhibit disrupted receptor trafficking. We sought to further investigate autophagy's role in GPCR signal attenuation. Considering that our cells are grown in nutrient replete media, we asked whether deletion of selective autophagy receptors (SARs) could recapitulate the upregulation of Ste2 signaling seen in Atg8Δ. We noticed modest increases in receptor abundance at the plasma membrane (PM) in deletions of several proteins including Atg36, a SAR involved in pexophagy, and Cue5 which mediates ubiquitin dependent autophagy. This prompted us to wonder if the observed increase in receptor output in Atg8Δ cells was due to autophagy promoting endocytosis. To test this, we created a HaloTag-Ste2 fusion protein that would be competent to visualize only the cohort of receptor that is present on the PM. We then labeled Halotag-Ste2 and fixed the cells at various timepoints throughout the pheromone response. We measured the mean membrane fluorescence intensity of Halotag-Ste2 at each timepoint and found no significant difference in PM receptor abundance between WT and Atg8/SAR deletions. This indicates that rates of endocytosis are not responsible for increased levels of Ste2-GFP in autophagy protein deletions and supports the conclusion that the observed disruption is driven by changes in delivery to the vacuole. Autophagy is not currently understood as a regulator of GPCR signaling. Therefore, this research represents a frontier in cellular biology that could uncover novel functions for the autophagic machinery and an overlooked mechanism of GPCR desensitization.

B253/P0831

Elucidating the Kinetics of EGFR Trafficking with an Inducible "Pulse-Chase" Method

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The human epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that plays a major role in the regulation of cellular proliferation, differentiation, and motility. EGFR activation is dependent on the binding of one of its seven known ligands, leading to receptor dimerization and signal transduction. Upon activation, EGFR is rapidly internalized and can be targeted for lysosomal degradation or recycling back to the plasma membrane. Despite several studies on the internalization of EGFR upon ligand activation, the kinetics by which ligand-bound EGFR is trafficked for degradation or recycling remain unknown, with studies focusing on only a few of the seven different ligands of EGFR. Additionally, there are conflicting studies that provide contradictory mechanisms of EGFR trafficking. For instance, some studies suggest that activation of EGFR by TGFα leads to degradation, while others postulate that TGFα disassociates from the receptor in low pH environments, allowing for recycling of EGFR to the plasma membrane. To provide clarity to this field, we have devised an approach that lends itself to a quantitative study of EGFR trafficking using physiologically relevant receptor expression levels and concentrations of ligand. In brief, we generated a binary, inducible EGFR fusion construct in which an amber stop codon (ASC) was added before a monomeric StayGold (mSG) fused to the N-terminus of human EGFR (TAG-mSG-EGFR). Our construct is expressed along with an engineered tRNA and tRNA synthetase (GCEXpress) in Chinese hamster ovarian (CHO) cells lacking endogenous EGFR expression. Using this system, we can titrate receptor expression levels in live cells and turn on or off expression as needed to study receptor trafficking without constitutive background synthesis of EGFR. This novel method allows us to overcome the limitations of previous studies and is applicable to the study of trafficking kinetics with other receptors.

B254/P0832

Elucidating the role of Spectrin ßII in endosomal morphology regulation

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Cells internalize various substances from the extracellular environment, thereby influencing their fundamental activities. Endosomes play a central role in sorting these internalized materials and transporting them to other organelles. Based on their functions and morphology, endosomes are categorized into early endosomes, late endosomes, and recycling endosomes. Interestingly, endosomes display diverse morphologies. Therefore, understanding this morphological heterogeneity, as well as its regulatory mechanisms, is crucial for elucidating endosomal functions. However, due to their small size and dynamic intracellular movements, the mechanisms regulating endosomal morphology remain largely unclear. Spectrin, known as a scaffold proteins, forms a meshwork underneath the plasma membrane, thereby maintaining cellular morphology. Spectrin exists as an α/β heterodimer, and β-Spectrin contains a pleckstrin homology (PH) domain that binds acidic phospholipids as well as an actin-binding domain, both of which are thought to be important for its membrane association. While Spectrin is well known for its functions as the plasma membrane, its localization and functions on other organelle membranes remain poorly understood. To test this, we focused on Spectrin βII (gene name: SPTBN1), which is highly expressed in HeLa cells and possesses multiple domains that mediate interactions with intracellular proteins, and we analyzed its subcellular localization and function. To investigate the intracellular localization of Spectrin βII, we first performed co-immunostaining using antibodies against Spectrin βII together with various organelle marker proteins. This analysis suggested that Spectrin βII localizes to multiple organelles; however, its colocalization with transferrin receptor (TfnR) was particularly high. These results indicate that Spectrin βII is strongly localized to recycling endosomes. Furthermore, SPTBN1 knockdown (βIIKD) cells exhibited an increased number of tubular recycling endosomes. Since endosomal tubulation is regulated by actin-associated proteins and BAR domain proteins, we examined the distribution of these membrane-

deforming factors using a system in which endosomes were enlarged by overexpressing Rab5QL. In wild-type cells, these factors localized to discrete puncta on endosomal membranes, whereas in βIIKD cells, they spread across a broader membrane area. These findings suggest that Spectrin βII regulates the localization of membrane-deforming factors on endosomal membranes. In this presentation, we will discuss a novel physiological role of Spectrin as a "brake" in endosomal morphological dynamics, based on our recent findings.

B247/P0825

Endocytic Machinery are Primed at Neuronal Synapses through Enrichment of PI4P and PS at Endocytic Zones

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Ultrafast endocytosis can retrieve membrane and synaptic vesicle protein within 50 ms after vesicle fusion. This recently discovered form of endocytosis utilizes the splice variant of dynamin 1 (Dyn1xA) and a BAR domain containing protein Syndapin 1. Both Dyn1xA and Syndapin 1 are primed at endocytic zones in synapses to accelerate the kinetics needed for ultrafast endocytosis. However, it is currently unclear how these proteins are targeted to the distinct membrane regions where ultrafast endocytosis occurs. Here we show that plasma membrane lipids, phosphatidylinositol-4-phosphate (PI4P) and phosphatidylserine (PS), are enriched at endocytic zones and mediate recruitment of Dyn1xA and Syndapin 1. Using genetically encoded lipid biosensors and super-resolution imaging with STED microscopy, we analyzed nano-scale organization of membrane lipids. We demonstrate that PI4P and PS are colocalized with Dyn1xA at endocytic zones, which is devoid of other major membrane signaling lipids. To maintain the endocytic zones PI4P abundance, we have shown that Synaptojanin1 localizes at endocytic zones and dephosphorylates the 5-phosphate of another membrane phosphoinositide PI(4,5)P2 to produce PI4P. The dephosphorylation of PI(4,5)P2 to PI4P is essential for generating the curvature needed for endocytosis. In addition, we have shown that PS can localize membrane bending and fission proteins such as Syndapin 1 using STED imaging and lipid binding mutants of Syndapin 1. STED imaging and time-resolved electron microscopy experiments demonstrate that Dyn1xA does not require its lipid-protein binding domain for localization and function during endocytosis. Instead, Dyn1xA localization at endocytic zones depends on binding to Syndapin 1 which recognizes PS through its membrane binding BAR domain. Together, these results indicate a unique lipid nano domain is formed at the endocytic zone and its formation is essential for the accumulation of endocytic proteins, allowing for the accelerated kinetics that are needed for synaptic endocytosis.

B249/P0827

GLUT Trafficking and Glucose Sensing Coordinate Insulin Secretion in Pancreatic beta-Cells: A Dysfunctional Axis in Diabetes

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Glucose transporters (GLUT1/2) mediate glucose uptake in pancreatic β-cells and are essential for initiating glucose-stimulated insulin secretion (GSIS). While their expression and membrane localization have been studied, the dynamic regulation of GLUT trafficking in response to glucose remains less understood in β-cells. In this study, we show that GLUT recruitment to the plasma membrane (PM) of β-cells increases with rising glucose concentrations and is closely coupled to clathrin-mediated endocytosis. Disruption of endocytosis,

either through dominant-negative Dynamin2-K44A expression or pharmacological inhibition with Pitstop2, alters GLUT dynamics and significantly reduces glucose uptake under high-glucose conditions. Supporting these findings, β-cells from type 2 diabetes (T2D) human islets exhibit impaired GLUT trafficking and endocytosis, as evidenced by both functional assays and single-cell RNA sequencing. These defects coincide with reduced insulin granule availability at high glucose concentrations. Furthermore, our data reveals a glucose-dependent spatial association between surface GLUTs and a subset of insulin granules primed by Munc13-1, suggesting a functional link between glucose uptake and insulin granule mobilization. Collectively, our findings highlight the critical role of GLUT trafficking and clathrin-mediated endocytosis in sustaining insulin secretion and suggest that targeting these processes may help restore β-cell function in T2D.

B237/P0815

Mechanochemical feedback defines a unified mechanism that couples synaptic vesicle exocytosis to endocytosis

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The coupling between exocytosis and endocytosis of synaptic vesicles (SVs) at neuronal synapse underlies the fundamentals of neurotransmission, the malfunctions of which contribute to many neuronal diseases. Despite great efforts, the mechanism of SV exo-endocytosis coupling remains controversial. After a single AP, the SV endocytosis is ultrafast and driven by the high-density dynamin/syndapin droplet at the periphery of active zone. However, AP intensity alters the timescale, vesicle size, and key players of SV endocytosis greatly; hence, the physiological relevance of ultrafast endocytosis and whether distinct mechanisms drive SV endocytosis under different conditions are questioned. Combining theoretical modeling and experiments, we present evidence that the dynamin/syndapin droplet locally induces the membrane invagination from the membrane pits, initially shaped by the SV exocytosis-mediated compression; thus, the high-density dynamin within the droplet accelerates the curvature-mediated positive feedback and ensures ultrafast endocytosis. This mechanism underlies both the during- and after-stimulation endocytosis upon the AP trains with the frequency- and duration-dependence that reconcile the disparate observations, including activity-dependent bulk endocytosis (ADBE). Our finding suggests a unified framework for different forms of SV exo-endocytosis coupling and sheds light on mechanical causes of neuronal diseases.

B255/P0833

Next-Generation GTP Biosensors to explore the role of GTP Localization in Macropinocytosis

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Cancer cells undergo metabolic adaptations to survive in the hostile and nutrient-deprived tumor microenvironment. A key adaptation is aberrant macropinocytosis, a conserved endocytic process that engulfs extracellular macromolecules to sustain tumor metabolism and growth. During macropinocytosis, cells generate large Actin-driven membrane ruffles which ultimately form intracellular vesicles, or macropinosomes. Intracellular macropinosome mature and fuse with lysosomes, wherein the contents are degraded to recycle extracellular nutrients. This process is associated with poor prognosis, resistance to standard of care therapies, and metastatic potential, highlighting a potential therapeutic vulnerability. Macropinocytosis is regulated by small GTPases such as Rac1, which must be in in the active GTP-bound state to trigger downstream signaling and cytoskeletal remodeling. Our group previously reported that Rac1 and other Rho GTPases are sensitive to

loss of intracellular GTP and are regulated by local GTP availability in metastatic cancer cells. Therefore, we investigated the role of GTP biosynthesis in macropinocytosis by inhibiting inosine monophosphate dehydrogenases (IMPDH1/2), which disrupts GTP production and reduces Rac1–GTP loading. We found a significant reduction in capacity for macropinocytosis in melanoma cells treated with IMPDH inhibitors, which corresponded to a substantial reduction of melanoma growth in vivo in a clinically relevant genetic mouse model. To further explore the connection between micropinocytosis and GTP metabolism, we updated previously published GTP biosensors (termed GEVALs) to have improved properties for timelapse live-cell imaging. Using several biochemical and cell-based methods, we established that these second generation GEVAL sensors can detect subcellular fluctuations in free GTP pools. Future studies will uncover the relationship between GTP dynamics, Rho GTPase activity, and macropinosome formation while further evaluating the therapeutic potential of targeting this pathway for anti-cancer treatment.

B245/P0823

PDGFRa/b Heterodimer Activation Negatively Affects Downstream ERK1/2 Signaling and Cellular Proliferation

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The platelet-derived growth factor receptor (PDGFR) family of receptor tyrosine kinases allows cells to communicate with one another by binding to growth factors at the plasma membrane and activating intracellular signaling pathways to elicit responses such as migration, proliferation, survival and differentiation. The PDGFR family consists of two receptors, PDGFRa and PDGFRb, that dimerize to form PDGFRa homodimers, PDGFRa/b heterodimers and PDGFRb homodimers. Here, we tested the hypothesis that differential internalization and trafficking dynamics of the various PDGFR dimers underlie differences in downstream intracellular signaling and cellular behavior. Using a bimolecular fluorescence complementation approach that allowed us to visualize and purify PDGFRa/b heterodimers for the first time, we found that these receptors heterodimerize relatively quickly in response to PDGF-BB ligand treatment, with a peak of receptor autophosphorylation following 5 minutes of ligand stimulation. Moreover, we demonstrated that PDGFRa/b heterodimers are rapidly internalized into early endosomes, particularly signaling endosomes, where they dwell for extended lengths of time. We showed that PDGFRa/b heterodimer activation does not induce downstream phosphorylation of ERK1/2 and significantly inhibits cell proliferation. Further, we characterized the PDGFR dimer-specific interactome and identified MYO1D as a novel protein that preferentially binds PDGFRa/b heterodimers. We demonstrated that knockdown of MYO1D leads to retention of PDGFRa/b heterodimers at the plasma membrane, resulting in increased phosphorylation of ERK1/2 and increased cell proliferation. Collectively, our findings impart valuable insight into the molecular mechanisms by which specificity is introduced downstream of PDGFR activation to differentially propagate signaling and generate distinct cellular responses.

B250/P0828

Regulation of early endosome trafficking and organization via the control of iPLA2 localization and activity by Rab5

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Calcium-independent phospholipase A2 (iPLA2) is involved in membrane remodeling and vesicle formation by catalyzing the hydrolysis of phospholipids at the sn-2 ester bond, resulting in the production of lysophospholipids and free fatty acids. In this study, we examined the roles of Rab proteins in regulating the localization and activity of iPLA2. Live-cell imaging showed that iPLA2-GFP mainly localizes to large perinuclear vesicles. Removal of N-terminal Ankyrin repeats disrupts this localization, highlighting their essential role in directing iPLA2 to specific subcellular compartments. iPLA2 exhibits strong colocalization with Rab5 on perinuclear vesicles and directly binds Rab5 through its ANK-NBM domains. This interaction does not depend on Rab5's activation state (GTP/GDP-bound), indicating iPLA2 is not a typical Rab5 effector. iPLA2 activity is necessary for endosome fusion and the organization of perinuclear endosomes. Blocking iPLA2 with BEL or mutating its catalytic site impairs EEA1 vesicle trafficking and causes their buildup near the nucleus. Rab5 binding to iPLA2 suppresses its enzymatic activity, consistent with their lack of colocalization in Rab5CA-induced enlarged endosomes, which contain EEA1 instead. This suggests iPLA2 and EEA1 might compete for Rab5 binding. Our findings propose that the interaction between Rab5 and the ANK repeat domain of iPLA2 could significantly influence early endosome trafficking.

B234/P0812

Regulation of WASP-mediated actin assembly by Rho GTPases and SH3 proteins during Ustilago maydis endocytosis

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Rho GTPases are major regulators of actin-mediated cellular processes including clathrin-mediated endocytosis. Understanding how Rho GTPases contribute to the spatio-temporally regulated endocytic pathway has been difficult due to functional redundancy and regulatory network complexity. A key endocytic actin regulator, the Wiskott–Aldrich syndrome protein (WASP), activates the arp2/3 complex, an actin nucleator that initiates branched filament formation. The classic model of WASP regulation primarily relies on Rho GTPases such as Cdc42 and Rac, PIP2, and Src Homology 3 (SH3) domain interaction networks. How these regulatory components coordinate their functions in vivo is still poorly understood. In Schizosaccharomyces pombe and Saccharomyces cerevisiae, WASP lacks the autoinhibitory Cdc42/Rac interactive binding (CRIB) domain. WASP regulation in these species primarily depends on SH3 proteins, Sla1 and Bbc1, which inhibit WASP activity. Using comparative sequence analysis, we discovered that the Ascomycete fungi, which include S. cerevisiae and S. pombe, have lost the CRIB domain in their WASPs. Therefore, Rho GTPase regulation of WASP cannot be studied in Ascomycota. To overcome this limitation, we chose the Basidiomycete fungus, Ustilago maydis, which retains a CRIB-containing WASP and allows easy genome editing and quantitative live-cell imaging. We then investigated the functions of candidate WASP regulators, including the Rho GTPases Cdc42 and Rac1, the RhoGEF Intersectin, RhoGAP Ecm25 and the SH3 proteins, Sla1 and Bbc1. Several mutants displayed distinct cell shape and cell division phenotypes. For instance, CRIB and RhoGAP mutants are rounder, while Sla1 and Cdc42 mutants have cell separation defects. Unlike in Ascomycete models, Cdc42 and Intersectin deletions in U. maydis are viable. By quantifying endocytic coat and actin marker protein dynamics in deletion mutants we found that Rac and Cdc42 exhibit distinct endocytic roles: both influenced endocytic timing, whereas only Cdc42 deletion reduced actin accumulation and blocked coat internalization. Intersectin deletion mutants failed to internalize the endocytic coat and had severe actin organization defects. The deletion phenotypes of SH3 proteins Sla1 and Bbc1 suggest similar functions to those of their homologs in S. cerevisiae. Rho GTPase signaling and cell polarity are linked in many organisms, and the cell shape defects observed in our mutants suggest a likely endocytosis-polarity connection. Together, our findings reveal a Rho GTPase–SH3 dual regulatory system that coordinates WASP activity during endocytosis. This work

demonstrates that U. maydis is a powerful yet simple system to dissect complex and ancient actin assembly mechanisms during clathrin-mediated endocytosis.

B256/P0834

Revealing Immunological and Neurological Phenotypes in a VPS45 E238K Mouse Model

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Vacuolar protein sorting 45 homolog (VPS45), a member of the Sec1/Munc18 (SM) family, controls vesicle trafficking in the endosomal system. Mutations in the human VPS45 gene are associated with severe congenital neutropenia (SCN) type 5 with bone marrow fibrosis, recurrent life-threatening infections and early death without hematopoietic stem cell transplantation. Several of the patients also exhibited neurological deficits such as developmental delay, motor, and visual impairment. We developed and characterized a novel mouse model of VPS45 neutropenia by CRISPR/Cas9-mediated knock-in of the pathogenic VPS45 E238K mutation. The Vps45E238K mice reveal unique consequences of this mutation in vivo, with disturbances in neutrophil number and function, and in lymphocyte number, along with significant impacts on birth ratios and survival. Ongoing cell biological studies in the mouse embryonic fibroblasts (MEFs) are examining defects in trafficking of cargos through the endocytic pathway, and further characterization of the Vps45E238K mouse suggests alterations in various cell types in the brain. By probing the cellular mechanisms and affected cell types, we aim to define how the VPS45 mutation leads to cellular dysfunction, ultimately guiding the development of potential therapies.

B242/P0820

Selenoprotein S Role in Endoplasmic Reticulum Ca2+ Flux

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Selenoprotein S (selenos) is a small transmembrane protein located in the endoplasmic reticulum. Its function and structure remain incompletely characterized, yet genetic variations in the SELENOS gene are associated with multiple health conditions, including cardiovascular pathologies. The objective of this study was to investigate the role of selenos in regulating Ca^{2+} flux through its potential interaction with Sarco/Endoplasmic Reticulum Ca^{2+}-ATPases (SERCAs). To examine the selenos interactome, we performed proteomic analyses followed by in vivo pull-down assays. These experiments identified an interaction between selenos and SERCA2, which is responsible for pumping Ca^{2+} from the cytoplasm into the endoplasmic reticulum lumen. We further observed interactions of selenos with additional isoforms, including SERCA1a, SERCA1b, and SERCA2a, which are expressed in a tissue-specific manner. To map the binding interface, we aligned the selenos sequence with small regulatory proteins of SERCA, known as regulins. This analysis predicted a candidate binding site for selenos. To validate this prediction, we applied unnatural amino acid-based photo-crosslinking combined with in vivo pull-downs. In conclusion, our findings demonstrate that selenos associates with multiple SERCA isoforms and may influence Ca^{2+} flux in the endoplasmic reticulum. These results suggest a mechanism by which genetic variation in SELENOS could affect cardiovascular physiology and provide a basis for exploring selenos-SERCA interactions as potential therapeutic targets.

B243/P0821

Sorting of endocytic cargos by yeast Gga proteins at the trans-Golgi network

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Endocytosis is the process by which cells internalize extracellular materials and membrane proteins via clathrin coated vesicles (CCVs) formed by membrane invaginations. Internalized endocytic cargos are first delivered to the sorting compartments and then recycled back to the plasma membrane or brought to the late endosomal compartment en route to the lysosome/vacuole for degradation. It has been reported that in mammalian cells the endocytic sorting compartments exist in the early endosomes but they exist within the trans-Golgi network (TGN) in plant cells. Recent studies using super-resolution confocal microscopy have demonstrated that the TGN is not a homogeneous organelle and can be divided into sub-domains that produce specific carriers for efficient cargo sorting. Recently, we have shown that endocytic early/sorting compartment in budding yeast exists as an independent sub-compartment within the TGN and endocytic cargos are first transported there, then transported to the yeast Rab5-residing endosomal compartment. Additionally, activity of Vps21, yeast Rab5, which is the key regulator of early endosome, is cooperatively regulated by PI4-kinase Pik1, Rab11 homologue Ypt31/32, and clathrin adaptors Gga1/2. However, molecular mechanisms regulating the Vps21 activity and cargo sorting at the TGN remain largely unclear. In this study, we generated domain deletion mutants of Gga2 and examined their effects on transport from the early/sorting compartment. We found that these mutants have a different effect on cargo transport from the early/sorting compartment to the vacuole/lysosome or the plasma membrane. Gga proteins are known to interact with different TGN-residing proteins through each domain. Our result suggests that Gga proteins have multiple functions in the endocytic pathway and regulate cargo sorting at the early/sorting compartment.

B251/P0829

The ARP2/3 complex regulation of clathrin dynamics is induced through ACK

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Clathrin-mediated endocytosis (CME) is an important cellular process that plays a role in cell-cell communication, signaling, and migration. In addition to the canonical clathrin coated pits, clathrin can form flat lattice structures on the membrane. Previously, Arp2/3 has been shown to be involved in the breakdown of large flat clathrin lattices which had formed while branched actin formation was transiently inhibited. Consistent with this finding, we can induce accumulation of large, long-lived clathrin structures in mouse fibroblasts (JR20 cells) via conditional knockout of Arpc2. Identifying factors involved in the regulation of clathrin dynamics is an area of ongoing research. Our JR20 cells present an interesting model to study this pathway because Arpc2 KO cells form large, highly stable clathrin structures, which we refer to as plaques, which parental JR20 cells do not readily maintain under typical experimental conditions. Phospho-proteomic data comparing Arpc2 KO and control JR20 cells revealed that many CME-related proteins have increased levels of tyrosine-phosphorylation upon Arpc2 deletion. Immunofluorescence shows that plaques co-stain with a pan-phospho-tyrosine antibody. Alternatively, plaques which form naturally in ITGB1 null GD25 cells do not costain for phospho-tyrosine. This suggests the tyrosine phosphorylation drives ARP2/3 branched actin plaque resolution. Based on the phospho-proteomic data, we have identified activated-Cdc42 kinase (ACK), a non-

receptor tyrosine kinase, as a potential plaque sensor upstream of Arp2/3 activation. Exogenously expressed mScarlet-tagged WT ACK shows preferential binding to large clathrin plaques over pits when imaged via TIRF microscopy. Additionally, ACK KO, small molecule inhibition, or overexpression of a kinase dead mutant results in increased plaque accumulation in JR20 cells. Together, these data provide an interesting potential pathway wherein ACK senses clathrin plaques and if activated, its kinase activity stimulates Arp2/3 branched actin activity and plaque resolution.

B239/P0817

UVRAG and the VPS34 complex II regulates macropinocytosis via the synthesis of PI(3)P on the plasma membrane

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Macropinocytosis is central to critical macrophage immune functions including wound healing, antigen presentation, and resolution of inflammation. Macropinocytosis also facilitates nutrient uptake and growth in macrophages, T cells, and cancer cells. Here, CRISPR/Cas9 whole-genome screens identified and ranked genes regulating uptake of Lucifer yellow, a fluorescent dye taken up non-specifically by pinocytosis, in murine bone-marrow derived macrophages (BMDM). UVRAG and other members of the VPS34 complex II (VPS34-II), which catalyze PI(3)P synthesis from phosphatidylinositol, were identified as top positive regulators of macropinocytosis. Targeted disruption of Uvrag and Pik3r3 revealed they were required for efficient macropinocytosis with UvragsgRNA BMDM having fewer but larger macropinosomes. In contrast, disruption of Atg14, encoding a unique component of VPS34 complex I, increased the formation of macropinosomes. Oblique plane illumination and lattice lightsheet microscopy of BMDM expressing 2xFYVE-mScarlet revealed PI(3)P in macropinocytic cups on the plasma membrane prior to cup closure as well as two types of PI(3)P vesicles: 1) large (0.5-3 µm) nascent macropinosomes, and 2) smaller (<0.5 µm) PI(3)P vesicles that moved toward the plasma membrane. Staining of PI(3)P in fixed cells using a fluorescent SNAP-2xFYVE probe revealed that PI(3)P concentrations in WT BMDM increased in response to CSF1 stimulation. UvragsgRNA and Atg14sgRNA BMDM had lower and higher PI(3)P, respectively. This work defines a role for UVRAG and VPS34-II in macropinosome formation and identifies the synthesis of PI(3)P as a key molecular event promoting macropinosome cup closure. In addition, our screens identified numerous genes encoding FYVE- and PX-domain containing proteins that may facilitate docking of secretory or recycling vesicles at the plasma membrane to deliver cargo that promotes cup closure or downstream trafficking and maturation of macropinosomes.

B241/P0819

VPS45 binding sites regulate Rabenosyn-5 and Syntaxin 16 binding in endosomal membrane trafficking

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Membrane trafficking is required for cell growth, secretion, signaling, and survival. Many proteins regulate vesicle trafficking and ensure cargo is delivered to the correct place at the appropriate time. SNARE proteins, located on the vesicle and target membranes (v-SNAREs and t-SNAREs, respectively), control membrane fusion

through the assembly of a four-helix SNARE complex. VPS45, a Sec1/Munc18 (SM) protein, is a regulator of endosomal membrane trafficking through its interaction with SNARE proteins. VPS45 binds the syntaxin type t-SNARE, Syntaxin 16, through two binding modes. The first interaction site is between the N-peptide of Syntaxin 16 and a hydrophobic pocket in VPS45. The second binding mode involves the cleft of VPS45 and the Habc domain and SNARE motif of Syntaxin 16. It is hypothesized that the interactions between VPS45 and Syntaxin 16 regulate membrane fusion. VPS45 also interacts with the endosomal Rab5 effector, Rabenosyn-5. The function of this complex and its role in membrane trafficking is currently under investigation. Using recombinantly expressed, purified proteins, we examine interactions, and any possible competition, between VPS45, Rabenosyn-5, and Syntaxin 16. We are testing the role of the VPS45 hydrophobic pocket in these interactions using a variety of VPS45, Rabenosyn-5, and Syntaxin 16 mutants.

Membrane Biology and Trafficking - Exocytosis and secretion mechanisms

B260/P0838
Dynamic early recruitment of GAK-Hsc70 regulates coated pit maturation
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Clathrin-mediated endocytosis (CME) begins with the assembly of clathrin onto the plasma membrane. These structures grow and stabilize to form clathrin-coated pits (CCPs), which invaginate and accumulate cargo. Finally, through membrane fission, CCPs detach to form clathrin-coated vesicles (CCVs). Mechanisms governing the transition of CCPs from flat-to-curved structures have been a matter of debate. GAK and its chaperone protein, Hsc70, are well-known to mediate clathrin release from CCVs and several studies have observed a late burst of GAK recruitment as CCVs form. Other studies have proposed that early recruitment of GAK-Hsc70 could function to provide the necessary energy source to remodel nascent flat clathrin lattices, replacing hexagons with pentagons and enabling a gain of curvature and invagination of the growing CCP; however, direct functional evidence is lacking. Here we show that GAK knockdown inhibits CCP stabilization and invagination. Furthermore, mutations in the J domain of GAK that abolish Hsc70 recruitment to and activation at CCPs, lead to the accumulation of GAK at CCPs, hinder CCP stabilization and invagination and result in a striking increase in the proportion of highly transient, abortive CCPs. These findings support the hypothesis that GAK-Hsc70 promotes the turnover and remodeling of nascent clathrin assemblies required for curvature development during CME.

B262/P0840
Exocyst undergoes multiple conformational changes to regulate SNARE binding, complex formation and fusion
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SNARE-mediated vesicle fusion during exocytosis is regulated by the exocyst, a multi-subunit hetero-octameric tethering complex. To carry out its functions with exquisite temporal and spatial control, exocyst interacts with a multitude of exocytic factors, including SNAREs, the Sec1/Munc18 (SM) protein, small Rab and Rho GTPases, PI(4,5)P2 and the type V myosin. Exocyst interacts individually with all three exocytic SNAREs, and we show that they exert tight control over several stages of SNARE complex assembly to facilitate membrane fusion. The

cryoEM structure of yeast exocyst indicates a compact, fairly static conformation; however, multiple recent studies demonstrate that exocyst undergoes several distinct structural changes involving the Exo70 and Exo84 subunits, which activate the complex for tethering. We also determined that exocyst function is regulated through multiple phosphorylation sites, which affect binding to SNARE partners. Other conformational changes are currently being investigated through biochemical and structural studies. We propose that exocyst conformational changes constitute a series of activation steps in order to efficiently tether, bind to SNAREs and accelerate membrane fusion.

B258/P0836

Investigating the role of the Sec1 regulator in SNARE mediated vesicle fusion in yeast

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Exocytosis is an essential cellular process that depends on the precise coordination of vesicle tethering and membrane fusion to maintain growth, secretion, and cellular homeostasis. Central to this process are SNARE proteins, which assemble into a stable four-helix bundle to generate the force necessary for membrane fusion. In Saccharomyces cerevisiae, Sec1, a member of the Sec1/Munc18 (SM) protein family, is indispensable for polarized secretion. Additionally, exocyst, a conserved hetero-octameric tethering complex, responds to polarity cues at vesicle docking sites and undergoes conformational changes that activate it for regulation of SNAREs to promote membrane fusion with the plasma membrane. However, the molecular mechanisms by how Sec1 interacts with both SNARE complexes and exocyst remain elusive. It is unclear whether Sec1 and exocyst cooperate or compete during vesicle fusion. Our study is focused on investigating these possible mechanisms by characterizing the interactions between purified yeast Sec1, SNARE proteins, and exocyst complexes (wild-type and activated mutants), with the ultimate goal of elucidating regulation of SNAREs during exocytosis.

B259/P0837

Neuropilin-2 trafficking and polarity in kidney epithelial cells are regulated by semaphorin-plexin signaling

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Paracrine communication between the microvasculature and epithelial cells is a key factor in tissue differentiation and homeostasis. Crosstalk between these cell types holds therapeutic potential for metabolic diseases, which are known to impair epithelial barriers. We investigated the regulation of neuropilin-2 (Nrp2), a co-receptor that interacts with various receptors in other cell types, but whose role in epithelial cells remains poorly understood. This study aimed to determine whether Nrp2 surface expression in epithelial cells is regulated by semaphorins—paracrine signals that we previously showed originate in adjacent endothelial cells. Methods: We assessed Nrp2 surface expression via surface biotinylation in polarized kidney proximal tubule epithelial cells (RPTEC). Gene silencing was performed using lentiviral shRNA vectors. Surface proteomics was used to analyze receptor expression Results: The presence of Nrp2 at the plasma membrane of RPTEC cells was decreased by 46 ±4% ($p < 0.01$) after treatment with 200 ng/mL Semaphorin-3E (Sema3E) at expense of the intracellular pool. This indicates that Nrp2 trafficking in RPTEC is susceptible to regulation via Sema-Plexin signaling, a paracrine pathway that we previously showed acts under high glucose conditions. Conversely, we observed that high glucose treatment abolished the polarized distribution of Nrp2 from 67 ±2% apical to non-

polarized (54 ±3% apical / 46 ±3% basolateral, $p<0.05$). A similar loss of polarity occurred when silencing Sema3G ($p<0.01$). To further explore the implications of semaphorin signaling, we silenced the receptor PlexinB2 and added semaphorins to RPTEC. We observed that semaphorin decreased surface expression of the tight junction protein Claudin-2 (32± 5%, $p<0.05$), and this was abolished by Plexin-B2 silencing ($p<0.05$). Neuropilins are known to act as co-receptors of Plexins in other cell types, and also of a number of other receptors. We explored the receptors present in RPTEC cells via surface proteomics. We found close to 15 receptors with a marked basolateral distribution, known to function with neuropilins in pathways of cell-cell communication, adhesion, and signaling. Conclusion: Nrp2 is expressed at the plasma membrane of RPTEC epithelial cells along with basolateral co-receptors. Nrp2 had apical distribution, shifting more basolateral by the action of semaphorins. We speculate that Nrp2 may regulate semaphorin-plexin modulation of epithelial tight junctions.

B261/P0839

Transporters at the host-parasite interface mediate inter-kingdom host microbial interactions

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Cryptosporidium spp. are widespread enteric parasites that cause life-threatening diarrhea in immunocompromised hosts. The parasite sits at the apex of intestinal epithelial cells, surrounded by the host membrane and encased in a vacuole that is subtended by a multimembrane structure, the feeder organelle (FO), which forms the host parasite interface. The FO houses transporters that mediate nutrient acquisition from the host. Cryptosporidium infection is influenced by the host microbiota, and microbial metabolites can both enhance and inhibit parasite growth. We have previously shown that indole and secondary bile acids, including deoxycholic acid (DCA) and lithocholic acid, inhibit the parasite development. Through forward genetic screening, we recently identified a multidrug-resistance-associated ABC transporter (CpMRP1) significantly associated with C. parvum infectivity. Epitope tagging localized CpMRP1 to the FO, and the AlphaFold predicted its canonical exporter architecture featuring two globular nucleotide-binding domains and transmembrane regions with long intracellular loops that form the substrate binding pocket. We demonstrated that polymorphisms in the intracellular loops mediate C. parvum susceptibility to the microbiota-derived metabolite DCA, revealing a mechanistic link between parasite genotype and microbiome-mediated inhibition. The C. parvum genome also encodes ~ 20 predicted ABC transporters that may also mediate resistance to toxic metabolites, although their precise roles remain poorly understood. We have developed a CRISPR-based screening platform targeting all C. parvum ABC transporters to systematically assess their contributions to parasite survival and adaptation. We will compare parasite fitness in antibiotic-treated and untreated mice, enabling dissection of transporter-mediated responses to microbial metabolites. These studies highlight new roles for Cryptosporidium transporters in exporting xenobiotic metabolites produced by the microbiota, thus contribute to pathogenicity.

B257/P0835

TRPML1 Activation by Reactive Oxygen Species is Critical for the Extracellular Digestion of Lipoprotein Aggregates by Macrophages

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The digestion of aggregated low-density lipoprotein (agLDL) by macrophages and the subsequent accumulation of excessive cholesterol esters in these macrophages in arteries plays a major role in the development of atherosclerosis. However, because agLDL is tightly crosslinked to the extracellular matrix, it cannot be digested by conventional endocytosis or phagocytosis mechanisms. Our laboratory discovered a novel mechanism by which macrophages digest agLDL and generate free cholesterol in an extracellular, acidic, hydrolytic compartment known as the lysosomal synapse. Macrophages form a tight seal around agLDL through actin polymerization and deliver lysosomal contents into this space in a process termed digestive exophagy. Vacuolar ATPase on the plasma membrane lowers the pH of the lysosomal synapse, enabling lysosomal acid lipase activity. Lysosomal acid lipase degrades cholesteryl esters in agLDL, generating free cholesterol that accumulates in macrophages, an important hallmark of atherosclerotic plaque. Our laboratory has begun to characterize the signaling pathways and machinery that regulate digestive exophagy. We recently identified release of Ca2+ from the ER as a critical step in digestive exophagy. We also observed that, upon addition of agLDL, macrophages exhibit a striking approximately 10-fold increase in Ca2+ "flashing," defined as a transient elevation of intracellular Ca2+. One role for ER Ca2+ release is refilling lysosomal Ca2+ stores. Interestingly, both activation and inhibition of the lysosomal cation channel, TRPML1, inhibit digestive exophagy. TRPML1 plays a role in lysosome exocytosis, so we are investigating its role in digestive exophagy. TRPML1 is activated by reactive oxygen species, which is of particular interest given that high levels of agLDL oxidation in atherosclerotic lesions are associated with poor patient outcomes. My preliminary results show marked differences in how macrophages interact with oxidized agLDL compared with non-oxidized agLDL, doing more lysosome exocytosis, but less actin polymerization. These results raise the possibility that agLDL oxidation serves as a key directional cue for initiation of lysosome exocytosis during digestive exophagy. We hope that expanding our understanding of the mechanisms of digestive exophagy will help identify protein targets for therapeutics to slow atherosclerosis.

Membrane Biology and Trafficking - Lipid rafts and microdomains

B193/P0773
Combined In Vitro and In Silico Perspectives on Hopanoid-Enriched Bacterial Microdomains
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The increase in the number of antibiotic-resistant bacteria requires new antimicrobial strategies targeting primary cellular structures. Lipid domains are distinct regions of the membrane, emerging through the lateral mixing of lipids with different shapes, degrees of saturation, charge, and interaction propensities. These regions display compositional and mechanical heterogeneity that modulates protein sorting, reaction kinetics, transport, and stress responses, thus shaping cellular function. In microbes, domain stability has been linked to the presence of anionic phospholipids, carotenoids, or isoprenoid lipids such as hopanoids (HOPs), yet rigorously validated evidence that clarifies their functional roles is limited. Here, we present an optimized composition of a bacterial membrane model that spontaneously forms visible microdomains both in vitro and in silico. Giant unilamellar vesicles (GUVs) composed of a bacterial lipid mixture mimicking Methylobacterium extorquens exhibited coexisting domain and non-domain regions observable by confocal microscopy. Matching coarse-grained molecular dynamics simulations independently reproduced lipid partitioning, providing a coherent in vitro/in silico view of bacterial domain formation. We characterize domain-enriched membranes by measuring the area per lipid, the bilayer thickness, the acyl chain order parameters, the lateral diffusion coefficients, the bending rigidity, and the compressibility modulus. Our analysis revealed clear bilayer partitioning into domain and non-domain regions with significant local differences: domain regions were more tightly packed, thicker, and more ordered, with markedly slower diffusion and higher bending rigidity

compared to adjacent non-domain regions. Furthermore, membranes containing microdomains showed higher overall bending rigidity relative to other homogeneous fluid membranes. A major advance of this work is the delineation of hopanoid-enriched ordered regions. HOP enrichment within domains, together with higher chain order, thickness, and rigidity, supports the hypothesis that hopanoids may function as sterol analogs in bacteria. Developing an optimized model of bacterial microdomains is crucial for interrogating their formation, dynamics, and effect on antimicrobial binding. Because the efficacy of many membrane-active antiseptics depends on local packing, curvature, and elasticity, both strategic disruption of microdomains and purposeful targeting of domain-specific agents may enhance drug effectiveness. Our model quantifies key mechanics and structure in hopanoid-enriched membranes, establishing baselines for selectivity tests and informing studies of membrane-active compounds.

B190/P0770

Flotillin-mediated endocytosis is hijacked to mediate the intercellular spreading of L. monocytogenes

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Listeria monocytogenes (L. monocytogenes), the causative agent of listeriosis, is a food borne pathogen that can cross the intestinal, placental and blood-brain barriers by spreading intercellularly. The bacteria achieves this by manipulating the host cell actin network to create finger-like actin-rich projections (listeriopods) that are endocytosed into recipient cells. Caveolin-mediated endocytosis has been the only mechanism known to mediate this endocytic process, however, cell-to-cell spreading was not entirely eliminated in cells depleted of caveolin-1, suggesting a secondary mechanism may be utilized for the uptake of L. monocytogenes in neighbouring cells. To explore the potential of other endocytic processes being involved we examined flotillin-1, a membrane-associated protein that participates in clathrin-independent endocytosis. We found flotillin-1 localized at cell-to-cell spreading sites and cells devoid of flotillin-1 had significantly less bacterial transfer. Additionally, cells that had flotillin-1 blocked from binding to the membrane, thus suppressing flotillin-1's endocytic ability, also had significantly less cell-to-cell spreading. Next, we explored if the functions of flotillin-1 were analogous to (or coordinated) with caveolin-1 by co-localizing the proteins. We found both proteins accumulated to the same sites (although with distinctly localized puncta). Plaque assays assessing infection foci in cells depleted in both caveolin-1 and flotillin-1, revealed a further reduction of bacterial spread compared to cells with caveolin-1 knockdowns alone. Notably, many cells exhibited a complete blockage of intercellular bacterial transfer. This work demonstrate that flotillin-1 has a role as a secondary endocytic mechanism for the internalization of L. monocytogenes.

B191/P0771

Jurkat T cell activation requires raft-associated LAT condensates

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T cells are central effectors of adaptive immunity, mediating critical defenses against infections and cancer. T cell activation must be tightly regulated to elicit proper immune responses while avoiding aberrant activation that could produce autoimmunity. Despite decades of research, the regulatory mechanisms of normal and aberrant T cell activation are not fully understood. Recently, using both in vitro membrane models and Jurkat cells we reported coupling between ordered, cholesterol-rich domains known as membrane rafts and protein

condensates driven by multivalent interactions between the transmembrane adaptor Linker of Activation of T-cells (LAT) and its effectors Grb2 and Sos1. Such coupling is mediated via the raft-preferring transmembrane protein LAT and is critical for transduction of T-cell signaling. To investigate how LAT condensates cooperate with membrane rafts to regulate T-cell signaling, we mutated the LAT transmembrane domain (TMD) to target it to nonraft domains and found both LAT phosphorylation and downstream MAPK signaling were completely abrogated. Thus, raft association of LAT and its associated protein condensates is essential for T cell signaling. We hypothesized that membrane rafts facilitate activation by enriching activating signals (e.g. Lck and ZAP70 kinases) and excluding inhibitory proteins (i.e. phosphatases like CD45), thereby promoting robust LAT phosphorylation, its subsequent condensation, and downstream signal propagation. Testing a panel of LAT TMD mutants whose raft affinity was systematically varied, we showed that LAT's raft affinity was tightly and linearly correlated with its phosphorylation. Conversely, targeting the phosphatase CD45 to membrane rafts reduces both LAT condensate density and downstream T cell signaling. Together, these results suggest that membrane rafts serve as a signaling platform to regulate LAT phosphorylation and condensation, which in turn tunes immune signaling.

B192/P0772

Role of the nuclear pore complex in nuclear envelope budding during leukotriene secretion in activated neutrophils

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Neutrophils are first responders to sites of injury and infection and play essential roles as mediators of inflammation. They reach injured and infected sites by recognizing gradients of chemical cues such as the peptide N-Formylmethionyl-leucyl-phenylalanine (fMLF) and migrating directionally towards them. fMLF activation also leads to the secretion of the secondary chemoattractant Leukotriene B4 (LTB4), which relays fMLF-mediated responses to neighboring cells and increases the recruitment range of neutrophils. LTB4 is synthesized through the concerted action of 5-Lipoxygenase (5-LO), 5-LO Activating Protein (FLAP), and Leukotriene A4 Hydrolase. Following stimulation, these LTB4 synthesizing enzymes are packaged in nuclear envelope (NE)-derived multivesicular bodies (MVBs) that fuse with the plasma membrane and release LTB4-containing exosomes. LTB4 secreted from exosomes relays the chemotactic signal to neighboring neutrophils by binding to specific receptors on the cell surface. Using FLAP-tGFP expressed in neutrophil-like HL60 cells, we found that NE budding is rapid and dynamic, occurs at the front of the nucleus in migrating cells before maturing into NE-derived MVBs that traffic to the back of cells and fuse with the plasma membrane. Intriguingly, despite their nuclear origin, NE buds are single membrane structures that harbor inner nuclear membrane proteins. As the formation of Nuclear Pore Complexes (NPCs) during interphase requires an inside-out fusion of the inner and outer nuclear membranes, we investigated whether NPC proteins play a role during LTB4 synthesis in activated neutrophils. Using immunofluorescence, we observed the presence of NPC proteins in the NE-buds and vesicles of migrating neutrophils. We performed a co-immunoprecipitation assay on NE isolated from activated neutrophils and found that the LTB4 synthesizing protein FLAP pulled down nucleoporin 210 (Nup210), an NPC transmembrane protein thought to play a role in membrane fusion during NPC assembly. Using expansion microscopy, we observed the presence of Nup210 in NE-derived MVBs. These findings suggest that NPC proteins are involved in the formation of NE buds during LTB4 synthesis in activated neutrophils. We are currently working on shRNA-mediated knockdown of Nup210 to assess its functional relevance for NE budding. We hypothesize that NPC proteins, including Nup210, act in concert with FLAP to promote membrane curvature and stabilize intermembrane fusion during the initiation of NE budding. This work will add to our growing knowledge of the basic molecular mechanisms that govern neutrophil migration and signaling during inflammation.

Membrane Biology and Trafficking - Membrane contact site structure and dynamics

B202/P0782
Adipogenin Shapes the Development of Lipid Droplets by Binding a Dodecameric Seipin Complex
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The microprotein adipogenin (Adig) is highly expressed in adipose tissue and testis. Here, we showed that Adig directly interacts with seipin to form a stable, rigid complex. We resolved the structure of the seipin-Adig complex at ~3.0 Å overall resolution and discovered that mammalian seipin assembles into two distinct oligomeric forms: undecamers and dodecamers. Notably, Adig was selectively bound to the dodecameric form, enhancing seipin assembly by bridging and stabilizing adjacent subunits. Functionally, this complex reshapes the nascent lipid droplet (LD) formation and promotes LD growth. In vivo, adipocyte-specific overexpression of Adig increased fat mass and enlarged LDs, whereas Adig deletion impaired triglyceride accumulation in brown adipose tissue. These findings establish Adig as a critical modulator of lipid storage through its structural and functional interaction with seipin.

B199/P0779
Analyzing the Role of FszA and FszB Proteins in the Mitochondrial Dynamics of Dictyostelium discoideum
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Mitochondria play an important role in the energy production and apoptosis of cells, therefore dysfunction of mitochondria could be detrimental. Mitochondrial dynamics such as fission, fusion, and motility determine the organelle's structure and can indicate the overall health of the cell. Dictyostelium discoideum, a well-established model for mitochondrial dynamics, contains two GTPase proteins that have been predicted to mediate mitochondrial dynamics, FszA and FszB. In this study, we will overexpress GFP tagged FszA and FszB proteins to gain insight into their role in the mitochondrial dynamics of D. discoideum. Through live imaging, we will quantify mitochondrial fission events, fusion events, motility, and localization of proteins with respect to fission and fusion events. Preliminary results show that the overexpression of the FszA protein in D. discoideum did not significantly change the amount of mitochondrial fission and fusion events occurring compared to the mitochondrial dynamic events of the control Ax4 strain. The results of this study will give insight into the underlying mechanism behind mitochondrial dynamics which could also shed light on neurodegenerative diseases that appear to have dysfunctional mitochondrial dynamics.

B200/P0780
Drosophila Ipo9 promotes H2Av exchange between LDs and H2Av import into the nucleus
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Lipid droplets (LDs) are cellular fat storage organelles that have emerged as important regulators of protein availability. In Drosophila melanogaster, LDs sequester canonical histones H2A and H2B, as well as the variant

H2Av, via the anchoring protein Jabba. By transiently binding these histones, Jabba protects them from degradation during oogenesis; for H2Av, this binding also slows its nuclear import during embryogenesis. Even in the absence of Jabba, about a quarter of these histones remains in the ooplasm, presumably protected from degradation by additional factors. A candidate is the nuclear import factor Importin 9 (Ipo9) because the mammalian ortholog is known to reside in the cytoplasm and to directly bind to H2A-H2B dimers. Using CRISPR, we tagged the endogenous Ipo9 with HA-mScarlet and determined its intracellular distribution. During oogenesis and early embryogenesis, Ipo9 is present in both nuclei and the cytoplasm, consistent with a cytoplasmic role. When we tested H2A, H2B, and H2Av levels in oocytes lacking both Jabba and Ipo9, they were indistinguishable from those of Jabba single mutants; thus, Ipo9 does not make a substantial contribution to histone protection. We did, however, identify a different cytoplasmic role for Ipo9: in the nurse cells of full-body Ipo9 knockouts, H2Av fails to exchange between LDs, suggesting that Ipo9 shuttles H2Av (and possibly the other histones) between LDs. In ongoing work, we are evaluating whether this exchange role is cell autonomous in the germline. We also assessed if Ipo9 contributes to the nuclear import of H2Av. In wild-type embryos, reducing the dosage of Ipo9 had little effect on nuclear H2Av levels. It was previously shown that in Jabba mutant embryos H2Av is imported at a faster rate than in the wild type as newly synthesized H2Av is not retained in the cytoplasm, i.e., on lipid droplets. In Jabba mutants, Ipo9 dosage reduction decreases nuclear H2Av levels by about half, suggesting that under these conditions Ipo9 is limiting for H2Av nuclear import. Finally, we found that Ipo9 modulates the embryonic DNA damage response. In embryos, DNA damage leads to so-called “nuclear falling’’, with damaged peripheral nuclei being disposed of by moving them into the embryo interior. Nuclear falling is enhanced in Jabba mutants as well as in mutants with increased H2Av dosage, suggesting that overaccumulation of nuclear H2Av results in DNA damage. Intriguingly, in the Jabba mutant background, reduced Ipo9 dosage decreased nuclear H2Av levels but increased nuclear falling. Future work will test if this increase is due to insufficient nuclear H2Av levels or reflects the contribution of other Ipo9 cargo, such as H2A or H2B.

B195/P0775

Exploring the Role of a Putative Endoplasmic Reticulum Protein in Membrane Contact Site Formation in Toxoplasma gondii

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Toxoplasma gondii is an obligate intracellular apicomplexan parasite with a high seroprevalence in human populations worldwide. During its infective stages, T. gondii undergoes rapid replication and dissemination through a tightly regulated lytic cycle that depends on calcium signaling and the coordinated activity of organelles involved in calcium storage and exchange, including the endoplasmic reticulum (ER), mitochondrion, and apicoplast. Membrane contact sites (MCSs) between these organelles function as critical hubs for inter-organelle communication and metabolite transfer; however, their roles in apicomplexans remain poorly understood. A previous study from the Huet lab identified TGME49_232050 as a putative ER-associated MCS protein using a split-TurboID proximity labeling approach. This protein was also shown to contribute significantly to parasite fitness in a genome-wide CRISPR screen. We hypothesize that TGME49_232050 plays an essential role in regulating the T. gondii lytic cycle. To investigate this, we are developing a conditional knockdown (cKD) strain employing the 3’ auxin-inducible degron system, as previous attempts to integrate an inducible promoter were unsuccessful. The resulting cKD strain will be analyzed using phenotypic assays, including replication and plaque assays, to assess its role in the lytic cycle. Additionally, we plan to determine the subcellular localization of TGME49_232050 through epitope tagging. Elucidating the role of TGME49_232050 in T. gondii may provide novel insights into calcium-dependent signaling mechanisms and identify new therapeutic targets for toxoplasmosis.

B198/P0778

Human cytomegalovirus controls bioenergetics via regulation of mitochondrial ultrastructure and protein networks

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The objective of this study is to investigate the mechanism of mitochondrial remodeling during human cytomegalovirus infection and its impact on protein interaction network and metabolism. Alterations in mitochondrial functions and cellular metabolism are hallmarks of many viral infections. During human cytomegalovirus (HCMV) infection, the traditional structure-function relationship of mitochondria is flipped, displaying enhanced bioenergetic output in conjunction with mitochondrial fragmentation. This phenomenon has remained a conundrum, with limited mechanistic explanation. Here, we characterize the underlying mechanisms by integrating in-depth ultrastructural analyses by cryo-ET with virology, and proteomics. For the first time, we have defined the geometrical parameters of mitochondrial ultrastructure through the entire viral replication cycle by in situ cryo-correlative light and electron microscopy (cryo-CLEM) and discovered that HCMV temporally induces significant changes in mitochondrial membrane architecture. Our work captured three-dimensional ultrastructures of several infection-driven mitochondrial remodeling events such as inter-mitochondrial contacts (IMCs) that facilitate membrane potential exchanges to maintain mitochondrial health. We also report the ultrastructure of infected mitochondria undergoing peripheral fission, characterized by alignment and continuation of membranes between two distinct mitochondria. To understand the mechanisms that drive these ultrastructural changes, we sought to define connected alterations in intra-mitochondrial protein interaction networks during infection. For this we established a workflow that allows integrating mitochondrial enrichment with thermal proximity coaggregation assay mass spectrometry (TPCA-MS). This workflow afforded increased detection of membrane-bound mitochondrial protein complexes and knowledge of their assembly and disassembly dynamics. To further delve into the viral factors that modulate mitochondrial dynamics, we utilized both WT and ΔUL13 HCMV, as we have shown that HCMV viral protein pUL13 can upregulate oxidative phosphorylation. Our analysis established that pUL13 interacts with another uncharacterized HCMV viral protein, which we showed to localize to the mitochondria late in infection. Furthermore, we demonstrate that the absence pUL13 is leads to the disruption of assembly of protein complexes involved in ETC complex I assembly. Altogether, our results provide insights into viral strategies for rewiring mitochondrial structure-function relationships by temporal regulation of cristae architecture and protein complex formation.

B203/P0783

Investigating the Role of Lysosomal Tubulation in NPC Disease

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Niemann-Pick type C (NPC) disease is a rare, fatal, neurodegenerative disorder caused by a mutation in the Npc1 or Npc2 genes. These genes encode for lysosomal proteins that are known to bind and transport cholesterol, and mutations in these proteins result in the accumulation of cholesterol and other lipids in the lysosomes of various cell types and tissues. The median age of death in NPC disease patients is 16 years old. The NPC1 protein is located in the membranes of lysosomes and late endosomes, while NPC2 is soluble in these compartments. Cholesterol is delivered to lysosomes packaged in low-density lipoproteins (LDL). Studies

have demonstrated that NPC2 transfers cholesterol to NPC1 and facilitates the passage of cholesterol through the glycocalyx, allowing it to be inserted into the luminal leaflet. It is known that 30% of lysosomal cholesterol is directly transported to the endoplasmic reticulum, but the mechanism of this transport remains unknown, and this is the defect in NPC disease. The Vaughan lab proposes that delivery of LDL to lysosomes triggers the formation of hollow tubular structures from the lysosomal membrane, which exhibit dynamic movement and likely interact with other cellular compartments. We have shown that lysosomal tubulation is not triggered by cholesterol, but rather by an oxysterol found in LDL, 27-hydroxycholesterol (27-HC). In fact, ongoing work with Dr. Daniel Wüstner has shown that NPC2 binds 27-HC in a concentration-dependent manner. We are currently investigating how mutations in NPC2 impact binding to 27-hydroxycholesterol and subsequent lysosomal tubulation and lysosomal cholesterol export. Existing treatments for NPC patients are not curative, nor do they directly address the cellular mechanisms that are defective in NPC disease. Thus, a deeper understanding of the lipid transport mechanisms and functions of NPC1 and NPC2 proteins is necessary for the study of NPC disease and other lysosomal disorders.

B201/P0781

Junctophilin-3: Master Architect of Lipid and Ca2+ Signaling Nanodomains at Neuronal ER-PM Junctions.

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Junctophilins (JPH1-4) are specialized ER-PM tethering proteins that simultaneously bind plasma membrane phospholipids, ion channels, ER membrane components, and regulatory proteins to create distinct signaling microenvironments. While muscle junctophilins (JPH1-2) are well-characterized for excitation-contraction coupling, neuronal JPH3 and JPH4 remain understudied despite their clinical importance—expansions in human JPH3 cause Huntington's Disease-like 2 (HDL2). Yet the molecular architecture of JPH3-containing nanodomains remains undefined. Here, we combine bespoke monoclonal antibody development, cross-linked proteomics, super-resolution microscopy, and live-cell imaging to establish the molecular organization and function of JPH3-driven ER-PM junctions. Proteomic analysis of the JPH3 interactome revealed co-enrichment with ER-PM tethers (KV2.1, JPH4, VAPB) and two functionally distinct protein categories: Ca^{2+} signaling machinery (CaV1.2, CaV2.1, IP3R1, RyR2) and lipid transport/metabolism proteins. Functional validation using Fluo-4 calcium imaging in electrically stimulated mouse cortical neurons demonstrated that JPH3 knockout significantly reduced somatic Ca^{2+} responses to initial stimulation compared to wild-type controls. Super-resolution microscopy revealed preferential co-localization of Ca^{2+} signaling proteins with JPH3 versus KV2.1, another neuronal ER-PM tether that organizes distinct Ca^{2+}/PKA signaling nanodomains, suggesting specialized functional roles for different junction types. To investigate JPH3's role in plasma membrane lipid regulation, we employed complementary approaches: overexpression studies with lipid biosensors in JPH3-negative HEK293T cells, and lipid-sensitive dyes/biosensors in wild-type versus JPH3 knockout neurons. JPH3 overexpression in HEK293T cells increased phosphoinositide levels (PI(4)P and PIP2) while decreasing cholesterol at the plasma membrane. Strikingly, JPH3 knockout neurons exhibited dramatically elevated plasma membrane cholesterol compared to wild-type, revealing a novel regulatory function for JPH3 in lipid homeostasis. These findings establish JPH3 as a critical organizer of specialized ER-PM nanodomains that coordinate both Ca^{2+} signaling and lipid metabolism. Our results suggest that disruption of these nanodomains in HDL2 may contribute to pathogenesis through dual mechanisms: altered Ca^{2+} dynamics and perturbed lipid homeostasis. This work provides the first comprehensive molecular characterization of JPH3-containing junctions and identifies potential therapeutic targets for HDL2.

B204/P1810

Lysosomal PI(4)P Signaling Links PIKfyve Dysfunction to Organelle Repair and Mitochondrial Homeostasis

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Lysosomes are vital organelles that regulate cellular catabolism, autophagy, and lipid metabolism, and their dysfunction contributes to a wide spectrum of diseases, including neurodegeneration. At the lysosomal surface, the phosphoinositide kinase PIKfyve, the phosphatase Fig4, and the scaffold protein Vac14 form a regulatory complex that plays a central role in phosphoinositide metabolism. However, a paradox has emerged: while loss-of-function mutations in this complex cause severe neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), Yunis-Varón syndrome and Charcot-Marie-Tooth disease, pharmacological inhibition of PIKfyve has shown therapeutic benefits in models of neurodegeneration. In this study, we seek to resolve this paradox by investigating how disruption of the PIKfyve complex reshapes lysosomal lipid signaling and organelle homeostasis. Using genetic and pharmacological perturbations of the PIKfyve complex, combined with advanced imaging and molecular biology approaches, we demonstrate that disruption of the PIKfyve complex leads to the accumulation of phosphatidylinositol 4-phosphate (PI(4)P) on lysosomal membranes. This occurs through ULK1-dependent trafficking of the PI(4)P-synthesizing enzyme PI4KIIα from the trans-Golgi network to lysosomes following reduced mTORC1 activity. Elevated lysosomal PI(4)P mediates two distinct adaptive responses: (i) it promotes lysosomal membrane repair by recruiting lipid transfer proteins to deliver cholesterol and phosphatidylserine to lysosomes, and (ii) it enhances ER-lysosome-mitochondria contact formation, driving mitochondrial fragmentation and enhancing respiratory capacity. Importantly, we show that pharmacological inhibition of PI4KIIα and/or ULK1 reverses these phenotypes, underscoring the central role of this pathway in stress adaptation. In conclusion, our study identifies a novel mechanism by which PIKfyve complex dysfunction triggers adaptive PI(4)P remodeling to coordinate lysosomal membrane repair and enhanced mitochondrial function. These findings provide mechanistic insight into the paradoxical effects of PIKfyve disruption and highlight new avenues for targeting phosphoinositide signaling in neurodegenerative disorders.

B196/P0776

Specialized contact sites regulate the fusion of chlamydial inclusion membranes

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The intracellular bacterial pathogen Chlamydia trachomatis (Ctr) replicates within a membrane-bound compartment called the inclusion. Upon infection with several chlamydiae, each bacterium creates its own inclusion, resulting in multiple inclusions within each host cell. Ultimately, these inclusions fuse together in a process that requires the chlamydial protein IncA. Here, we show that inclusions form unique contact sites (inclusion contact sites, ICSs) prior to fusion, that serve as fusogenic platforms in which specific lipids and chlamydial proteins concentrate. Fusion depends on IncA clustering within ICSs and is regulated by PI(3,4)P2 and sphingolipids. As IncA concentrates within ICSs, its C-terminus likely interacts in trans with IncA on the apposing membrane, securing a high concentration of IncA at fusion sites. This regulatory mechanism contrasts with eukaryotic or viral fusion systems that are either composed of multiple proteins or use a change in pH to initiate membrane fusion. Thus, our study demonstrates that Chlamydia-mediated membrane fusion is

primarily regulated by specific structural domains in IncA and its local organization on the inclusion membrane, which is affected by the host cell lipid composition.

B197/P0777
Vacuolar Phosphatidylinositol 3,4,5-trisphosphate controls fusion through binding Vam7, and membrane microdomain assembly
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Membrane trafficking is regulated by phosphoinositides (PI) and their modification by kinases, phosphatases, and phospholipases. The endolysosomal pathway is primarily controlled by PI3P, PI(4,5)P2 and PI(3,5)P2, whereas a role for PI(3,4,5)P3 is less clear. We report that yeast vacuoles produce PI(3,4,5)P3 through class III PI 3-kinase activity. In vitro assays showed that adding dioctanoyl (C8) PI(3,4,5)P3 or the PI(3,4,5)P3-binding domain Grp1-PH blocked fusion. Furthermore, modifying endogenous PI(3,4,5)P3 with the phosphatase PTEN also blocked fusion. Fluorescence microscopy showed that PI(3,4,5)P3 was enriched at vertex microdomains, which was blocked by PTEN, C8-PI(3,4,5)P3, and the class III PI 3-kinase inhibitor SAR405. Importantly, blocking or eliminating PI(3,4,5)P3 prevented the vertex enrichment of Ypt7 and the HOPS subunit Vps33. Finally, we show that the soluble SNARE Vam7 binds PI(3,4,5)P3 and that PTEN abolished trans-SNARE pairing between partner vesicles. Together these data indicate that vacuolar PI(3,4,5)P3 coordinates the assembly of microdomains and SNARE function.

B194/P0774
Zinc finger protein (ZFR1) is required for tight pairing but not membrane pore formation in mating Tetrahymena
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In the model ciliate, Tetrahymena thermophila, membrane fusion during fertilization is catalyzed by the ancestral gamete fusion protein, HAP2/GCS1, with at least two additional proteins, namely, GFU1 and GFU2 (Gamete FUsion 1 and 2) being essential for membrane pore opening and/or expansion. Mutant mating cells lacking either HAP2/GCS1, GFU1 or GFU2 can recognize each other and adhere to form pairs but subsequently fail to form fusion pores and complete the mating. Interestingly, these pairs are also mechanically unstable indicating their role in pore formation and in the maintenance of tight junctions between mating cells. We demonstrate that another protein, the zinc finger-containing protein (ZFR1), expressed specifically in the conjugation stage, is also required for formation of stable pairs in T. thermophila. Surprisingly, ZFR1 is not required for membrane pore formation. Based on the structure from AlphaFold2 and Phyre2 predictions, ZFR1 appears to be a membrane-bound E3 ubiquitin ligase, putatively responsible for the turnover and kinetic control over essential conjugation-specific factors. A possible role for ZRF1 in the expansion and/or maintenance of fusion pores at the interface between mating cells is also considered.

Membrane Biology and Trafficking - Plasma membrane organization and dynamics

B170/P0750
Actomyosin cortex integration with plasma membrane topography over the one-cell Drosophila embryo
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The actomyosin cortex governs shapes and interactions of cells and is regulated by associated Arp2/3 actin networks. Schematic models typically depict a thin actomyosin cortex below a flat plasma membrane (PM), but most animal cells have complex PM topographies. How actomyosin networks generate cortical tension in association with highly folded PM, and how they are regulated in such contexts, remains unclear. To study these questions, we examined the one-cell Drosophila embryo with quantitative live-imaging, genetic perturbations, inhibitor injections, and laser cutting experiments. We found actomyosin networks intertwined across PM folds of the one-cell embryo surface. Additional evidence indicated that a composite material of actomyosin networks and PM folds conveys cortical tension: (i) the PM topography oscillated between expanded and condensed states in concert with known assembly-disassembly cycles of cortical myosin; (ii) the folded PM gained myosin-dependent tension with each cycle; (iii) with perturbations of myosin activity, the PM folds expanded with excessive heights and widths, and gained compressive stress; and (iv) with partial F-actin inhibition, actomyosin networks and PM folds co-aggregated into fragmented patches with myosin dependence. Additional experiments showed that regulation of the actomyosin cortex by Arp2/3 involved changes to the associated PM topography. Injection of fluorescent dextran into the extracellular space showed that the cyclic expansions between PM folds occurred primarily on the cytoplasmic side. These expansions coincided with initial cortical assembly of myosin, but also with cyclic accumulations of the Arp2/3 activator SCAR, and F-actin. Perturbation of Arp2/3 hindered the expansions between PM folds and altered the associated myosin localization. Instead of a relatively thick distribution of myosin over broader depths of normal PM folds, myosin became focussed at an abnormally thin plane with dense PM, from which misshaped PM folds emanated above and below. This abnormal plane displayed excessive tension, and the surfaces of Arp2/3-depleted embryos exhibited unusual inward contractions. Overall, our study characterizes a composite of actomyosin networks and PM folds that conveys cortical tension, and that regulation of cortical actomyosin activity by Arp2/3 involves modulation of the associated PM topography.

B181/P0761
Anionic Lipids and Membrane Trafficking Regulate Electrical Potential in Non-Excitable Tissue Cells
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The membrane potential of eukaryotic cells, traditionally described by the Goldman-Hodgkin-Katz model, has been considered spatially uniform and primarily governed by ion gradients. Here, we challenge this paradigm by demonstrating that non-excitable cells exhibit sustained and spatially heterogeneous membrane voltage gradients. Using a ratiometric voltage indicator, JEDI-2P-cyOFP1, we mapped membrane potential distributions in live cells and found that depolarization localizes to the leading edge, while hyperpolarization dominates the lateral regions. Our findings show that these voltage gradients correlate strongly with asymmetric distributions of anionic lipids, including phosphatidylserine (PS) and phosphatidylinositol phosphates (PIP3). Disrupting cytoskeletal integrity, ion transporters such as NHE1, or vesicle trafficking mechanisms led to a loss of voltage

asymmetry and lipid polarization, revealing a tightly regulated electromechanical feedback system. Furthermore, membrane potential dynamically responds to cell shape changes, substrate stiffness, external electrical fields, and cell cycle transitions. We developed a quantitative model incorporating ion fluxes and lipid distribution to explain the observed membrane potential gradients. This integrated framework emphasizes that membrane voltage is governed not only by ionic currents but also by lipid dynamics and mechanical signaling. Our work proposes a revised biophysical model where membrane potential acts as a global integrator of mechanical, metabolic, and electrochemical cellular states.

B186/P0766

Biochemical and Functional definition of a minimal Sonic Hedgehog (SHH) cytoneme deployment complex

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Study's objective: To elucidate the role of Signal peptide, CUB and EGF-like domain-containing protein 2 (SCUBE2) in cytoneme-mediated transport of the morphogen Sonic Hedgehog (SHH). We hypothesize that SCUBE2 may interact with the SHH deployment complex at cytoneme tips to facilitate ligand release. The deployment co-receptor complex contains the deployment receptor Dispatched (DISP), Cell adhesion molecule-related/down-regulated by oncogenes (CDON) and Brother of CDON (BOC) and augments the growth of cytonemes, via Myosin 10 (MYO10), for long range SHH signaling. Statement of methods: To gain a handle on the mechanistic role of SCUBE2 in cytonemes, functional assays that involved co-culturing SHH and SCUBE2 expressing NIH-3T3 and HEK293 cells respectively, were used to score cytoneme occurrence and numbers. These assays were repeated with SCUBE2 containing conditioned media to mimic physiological conditions. Co-immunoprecipitation experiments were performed to check the biochemical interaction of SCUBE2 with the SHH deployment machinery. Mouse embryonic fibroblast cells (MEFs) lacking Myo10 were used to test the dependence of cytoneme biogenesis on SCUBE2. It will be interesting to know the role of deployment machinery members in the interaction of the motor with SHH and what is its impact on the formation of cytonemes. Summary of the results: Our preliminary results show that secreted SCUBE2 increases the number of cytonemes in SHH expressing wild type NIH-3T3 and MEF cells. This increase in cytoneme numbers is compromised in MEFs lacking MYO10 function. Biochemically, SCUBE2 was found to interact with the SHH deployment complex. Statement of conclusion: SCUBE2 may be involved in the initial growth and subsequent stabilization of the cytonemes by mediating the interaction of SHH with the SHH deployment complex.

B174/P0754

Ca2+ dependent vesicular and non-vesicular lipid transfer controls hypoosmotic plasma membrane expansion

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Robust coordination of surface and volume changes is critical for cell integrity. Few studies have elucidated the plasma membrane (PM) remodeling events during drastic cell surface and volume alteration, especially regarding PM sensing and its subsequent rearrangements. Here, using fission yeast protoplasts, we reveal a Ca2+-dependent mechanism for membrane addition that ensures PM integrity and allows its expansion during acute hypoosmotic cell swelling. We show that MscS-like mechanosensitive channels activated by PM tension control extracellular Ca2+ influx, which triggers potential direct lipid transfer at endoplasmic reticulum (ER)-PM contact sites by conserved extended-synaptotagmins and accelerates exocytosis, enabling PM expansion

necessary for osmotic equilibrium. Defects in any of these key events result in rapid protoplast rupture upon severe hypotonic shock. Our numerical simulations of such hypoosmotic PM expansion further propose a cellular strategy that combines instantaneous non-vesicular lipid transfer with bulk exocytic membrane delivery to maintain PM integrity for dramatic cell surface/volume adaptation. We propose a cellular strategy that combines instantaneous non-vesicular lipid transfer with bulk exocytic membrane delivery to maintain PM integrity for dramatic cell surface/volume adaptation

B184/P0764
Cell surface RNAs and RNA-binding proteins mediate human papillomavirus cell entry
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Although RNA was traditionally considered confined to the nucleus and cytoplasm, recent work revealed that a subset of RNAs is glycosylated and displayed on the cell surface (cell surface glycoRNAs), raising fundamental questions about their functions and mechanisms. Emerging studies implicate these newly discovered molecules in immunity and cancer, yet their functional significance in pathogen infection remains unknown. Human papillomavirus (HPV), the most prevalent sexually transmitted virus and a leading cause of cancer, relies on a poorly defined entry pathway: following initial attachment, HPV engages an elusive endocytic receptor complex to mediate internalization via an underexplored endocytic route. Defining this receptor complex and the unique endocytosis pathway remains a major knowledge gap. In this study, we find that cell surface RNAs are critical for HPV infection, facilitating the sorting of virions to very early endosomes during entry. We confirm the presence of glycoRNAs across diverse HPV-permissive cell lines using metabolic labeling coupled with bioorthogonal chemistry, and we observe dynamic glycoRNA–HPV interactions during early entry. GlycoRNA neutralization and reconstitution infectivity assays indicate that cell surface glycoRNAs act as critical components of an HPV endocytic receptor across multiple cell types, and RNA-seq identifies specific glycoRNAs associated with very early stages of HPV entry. HPV affinity capture followed by immunoprecipitation–mass spectrometry identifies an early-entry–enriched cohort of RNA-binding proteins (RBPs), including DDX3X and Annexin A2, a known HPV co-receptor. Live cell staining and flow cytometry demonstrate their surface presentation and the modulation by HPV. Cell surface proximity labeling reveals that cell surface RNAs are in close proximity to HPV capsid proteins and form higher-order assemblies. Together, these findings support a model in which glycoRNAs, RBPs, and HPV virions assemble at the cell surface to form a previously unrecognized type of viral entry receptor complex. This work provides new mechanistic insight into HPV entry and highlights cell surface RNAs as a novel class of biomolecules that expand our understanding of endocytic trafficking and host–pathogen interactions.

B175/P0755
Cellular mechanisms of plasma membrane repair
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A defining feature of animal cells is the lack of a cell wall. While this absence affords animal cells distinct functional advantages compared to plant cells, it also renders animal cells uniquely susceptible to plasma membrane (PM) damage. Upon PM disruption, extracellular calcium floods into cells and stimulates the recruitment of repair factors to sites of PM damage. Failure to restore PM integrity after membrane damage is

associated with human diseases including muscular dystrophy and neurodegeneration. Despite the importance of PM repair in normal physiology and disease, the molecular mechanisms underlying PM repair remain incomplete. Using quantitative organellar proteomics and genome-wide CRISPR interference screens, we have identified sorcin, a previously uncharacterized gene, and EMRE, an essential component of the mitochondrial calcium uniporter, as new PM repair factors. We provide evidence that sorcin functions as a calcium-dependent tether that recruits endosomal sorting complex required for transport (ESCRT)-III to sites of membrane damage. We also demonstrate that EMRE prevents cell death by promoting the import of excess cytoplasmic calcium into mitochondria upon PM damage. Overall, we have identified novel factors required for PM repair and provide mechanistic insight into both the initial (repair) and terminal (lytic) phases of the mammalian response to PM damage.

B187/P0767
Characterization of TMEM16F-specific affibodies and their cellular effects
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The TMEM16 (Anoctamin) family comprises a group of transmembrane proteins involved in diverse physiological processes, including ion transport and phospholipid scrambling. TMEM16F (Anoctamin 6), a phospholipid scramblase and nonselective ion channel, plays a central role in membrane remodeling, blood coagulation, immune responses, and cell death pathways through its ability to externalize phosphatidylserine in response to elevated intracellular calcium levels. Consequently, modulating TMEM16F activity has emerged as a promising strategy for the development of new therapeutic applications. Despite the functional importance of TMEM16F, TMEM16F modulators have received little study. In a previous study, we generated TMEM16F-specific affibodies by biopanning a phage display library for affibodies that bind to brain-specific TMEM16F (hTMEM16F) variant 1. In this study, we selected six other affibodies from among the 38 previously sequenced affibody candidates and characterized them. After purification, we confirmed that two of these affibodies bound to human TMEM16F with high affinity. To provide functional insights into how these affibodies modulate TMEM16F activity, we tested whether they could exert functional effects at the cellular level. Finally, we show that TMEM16F affibody attenuated the neuronal cell death induced by glutamate and microglial phagocytosis, suggesting that these affibodies might have potential therapeutical and diagnostic applications.

B180/P0760
Complexome profiling charts the architecture of signaling at brain membranes
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Operation of the brain fundamentally relies on signaling processes at and across membranes that are mediated by molecular entities - defined as protein complexes and biochemical protein-protein interactions - and that are endowed with exquisite spatiotemporal specificity and dynamics. Organization and architecture of most signaling entities in their native configuration(s) are currently unresolved. Here we use high-resolution complexome-profiling of plasma membrane-enriched fractions obtained from adult mouse brain to resolve the native molecular appearance of proteins and their assemblies therein. This approach comprises gentle

solubilization and separation of proteins by broad range Blue Native gel electrophoresis, high-resolution cryo-microtome sampling and quantitative mass spectrometry (csBN-MS) followed by advanced bioinformatic analysis. The resulting complexome, termed BrainCOM, encompasses abundance-mass profiles of >5700 proteins, which exhibit an average of more than six distinct assemblies per protein reflecting a highly complex architecture of protein complexes and -interactions. We further demonstrate how this data can be used to (i) uncover the native appearance of protein complexes in structural analyses, (ii) identify novel functional interaction partners, (iii) resolve molecular distinctions for members of protein families as well as assembly preferences across protein classes, and (iv) reveal disease-relevant protein-protein interactions and associated pathways. In summary, BrainCOM is a valuable, comprehensive resource for structural and functional neuroscientists and establishes complex-formation as a general principle enabling speed and specificity of signaling processes in the brain.

B189/P0769

Computational model of S. pombe protoplast plasma membrane expansion under hypoosmotic stress

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Maintenance of cellular membrane integrity is essential for cell survival in response to rapid expansion such as occurs during hypoosmotic shocks. How the cell survives these events involves the stretching and, for large expansions, rapid delivery of lipids to the plasma membrane (PM). Few studies have elucidated how the PM senses and reorganizes in response to cell surface and volume changes. We developed a mathematical model of S. pombe protoplast expansion to interpret our hypoosmotic protoplast stress experiments, which incorporates the most important membrane delivery processes during this hypoosmotic expansion including membrane delivery by rapid endo/exocytosis, delivery of membrane by non-vesicular means including tricalbin (Tcb1/3) mediated lipid transfer, and eisosome stretching. The model incorporates water flux across the PM due to an imbalance between osmotic and Laplace pressures as well as factoring in the effects of cell growth. We assume that membrane tension increases/decreases the transfer of lipids to/from the PM from/to internal reservoirs. Additionally, the model incorporates the effect of Ca2+ influx due to high membrane strain on exocytosis, where the exocytosis rate increases in response to Ca2+ with a time delay. The model suggests that the presence of tricalbins gives the cell time to respond to this delayed exocytosis. We find a delay of ~5 seconds in the exocytosis response to Ca2+ captures the experimental trends of which cells survive for several Tcb1/3 mutants. We also investigate the effects of various drug (BFA, LatA) and genetic (pil1Δ, msy1/2Δ) treatments on the protoplast expansion, finding agreement with experiment. Our model provides a quantitative explanation for how fission yeast cells adapt to large hypoosmotic stress by utilizing both non-vesicular lipid transfer and exocytic membrane delivery to maintain PM integrity.

B183/P0763

Controlling the endocytic uptake of receptors using intrinsically disordered ligands

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Endocytosis plays a crucial role in determining receptor fate, regulating receptor signaling at the eukaryotic cell surface, and degradation and recycling of receptors. Determining how endocytosis is influenced by receptors

and their ligands is important to our understanding of cell signaling. Similarly, designing novel ligands that can control endocytosis could suggest new therapeutic approaches. Interestingly, recent work has suggested that attractive and repulsive interactions between intrinsically disordered proteins (IDPs) can modulate membrane curvature, a key step in endocytic uptake of receptors and their ligands. These findings suggest an opportunity to control endocytosis by designing IDP ligands and controlling the biochemical interactions between them. Here we show that phase-separable IDPs can drive endocytosis by promoting attractive interactions between receptors, whereas non-phase-separable IDPs escape from endocytic sites by creating repulsive forces between receptors. Specifically, we chose a model IDP that is known to undergo phase separation, the low complexity domain of Fused-In-Sarcoma (FUS LC), to examine how attractive forces between ligands influence endocytic uptake of receptors. Binding of FUS LC to a model receptor led to receptor clustering that promoted endocytic uptake. However, when these clusters became too large, they inhibited endocytosis by forming protein aggregates on the plasma membrane. To evaluate whether increased propensity to undergo phase separation led to internalization of receptors bound to ligands, we mutated glutamine residues within FUS LC, which are critical for phase separation via hydrogen bonding. When we strengthened attractive forces by replacing 12 serines with glutamines, we found the recruitment of receptors to endocytic structures increased. In contrast, endocytic uptake decreased when we attenuated attractive interactions between FUS LC ligands by replacing 8 glutamine residues with serines. As an example of an IDP that resists phase separation, we used an IDP taken from the AP180 protein, which has a high negative net charge. AP180's IDP has been known to resist phase separation and generate steric and electrostatic repulsion that promotes outward membrane bending, in opposition to endocytosis. We found that ligands that included AP180's IDP reduced the localization of receptors to endocytic sites, which is the opposite of our findings with ligands consisting of IDPs that have attractive interactions with one another. Taken together, these results suggest that by modulating the attractive and repulsive interactions between disordered proteins, we can exert control over the internalization of receptors by endocytic pathways.

B168/P0748

Effect of the Membrane Proximal Actin Cytoskeleton and Crowding on the Local Mobility of Protein Tracers in the Cell Membrane

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The plasma membrane is a highly dynamic interface where cells interact with their environment, organize signalling cascades, and regulate molecular trafficking. Critical to these functions is the diffusion of the membrane's constituent proteins and lipids, which enables them to encounter their functional counterparts rather than non-specific ones. The cortical actin meshwork and protein crowding have long been considered to be key regulators of diffusion at the membrane. In this study, we combined live-cell imaging with a minimal, reconstituted, in vitro system to directly test how actin fences and protein crowding shape the mesoscale diffusional transport of membrane proteins.Using Imaging-Fluorescence Correlation Spectroscopy (ImFCS) we were able to measure for the first time, the relationship between membrane proximal actin density and molecular mobility of proteins in the cell membrane. We developed a novel pipeline to measure the density of proteins at the cell membrane using Quantitative Microscopy, Super Resolution imaging and Bioinformatic analyses. We then systematically varied actin-based picketing and protein density in our in-vitro and in-silico model membrane systems and compared it with tracer mobility in live cells. Our analysis revealed that membrane-proximal actin density does not correlate with tracer mobility at the mesoscale. Instead, protein crowding within the bilayer dominates and can mask the influence of local actin structures. Our results reveal

that cortical actin density is not the primary determinant of diffusion in the cell membrane and emphasizes crowding as an intertwined regulator of molecular mobility in the plasma membrane.

B171/P0751

FabID: An antibody-based proximity biotinylation method for extracellular protein-protein interaction analysis

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Membrane proteins constitute over 30% of the human proteome and are central to cellular functions such as environmental sensing, signal transduction, and cell-cell interactions. Their extracellular protein-protein interactions (exPPIs), which are crucial for these processes. However, standard interaction assays—like yeast two-hybrid, immunoprecipitation with mass spectrometry, or protein arrays—are optimized for soluble intracellular proteins and are poorly suited for exPPIs that depend on membrane and extracellular contexts. BioID (proximity-dependent biotin identification) has been developed as an enzymatic proximity-labelling technology. We recently developed AirID, a novel enzyme for BioID applications. However, currently, BioID has not been conjugated to mAb and is used as a direct tool to analyse exPPIs through genetic insertions. This genetic approach requires the insertion of BioID enzymes between the domains of membrane proteins, which may have a substantial effect on membrane protein function. To overcome this, we engineered FabID, in which AirID is fused to the Fab fragment of an antibody. Specifically, EGFR-FabID—AirID fused to an anti-EGFR Fab—successfully biotinylated EGFR extracellular regions. LC-MS/MS analysis revealed numerous known EGFR interactors, along with novel candidate proteins, validating FabID as an effective exPPI mapping tool. Furthermore, these proximity exPPI networks were modulated by EGF ligand stimulation and by gefitinib (Iressa), an EGFR tyrosine kinase inhibitor, suggesting extracellular interactions are dynamically regulated by receptor activation states. These results indicate that the FabID strategy provides a robust, antibody-based strategy for analyzing exPPIs of receptor proteins without genetic modification, offering new insights into extracellular interactomes. Reference Yamada K, et al., “Proximity extracellular protein-protein interaction analysis of EGFR using AirID-conjugated fragment of antigen binding.” Nat Commun. 14;14(1):8301. 2023 Dec. doi: 10.1038/s41467-023-43931-7.

B169/P0749

Integrins and tetraspanins link CD36 to the actin cortex, regulating its organization and signaling

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Cell surface receptors dynamically organize into functional plasma membrane domains that integrate extracellular cues with intracellular responses. Cortical actin is known to influence cell surface receptor mobility and clustering thus regulating cellular signaling. Yet, the molecular mechanisms linking many receptors to actin remain poorly defined, particularly for receptors with short cytoplasmic tails. One such receptor is CD36, a scavenger receptor found on the surface of many different cell types including endothelial cells where it primarily acts as a negative regulator of angiogenesis in the presence of Thrombospondin-1 (TSP-1). Here, we found that CD36 is part of multimolecular complexes that couple CD36 to the actin cortex, a previously unrecognized function of these complexes. Using quantitative imaging, including 3 -color colocalization analysis, single molecule imaging and fluorescence speckle microscopy, we show that β1-integrin and the tetraspanins CD9, CD81, and CD151 recruit CD36 into actin enriched nanodomains at the cell

periphery. A single-point mutation in CD36 (G12V, in the N-terminal transmembrane domain) designed to perturb its interactions with CD9 differentially modified the association of CD36 with β1-integrin and the tetraspanins based on cellular location and reduced its coupling with actin. This resulted in redistribution of CD36 (G12V mutant) towards the center, an increase in receptor confinement and increased colocalization with caveolae. Further underscoring the importance of its dynamic organization at the cell surface, mutated CD36 (G12V mutant) was found to be unresponsive to TSP-1-mediated signaling through the Src family kinase Fyn. These findings establish integrin - tetraspanin assemblies as a molecular bridge between CD36 and cortical actin, revealing a potentially generalizable mechanism for plasma membrane and cytoskeleton coupling, which may fine-tune receptor signaling.

B182/P0762

Molecular determinants of caveolin membrane interactions

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Caveolins are the major structural proteins of small (60-80 nm), cholesterol-rich plasma membrane (PM) invaginations called caveolae. These structures participate in many diverse cellular processes including mechano-adaptation, lipid sorting, and signal scaffolding. Accordingly, caveolin mutations are associated with diseases of dysfunctional signaling and impaired lipid trafficking, highlighting the importance of these proteins in cellular organization and physiology. Critically, the molecular mechanisms by which caveolins control membrane organization and contribute to physiological processes are unknown, in part because the molecular architecture of these proteins has remained speculative. The recently determined cryo-EM structure of the Caveolin-1 8S (Cav1-8S) complex solved this knowledge gap, revealing that caveolin assembles into a unique, tightly packed 11-mer disc with a 14-nm diameter hydrophobic surface. Each monomer has a disordered N-terminus region that likely projects into the cytosol, while the C-termini assemble into a beta barrel at the center of the complex. These unusual physical characteristics suggest a novel mode of protein interaction with lipids and other proteins at the PM. To investigate caveolin-membrane interactions, we combined computational molecular modeling and model membrane experiments to reveal that Cav1-8S inserts deeply into one leaflet of a lipid bilayer via its large hydrophobic surface. This insertion leads to membrane curvature, induced by caveolin's ordering of lipid acyl chains, and sequestering cholesterol to its hydrophobic surface and beta barrel. The cellular context for these interactions and how they contribute to PM organization is unknown. Based on these predictions, we hypothesize that unique structural features of Cav1-8S bend cellular membranes, modulate membrane biophysical properties, and contribute to cholesterol homeostasis. We test these hypotheses through cell-based experiments and reconstitution of recombinantly purified Cav1-8S into biomimetic membrane models. Overall, our current findings suggest a novel mechanism by which Cav1-8S shapes membranes, sequesters cholesterol, and sorts lipids, uncovering how the unique properties of Caveolin-1 modulate the biophysical properties and homeostasis of the PM.

B176/P0756

Optimizing PH Domain-Based Biosensors for Improved Plasma Membrane PIP3 Measurements

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Phosphoinositide-binding pleckstrin homology (PH) domains interact with both phospholipids and proteins, often complicating their use as specific lipid biosensors. In this study, we introduced specific mutations into the phosphatidylinositol 3,4,5-trisphosphate (PIP3)-specific PH domains of protein kinase B (Akt) and general receptor for phosphoinositides 1 (GRP1) that disrupt protein-mediated interactions while preserving lipid binding, in order to enhance biosensor specificity for PIP3, and evaluated their impact on plasma membrane (PM) localization and lipid-tracking ability. Using bioluminescence resonance energy transfer (BRET) and confocal microscopy, we assessed the localization of PH domains in HEK293A cells under different conditions. While Akt-PH mutants showed minimal deviations from the wild type, GRP1-PH mutants exhibited significantly reduced PM localization both at baseline and after stimulation with epidermal growth factor (EGF), insulin, or vanadate. We further developed tandem mutant GRP1-PH domain constructs to enhance PM PIP3 avidity. Additionally, our investigation into the influence of ADP ribosylation factor 6 (Arf6) activity on GRP1-PH-based biosensors revealed that while the wild-type sensors were Arf6- dependent, the mutants operated independently of Arf6 activity level. These optimized GRP1-PH constructs provide a refined biosensor system for accurate and selective detection of dynamic PIP3 signalling, expanding the toolkit for dissecting phosphoinositide-mediated pathways.

B185/P0765

Paracrine regulation of epithelial tight junctions and claudin-2 by endothelial semaphorin 3F under normal and high glucose conditions

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Microvascular beds have been recognized as important sources of paracrine signals that maintain the homeostasis of surrounding tissues. This crosstalk can be disrupted by conditions such as diabetes or exposure to high glucose, as we have previously demonstrated. Our earlier work also showed that vascular endothelial cells secrete semaphorins, which play a role in regulating epithelial polarity. Notably, high glucose influences the secretion of these semaphorins. In this study, we aimed to determine whether the semaphorin isoform Sema3F, secreted by endothelial cells, maintains the epithelial barrier integrity of tight junctions by promoting the trafficking of claudin-2 to the cell surface. Methods: We assessed claudin-2 surface expression using surface biotinylation in either cultured Calu-3 epithelial cells or native renal tubule epithelia from diabetic Akita mice. To study cell-cell communication, we co-cultured HUVEC endothelial cells with Calu-3 cells using transwell permeable supports under normal (5.5 mM) or high glucose (25 mM). Sema3F was silenced in HUVEC cells using a lentiviral vector carrying shRNA. Results: Surface expression of claudin-2 in renal epithelia from diabetic Akita mice was reduced by 76 ± 11% ($p < 0.01$), indicating dysregulated tight junction protein trafficking. In vitro, treatment with recombinant Sema3F (200 ng/mL) decreased surface claudin-2 in Calu-3 cells by 55 ± 1% ($p < 0.01$), but not under high glucose. These changes paralleled tight junction maturation over two weeks, as measured by transepithelial electrical resistance. To investigate the role of endogenous Sema3F, we silenced its expression in HUVEC cells and co-cultured them with Calu-3 cells. The presence of HUVEC cells stimulated claudin-2 surface expression in Calu-3 two-fold and accelerated tight junction maturation ($p<0.01$). However, this stimulation was significantly diminished when Sema3F was silenced, indicating that endogenous Sema3F stimulated tight junctions in adjacent epithelial cells. Interestingly, under high glucose, Sema3F did not abolish the stimulatory effect of co-cultures. Conclusion: Semaphorin 3F secreted by endothelial cells stimulate tight junction maturation and increases claudin-2 surface expression in neighboring epithelial cells. On high glucose, this paracrine effect persists despite Sema3F silencing, suggesting the involvement of additional secreted factors.

B177/P0757

Plasma membrane asymmetry regulates cellular cholesterol homeostasis

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The plasma membrane is the interface between the external world and a cell's internal chemistry, and must therefore facilitate a multitude of parallel, tightly regulated tasks. To achieve this functional complexity, mammalian cells produce hundreds of lipid species, nearly all of which are asymmetrically distributed between the two leaflets of the plasma membrane bilayer. Further, the various organellar membranes have vastly different lipid compositions that are essential for their functions, and thus must be homeostatically maintained. Using quantitative lipidomics we determined the asymmetric distribution of all phospholipids in human erythrocyte plasma membranes. In addition to defining the asymmetric lipidomes of the two PM leaflets, we discovered that the cytoplasmic leaflet contains ~50% more phospholipids than the exoplasmic leaflet. We show that this imbalance of phospholipids in the plasma membrane is enabled by the large abundance of cholesterol (~40%) in the plasma membrane, which can rapidly flip between leaflets to buffer area and mechanical stresses. Through computational and experimental approaches, we find that the combination of cholesterol's preference for the more saturated exoplasmic lipids and the overabundance of lipids in the cytoplasmic leaflet yields a major enrichment of cholesterol in the exoplasmic leaflet. We show that this transbilayer distribution can be altered by manipulating the determinants of cholesterol asymmetry, namely the disparity in phospholipid composition and abundance between leaflets. Specifically, addition of phospholipids to the outer leaflet redistributes cholesterol to the cytosolic leaflet of the plasma membrane. This redistribution leads to cholesterol's recognition by cytoplasmic sensor proteins (i.e. GRAMD), which transfer it to the endoplasmic reticulum for esterification into cholesterol esters and storage in lipid droplets. Thus, transbilayer cholesterol asymmetry is a major contributor to cellular cholesterol homeostasis

B188/P0768

Role and regulation of SNAREs in specialized cell elimination

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Programmed cell death (PCD) is essential for development and homeostasis. Specialized cells, like neurons, have disparate structural and functional compartments. The molecular machinery governing their elimination is poorly understood. The plasma membrane, the physical barrier for cells, is also an elaborate and controlled signaling platform. We explore the local control and role of SNAREs (Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors), highly conserved and known in vesicle fusion. A direct role for SNAREs in PCD has not been described. We examine this in vivo in the scaffolding tail-spike epithelial cell (TSC) of the nematode C. elegans. This specialized cell dies in the embryo via a novel cell death program we named Compartmentalized Cell Elimination (CCE). During CCE, the TSC is remodeled to display three different elimination morphologies reminiscent of developmental neurite pruning. CCE takes place 10 times slower than canonical apoptosis, allowing for examination of cell death events at high spatial and temporal resolution. CCE, while caspase dependent, is genetically non-apoptotic. We report the first sign of CCE at the junction of the TSC soma and the proximal segment process and show that critical events taking place in this degenerative hub include caspase activation, Ca2+ spiking and mitochondrial exclusion. Strikingly, we find that in the absence of caspase, CCE initiates, with an early membrane nicking event, and the morphological hallmarks are indeed

observed. However, death cannot progress and the TSC reverts as intact, suggesting a caspase-independent mechanism directs the severing of the TSC process from its soma and the completion of CCE. We have recently observed that the SNARE target membrane component SYX-2/Syntaxin, known to play a role in wound healing and, from our work, phagosome sealing, localizes at the site prior to CCE initiation. Interestingly, we find that syx-2 loss-of-function mutants have a surviving intact TSC, suggesting a role in CCE initiation. In addition, we observe phosphatidyl serine (PS) exposure locally. PS is classically known as an “eat me” signal presented by apoptotic corpses but has also been shown as a “save me” signal following axonal fusion. This data motivates several intriguing questions we address: What other SNAREs are important for CCE? At specific steps/stages and in what order are SYX-2/Syntaxin and PS localized? What drives their spatiotemporal control and what are their specific roles in CCE initiation? Does CED-3/Caspase target SYX-2/Syntaxin? Our work will further define a novel functional membrane microdomain that directs specialized cell elimination and will provide novel insights into developmental neuronal pruning and neurodegeneration and cellular resilience.

B179/P0759

-The ALS- and FTD-associated proteins Annexin A11 and CHMP2B act sequentially in membrane repair

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Maintenance of plasma membrane and organellar integrity is essential for cell viability. Cells must recognise damaged membranes and orchestrate repair programmes to preserve compartmentalisation. A variety of cellular factors including ESCRTs, Annexins, stress granules, lipids and proteins allowing vesicle and organelle fusion with damaged membranes have been reported to contribute to membrane repair. However, whether these factors operate independently or together to repair membranes is unclear. Here, we expose temporal differences and interdependencies in the recruitment of ESCRT-III and Annexin proteins to sites of membrane damage. We show that while Annexins are recruited immediately to sites of damage, ESCRT-III assembles only after membrane permeability has been restricted. We show that ESCRT-III acts to shed plugs of damaged membranes from the cell and that FTD-and ALS-associated mutations in CHMP2B and ANXA11 compromise the repair process, suggesting integrated ‘sealing and healing’ model of events to restore membrane integrity.

B178/P0758

The cell cortex dictates plasma membrane blebbing through tension-dependent control of lipid scramblases

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The two leaflets of the plasma membrane (PM) are intrinsically asymmetric in lipid distribution. This asymmetry is lost during outward expansion of the PM and release of membrane blebs, processes involved in apoptosis, amoeboid migration, and immune cell evasion. During blebbing, rise in intracellular calcium activates the lipid scramblase TMEM16F, exposing phosphatidylserine on the cell surface, while calcium-dependent proteases release the PM from the underlying actin cortex. Although both processes are essential for bleb formation, their interconnection remains unclear. Using a combination of live-cell imaging approaches, we show that the actin cytoskeleton regulates scrambling and blebbing by controlling the PM’s mechanical state. Scrambling occurs only at regions of the PM detached from the cortex before blebs form. Detachment precedes scrambling, lowers PM tension, and spatially restricts scrambling to low-tension regions.

Weakening PM-cortex interactions enhances scrambling, while stabilization of the PM-cortex prevents both scrambling and blebbing. Scrambling is further promoted on soft substrates and suppressed on stiff substrates, underscoring the role of the mechanical environment. Our findings demonstrate that cortex detachment relieves mechanical constraints on the membrane, enabling lipid scrambling. We provide direct evidence that membrane tension governs this process and offer new insights into the mechanism of bleb formation.

B172/P0752

The ER-localized ABCG transporters upregulate free/accessible cholesterol in plasma membrane to promote protrusion formation and cell-cell fusion

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Cell-cell fusion is critical for the development and physiology of multicellular organisms. It is an asymmetric process in which an invading cell projects actin-propelled membrane protrusions into the receiving cell to promote cell membrane juxtaposition and fusion. Although the protein machinery at the site of fusion, known as the fusogenic synapse, has been extensively studied, the function of lipids in the fusion process remains largely unknown. Here, we identified a role for the cholesterol-efflux G1/G4 family of ATP-binding cassette transporters (ABC transporters) in cell-cell fusion. Overexpressing endoplasmic reticulum (ER)-localized ABCG transporters in Drosophila S2R+ cells increased the amount of free and accessible cholesterol in the plasma membrane (PM) and enhanced cell-cell fusion, whereas knocking down ABCG transporters in mouse myoblasts resulted in reduced free/accessible cholesterol in the PM and inhibited cell-cell fusion. We showed that the free/accessible cholesterol, but not the sphingolipid-sequestered cholesterol, promoted lipid packing disorder and decreased cortical stiffness, lowering the energy barrier for protrusion formation required for cell-cell fusion. Taken together, our studies uncovered a novel role for ABCG1/4 transporters in cell-cell fusion and highlighted a previously unrecognized function for free/accessible cholesterol in enhancing lipid packing disorder and facilitating protrusion formation in both insect and mammalian cells.

B173/P0753

The functional role of localized plasma membrane scrambling in immune cell activation

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Membrane asymmetry is a carefully controlled and energetically costly differential in lipid composition between the inner and outer plasma membrane (PM) leaflets. Asymmetry is created and maintained by lipid translocases (flippases) that force anionic and amino-phospholipids into the cytosolic leaflet, and released by lipid channels (scramblases) that shuttle phospholipids down their concentration gradient to disrupt PM asymmetry. The release of lipid asymmetry exposes the charged lipid phosphatidylserine (PS) to the external leaflet, where it is essential for apoptosis and blood clotting. Additionally, exposure of inner leaflet phospholipids has been observed during the activation of several immune cell types (mast cells, basophils, leukocytes). We confirmed these observations, showing exposure of PS and other inner leaflet phospholipids on the PM outer leaflet following antigen-mediated stimulation of T cells and mast cells. Importantly, in contrast to wholesale release of PM asymmetry by apoptosis, PS exposure in activated immune cells is focal, transient, reversible, and not associated with cell death. This lipid scrambling appears to be involved in

immune cell function, as inhibition of the scramblase TMEM16F attenuates antigen-activated mast cell degranulation. To investigate the mechanisms underlying these effects, we knocked out (KO) TMEM16F, the ubiquitous Ca2+-dependent scramblase / lipid channel, which is putatively responsible for PS and PE translocation during immune cell signaling. Indeed, we find that TMEM16F is essential for activation-associated lipid scrambling. Finally, we combine theory and cell experiments to explore the molecular nature and functional role of localized, transient membrane scrambling during mast cell activation and degranulation. Altogether, our findings suggest that localized scrambling is an essential component of signal transduction downstream of immune receptor activation.

Membrane Biology and Trafficking - Vesicle trafficking and sorting mechanisms

B213/P0791
Acute Disruption of COPII Assembly and ER-Export Function Using The mAID-Sec12 System
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Protein trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus is a critical step in protein secretion and distribution to membrane-bound organelles. ER-to-Golgi protein trafficking is driven by evolutionarily conserved coat protein complex II (COPII) proteins, which assemble at discrete ER exit sites (ERESs) to bud cargo-loaded export compartments in a dynamic process governed by the Sar1 GTPase and its guanine exchange factor, Sec12. Recent studies reveal that mammalian cells can organize ERESs into a clustered tubule network that is continuous with the ER via a narrow neck where COPII localizes. This intricate architecture likely facilitates efficient sorting and export of diverse secretory proteins, including bulky cargoes. However, the mechanism by which COPII machinery morphs the ER membrane into these interwoven export compartments remains unclear. This is largely due to the lack of a model to acutely disrupt COPII activity. Current approaches using RNA interference or chemical inhibition are too slow or lack the specificity needed to define the biological mechanism without capturing compensatory or off-target effects. To overcome these technical difficulties, we generated a homozygous mini auxin-inducible-degron (mAID)-tagged Sec12 RPE1 cell line via CRISPR genome editing to rapidly blunt Sar1 activation and COPII assembly. Auxin-treatment results in 95% reduction of mAID-Sec12 via proteasomal degradation within 3 hours, accompanied by the dissociation of endogenous and exogenous COPII from the ER membrane and loss of peripheral ERESs. Synchronized cargo release assays reveal a stark loss of cargo loading into ERESs and impaired trafficking. By achieving robust and acute disruption of peripheral ERESs, the mAID-Sec12 system serves as an ideal model for investigating the mechanisms generating mammalian ERES architecture and regulating the secretory pathway.

B216/P0794
Amino acid substitutions in Sec5 and Sec10 allosterically activate the exocyst
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The exocyst, a fundamental hetero-octameric tethering complex consisting of eight essential subunits, is allosterically activated by Sro7 and Rho GTPases. Sro7 activation increases the exocyst’s affinity for the Rab GTPase, thus enhancing its ability to tether vesicles to the plasma membrane. The Rho pathway involves Cdc42, a Rho GTPase, activating the input subunit Exo70 and enhances the exocyst’s ability to tether vesicles to the plasma membrane by increasing affinity for the v-SNARE Snc2. Spontaneous gain-of-function (GOF) alleles of the Sec5 and Sec10 subunits of the exocyst in the temperature-sensitive yeast mutants sec2-41 and cdc42-6

were identified via a GOF screen. To pinpoint the specific GOF mutations in both yeast mutants that rescue pathway defects, we performed site-directed mutagenesis and introduced individual point mutations derived from the spontaneous alleles obtained in the GOF screens. We structurally modeled the exocyst subunits and labeled the single point mutations obtained from the GOF screen. From this, we selected mutations that were in areas of subunit interactions and had amino acid property changes. Retesting of these single mutations in the sec2-41 and cdc42-6 strains confirmed that specific amino acid substitutions in Sec5 and Sec10 confer GOF activity. These results highlight mechanisms by which exocyst subunits can be allosterically activated. To continue this work, we are testing additional site directed point mutations from the spontaneous GOF alleles to identify the individual point mutations that rescue both pathways. We are also using fluorescent microscopy in visualizing areas of polarized exocytosis in yeast cells.

B232/P0810

Analysis of the Role of Rab32/38 in Bone Formation

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Human bones are maintained through constant remodeling. Osteoclasts resorb bone by secreting a variety of proteases and acids at the ruffled border. Osteoblasts form bone by producing and secreting bone matrix and promoting its mineralization. We recently reported that Rab38 is highly expressed among Rab proteins during osteoclast differentiation and is involved in the formation of lysosome-related organelles (LROs) (Noda et al., 2023). We also demonstrated that Rab32/38 double knockout (DKO) mice exhibit high bone density, which we attributed to reduced osteoclast bone resorption capacity, as LROs are involved in the secretion of acids and proteases (Tokuda et al., 2023). However, bone morphometry analysis revealed that Rab32/38 DKO mice have fewer osteoclasts and more osteoblasts in their femurs. The high bone density Rab32/38 DKO mice was not only resulted from decreased enzymes secretion but also a balance biogenesis between osteoclast and osteoblast. Since Rab38 expression is low in osteoblasts, we wandered what is the mechanism underlying the increased number of osteoblasts in Rab32/38 DKO mice. Semaphorin 4D (Sema4D) has been reported to be transported to plasma membrane, where the surface enzymes cleave it into two forms: a membrane-bound cleavage form remains on plasma membrane and a soluble form that is released from osteoclasts to inhibit osteoblast differentiation. As results, with comparable level of total Sema4D protein between WT and DKO, the cleaved form of Sema4D was reduced in cell lysates, and the concentration of the secreted form of Sema4D was reduced in the culture supernatant. To investigate how Rab32 regulated Sema4D transport, we examined the location of Sema4D and found it was primarily localized to the ER, partially to the Golgi, but not to Rab32-LRO membrane. However, Correlative Light & Electron Microscopy(CLEM) analysis revealed that Sema4D-positive ER was in contact with Rab32-LRO. The detail mechanism remained further elucidate, we propose that the ER-Rab32/38-LRO contact site may regulate the intracellular trafficking of Sema4D to plasma membrane, thereby modulating bone remodeling.

B222/P0800

Biochemical and structural characterization of pancreatic amylase and its distribution within secretory vesicles

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Pancreatic amylase is a non-zymogen enzyme whose premature activation within pancreatic tissue can lead to cellular damage and inflammation. To prevent this, amylase must remain inactive within specialized secretory vesicles until secretion. Despite its critical role in digestive physiology, the molecular composition and structural organization of these vesicles remain poorly defined. Dysregulation of amylase secretion has been implicated in pancreatitis and metabolic disorders such as diabetes and obesity. To investigate this, we used AR42J pancreatic acinar cell line as a model to investigate the molecular features of amylase-containing secretory vesicles. Through biochemical isolation, we distinguished these vesicles from other intracellular compartments and identified two different populations enriched in surface components such as heparan sulfate proteoglycans. Confocal and structured illumination microscopy revealed the intracellular localization of amylase and its spatial association with Syndecan-4, suggesting a role for this proteoglycan in vesicle identity or trafficking. Together, these findings provide a comprehensive biochemical and structural framework for understanding regulated amylase secretion in pancreatic acinar cells, particularly under conditions of compromised health. This work lays the foundation for future studies into vesicle biology and its implications in pancreatic disease.

B207/P0785

Capturing intercellular communication via tunneling nanotubes using cryo-electron tomography

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Tunneling nanotubes (TNTs) are thin intercellular bridges that mediate communication and the exchange of proteins, organelles, and nucleotides between neighboring cells. They are enriched in tumor cells, linked to chemotherapy resistance, and readily form in models of aggregation-based genetic diseases such as cystinosis and Parkinson's disease. Viruses can also induce and hijack TNT-mediated transport to enhance infection. Despite their broad relevance and attractive therapeutic potential, TNT morphology and function remain controversial due to the absence of clear morphological criteria and the limitations of light microscopy. Here, we establish three complementary systems to study TNT formation and function. First, we stimulate TNTs with the pseudorabies viral kinase US3, mimicking viral transmission. Second, to model chemotherapy resistance, we treat monocytic leukemia THP-1 cells with daunorubicin. Third, to model genetic disease, we co-culture Sanfilippo primary fibroblasts, which carry a lysosomal storage disorder mutation, with IC21 macrophages. Using live-cell imaging, we track cytoskeletal organization and bidirectional lysosome transport in all three contexts. We then apply correlative cryo-electron tomography (cryo-ET) to visualize mature TNTs in their native environment. TNTs display a rich ultrastructure, including actin, microtubules, intermediate filaments, ribosomes, and diverse organelles such as multivesicular bodies, autophagosomes, and lysosomes. Subnanometer-resolution microtubule reconstructions reveal mixed polarity, suggesting contributions from both donor and acceptor cells to transport. Together, these results provide the first high-resolution structural framework of TNTs, revealing their molecular complexity and offering new insights into how TNTs may mediate intercellular communication across diverse biological contexts.

B228/P0806

Coexpressed neuropeptides segregate into separate dense core vesicles

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In the Drosophila central complex, many peptidergic neurons express more than one neuropeptide encoded by different genes (Wolff T. et al 2025, Elife). To determine how cells distribute these neuropeptides, we have inserted nanotags into the proprotein domain of multiple neuropeptides, eliminating the need for neuropeptide-specific antibodies and enabling visualization of multiple neuropeptides in the same animal. We have successfully tagged 23 neuropeptides in Drosophila with nanotags, including four independently labelled with different nanotags to facilitate double labelling. Tachikynin (Tk) and Myosin inhibitory peptide (Mip) are coexpressed in various central complex neurons, and when labelled, these neuropeptides are observed in separate dense core vesicles (DCV) in axons. We confirmed that the segregation of different neuropeptides into distinct DCVs was a general phenomenon by examining other cell types that coexpressing small Neuropeptide F (sNPF) and Allostatin-C (Ast-C), Neuropeptide F (NPF) and Allostatin-A (Ast-A), or NPF and Ast-C. We aim to uncover the mechanisms underlying this sorting of neuropeptides by identifying the protein regions required for sorting and performing proteomic analyses of neuropeptides and their DCVs to identify interactors that mediate this process.

B217/P0795

Deciphering the mechanisms regulating PIEZO channel membrane trafficking

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PIEZO proteins are mechanotransductive ion channels that translate mechanical stimuli into cellular signaling, which are essential for almost all physiological processes. Over 100 pathogenic variants of PIEZO have been associated with at least 26 diseases and disorders, including congenital lymphatic dysplasia, distal arthrogryposis, and perinatal respiratory distress. Despite their clinical relevance, effective therapeutic treatments for PIEZO-associated pathologies remain largely elusive. A limitation to developing targeted treatments is the incomplete understanding of the cellular and molecular natures of the PIEZO channel, including the mechanism regulating PIEZO channel trafficking and localization. In this study, we leverage the C. elegans germline to investigate the cellular mechanisms governing PIEZO protein localization and membrane trafficking. Utilizing a high-resolution confocal imaging system, we characterized the trafficking dynamics of PEZO-1, the C. elegans PIEZO ortholog, endogenously tagged with multiple fluorescent markers. Our findings reveal that GFP::PEZO-1 colocalized with key membrane vesicle markers, including caveolin-1 GFP::CAV-1 and the recycling endosome regulator GFP::RAB-11, underscoring a conserved vesicular trafficking pathway of PEZO-1. Notably, knock-down rab-11 and a t-SNARE syx-4, disturbed GFP::PEZO-1 vesicle transportation to the plasma membrane, leading to its aberrant accumulation in the perinuclear region. These data suggested that PEZO-1 trafficking is dependent on RAB-11-dependent finely tuned vesicular transport machinery. Furthermore, we demonstrated that the structural integrity of PEZO-1 is required for its proper trafficking. Depletion of either the C-terminal ion pore or N-terminal transmembrane domains, as well as the gain-of-function PIEZO missense mutation R2405P, resulted in severe trafficking defects, vesicular mislocation, and impaired plasma membrane expression. Intriguingly, we uncover a regulatory role of sperm-derived signaling in PEZO-1 trafficking, wherein the absence of sperm, and its major signaling molecule, major sperm protein (MSP), abrogated PEZO-1 cortical enrichment, linking PIEZO channel dynamics to reproductive signaling and oocyte maturation.Collectively, our findings establish a fundamental cellular framework governing PIEZO protein trafficking and membrane localization. By identifying key vesicular trafficking regulators and structural elements of PEZO-1, our study demonstrates mechanistic insights into the regulation of PIEZO channel membrane dynamics. These findings may inform the development of therapeutic strategies for the medical conditions associated with PIEZO channel dysfunction

B233/P0811

Determining the Role of BLOC-2 in Melanosome Motilty and Biogenesis

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Lysosome-related organelles (LROs) are cell type-specific subcellular compartments that carry out specialized physiological functions. In disorders such as the Hermansky-Pudlak syndromes (HPS), mutations in genes encoding subunits of protein complexes that are essential for cargo delivery to LROs impair the biogenesis of several LROs, including melanosomes - the pigment-producing organelles in skin melanocytes and eye pigment cells. Despite progress, fundamental gaps remain in understanding the mechanisms of LRO maturation and the roles of the HPS-associated protein complexes. My research investigates the role of one of these complexes, Biogenesis of Lysosome-related Organelles Complex 2 (BLOC-2), in melanocytes to better understand the basic biology of LRO development. BLOC-2 is a three-subunit complex implicated in cargo delivery to melanosomes via tubular transport carriers (TTCs) that extend from early endosomes. Previous work shows that BLOC-2 is required for TTC stability and their efficient contacts with melanosomes that are imperative for cargo transfer, but how BLOC-2 mechanistically facilitates these contacts is unknown. My work demonstrates via live-cell imaging that BLOC-2 localizes predominantly to maturing melanosomes and that these organelles are highly motile, traveling long distances along microtubules (MTs) yet returning close to their starting points. Treatment with nocodazole significantly decreases this motility, indicating an essential role for MTs. I hypothesize that maturing melanosomes seek out TTCs via a search-and-capture mechanism mediated by BLOC-2 on the melanosomal membrane and subsequent interactions with microtubule motors. If my hypotheses are correct, my project will uncover a novel mechanism of cellular transport in which target organelles actively search for cargo carriers to facilitate contact events that may apply to other interorganellar transport systems. Findings from this project will also further understanding of the pathogenesis of HPS.

B220/P0798

Identifying unique cargoes or regulators of AP-1 sub-complexes in Saccharomyces cerevisiae

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Around a third of the proteome traverses the secretory pathway and is modified by enzymes in the Golgi apparatus. The localization of these Golgi enzymes is maintained by recycling machinery, such as adaptors and cytosolic coats, which mediate the vesicular delivery of Golgi residents to their functional compartments. The highly conserved heterotetrameric adaptor complex, AP-1 recycles resident Golgi membrane proteins from the late to medial Golgi. Interestingly, several subunits of the human AP-1 heterotetramer are encoded by multiple alternate genes. Mutations in specific AP-1 isoforms or their regulators lead to distinct human disorders, suggesting unique isoform-specific function. In yeast, only one subunit (mu) has two isoforms which can form two sub-complexes of AP-1. Like in human cells, the loss of either yeast isoforms results in distinct phenotypes. Taking advantage of the simplicity of the yeast system, we present evidence for differential cargos, regulators, and interactors of AP-1 sub-complexes. Using unbiased genetic screens, proximity labeling-MS, fluorescence microscopy, and in vivo functional experiments, we have identified unique candidates involved in maintaining Golgi pH as well as phosphoinositide and complex sphingolipid homeostasis as putative cargos and regulators of specific AP-1 mu isoforms. Our findings suggest that the yeast, and likely human AP-1 isoforms, perform

non-redundant functions in cargo recycling, allowing for nuanced regulation of cellular processes and signaling pathways.

B209/P0787
Lipid Transfer and Endosomal Sorting: Compensatory Pathways for Vps13 Deletion-Induced Sorting Defects
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Vps13 is a lipid transporter localized at membrane contact sites, where its hydrophobic cavity facilitates lipid transfer between organelles to maintain cellular lipid homeostasis. Deletion of Vps13 alters organelle morphology and impairs vesicle-mediated protein sorting. Understanding compensatory mechanisms for Vps13 deletion could provide insights into the cellular regulation of lipid homeostasis, potentially informing therapeutic approaches for diseases linked to similar defects. To identify genes involved in compensatory mechanisms, we conducted a genetic screen for molecular suppressors whose overexpression in S. cerevisiae restored proper sorting of the model protein cargo, Mup1. Our results revealed that overexpression of the transcriptional regulators Ino80, Mpe1, Soh1, Adr1, and Vma1 improved Mup1 trafficking, suggesting a link between transcriptional control and the regulated expression of molecular players in compensatory pathways. Additionally, overexpression of Cog2, a component of the oligomeric Golgi complex, and Vps3, a CORVET subunit, also enhanced Mup1 sorting, implicating a potential link between SNARE-related vesicular tethering and compensation for dysfunctional lipid transfer. While the precise molecular mechanism remains unclear, these findings offer a foundation for future investigation into the cellular compensatory mechanisms to facilitate lipid homeostasis and proper endosomal trafficking under conditions of reduced lipid transfer. Characterization of compensatory pathways of genetic suppressors may allow mapping of molecular frameworks that govern vesicular trafficking, lipid homeostasis, and membrane integrity, with implications beyond yeast models.

B231/P0809
Mechanism of cargo sorting during AP-2 dependent endocytosis
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Adaptor proteins govern endocytosis by coupling cargo recognition with clathrin recruitment. The adaptor protein 2 (AP-2) complex mediates internalization of receptors such as transferrin receptor (TfR) and epidermal growth factor receptor (EGFR). However, receptor uptake persists in AP-2-depleted cells, suggesting additional factors are involved. Here, we identify phosphoglycerate kinase 1 (PGK1), the glycolytic enzyme, as a co-adaptor that cooperates with AP-2 in receptor endocytosis. Combined knockdown of PGK1 and AP-2 caused synergistic defects in TfR and EGFR uptake. Mechanistically, PGK1 directly bound to the sorting motifs within TfR and EGFR cytoplasmic tails, and disruption of PGK1-AP-2 interaction significantly inhibited endocytosis. These findings demonstrate that PGK1 acts beyond its metabolic role to reinforce AP-2-mediated endocytosis. This cooperative adaptor mechanism highlights the moonlighting function of PGK1 in membrane trafficking.

B208/P0786
Mechanism of Intracellular ANGPTL3 and ANGPTL8 Trafficking

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Lipids circulate in the blood in lipoproteins including chylomicrons and very low-density lipoproteins (VLDLs). Lipoprotein Lipase (LPL) is the main enzyme that hydrolyzes the triglycerides from circulating lipoproteins into free fatty acids that can be taken up by cells. Without LPL, dangerously high levels of lipoproteins circulate in the blood, which can lead to cardiovascular disease. LPL inhibitors, known as angiopoietin-like (ANGPTL) proteins, have key roles in the regulation of lipid metabolism. ANGPTL3 is a potent inhibitor of LPL. ANGPTL8 can form a complex with ANGPTL3 which results in more efficient secretion of ANGPTL8 and greater inhibition of LPL than ANGPTL3 alone. Based on immunofluorescence staining of LAMP1 (lysosomal-associated membrane protein 1), I have found that ANGPTL8 when expressed alone, gets trafficked toward lysosomes. However, when ANGPTL8 is co-expressed with ANGPTL3, both ANGPTL3 and ANGPTL8 are effectively trafficked out of the cell. Using the Retention Using Selective Hooks (RUSH) assay, I confirmed that a population of ANGPTL8, when expressed alone, gets trafficked to lysosomes. I have also found that ANGPTL3 and ANGPTL8 are synthesized and form a complex in the endoplasmic reticulum (ER) and get trafficked out of the cell together. Thus, ANGPTL3 diverts a pool of ANGPTL8 from lysosomal degradation. Future goals include understanding why ANGPTL8 is unstable when it is not in complex with ANGPTL3, as well as visualizing the trafficking dynamics of the ANGPTL3/8 complex.

B211/P0789

Occludin Links Endosomal Trafficking to Mitotic Spindle Assembly and Epithelial Morphogenesis

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The emergence of vertebrates is thought to depend in part on lineage-specific innovations in cell division and trafficking, yet the genetic underpinnings remain poorly understood. We identify the vertebrate-specific tight junction protein Occludin (OCLN) as a critical regulator of epithelial morphogenesis through its unexpected role in coordinating vesicle trafficking and spindle function. Mammary glands lacking Ocln exhibit impaired epithelial branching due to reduced cell proliferation and surface expansion. Mechanistically, Ocln binds the Rab11 adaptor FIP5 to recruit recycling endosomes to the centrosome, thereby ensuring proper spindle assembly and orientation. Loss of either Ocln or FIP5 leads to prolonged prophase, spindle dysfunction, and failed nuclear and cytoplasmic division. These findings establish OCLN as a central link between endosomal trafficking and mitotic spindle dynamics. Together with our prior work showing roles for OCLN in SNARE-dependent secretion and lipid droplet release, this study reveals how a vertebrate-specific gene integrates trafficking and division machinery to control tissue growth and highlights an evolutionarily conserved vesicle-based mechanism essential for epithelial development.

B225/P0803

Patient mutations reveal an oligomerization interface that is critical for the regulation of PIKfyve-VAC14-FIG4 complex

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PIKfyve-VAC14-FIG4 complex is the sole source of PI(3,5)P2 and responsible for the majority of PI5P in cells. Both PI(3,5)P2 and PI5P are low abundance signaling lipids, and act by recruiting downstream effector proteins. Emerging studies suggest that these effector proteins regulate multiple cellular events including lysosome function, mTORC1 signaling, autophagy, receptor recycling from endosomes, transcriptional regulation, metabolic reprogramming in cancer cells, and regulation of the cGAS-STING pathway. Mutations in the PIKfyve-VAC14-FIG4 complex underline some neurological diseases. Here we focus on mutations in VAC14, which result in pediatric striatonigral degeneration. To determine the molecular defect of these mutations, I combine the published medium resolution Cryo-EM Map of the complex with AlphaFold3 predictions. When mapped onto this model, two of the patient mutations mapped to the putative VAC14-VAC14 oligomerization interface. To further probe the function of these mutants, I developed a quantitative assay to assess the function of these mutants in VAC14 KO cells, based on the ability of the mutants to rescue the large vacuoles that form due to loss of PIKfyve function. Moreover, using pull-down experiments, and fluorescence size exclusion chromatography, we found that these residues are essential for assembly of the PIKfyve-VAC14-FIG4 complex, and VAC14 oligomerization. In addition, we found that these mutants are defective in localization to membranes. To further probe the impact of these mutations on PIKfyve activity, we generated and tested the corresponding yeast mutants and found that there was a defect in the generation and turnover of $PI(3,5)P_2$. Moreover, similar to studies in human cells, the yeast mutants disrupted VAC14 oligomerization and resulted in the mislocalizaton of VAC14. Together, these findings demonstrate that VAC14 oligomerization interfaces are central to the structural integrity and regulatory function of the PIKfyve–VAC14–FIG4 complex. Moreover, these studies lay out a strategy for studies of additional patient mutations in the PIKfyve-VAC14-FIG4 complex.

B221/P0799

Rab11 and PI4KIIIα/ß differentially regulate extracellular vesicle trafficking at synapses

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Neuronal extracellular vesicles (EVs) are membrane-bound compartments containing proteins, lipids, and nucleic acids, and are implicated in intercellular communication and propagation and clearance of pathogenic proteins. Despite their importance, the mechanisms underlying neuronal EV trafficking remain poorly understood, due to uncertainty about where EV biogenesis and release occur within neuronal cell bodies, axons, and synapses, and the overlap between sharing trafficking pathways for EVs, synaptic vesicles and other cargo. Rab11, a small GTPase, functions as a molecular switch by recruiting effector proteins to regulate processes such as exocytosis, recycling, and maintenance of EV cargo pools at presynaptic terminals. Using the Drosophila neuromuscular junction (NMJ) as our model system, we investigated the roles of two unrelated Rab11-associated lipid kinases, PI4KIIIα and PI4KIIIβ, in neuronal EV trafficking. Although both kinases are responsible for synthesizing the phosphoinositide PI(4)P from phosphatidylinositol (PI), PI4KIIIα and PI4KIIIβ exhibit opposite phenotypes in EV trafficking. RNAi-mediated knockdown of PI4KIIIα significantly reduces EV cargo levels, while loss of four wheel drive (fwd), the Drosophila homolog of PI4KIIIβ, increased EV cargoes both pre- and postsynaptically. To further our understanding of the relationship between Rab11 and PI4KIIIα/PI4KIIIβ, we examined PI4KIIIα/PI4KIIIβ subcellular localization and assessed active Rab11 distribution using the bioengineered Rab11 activity reporter GFP-RBD11. PI4KIIIβ localization was Rab11-dependent, while PI4KIIIα localization was largely independent of Rab11. Together, these results suggest that different classes of Rab11 effector proteins and PI(4)P pools differentially regulate EV trafficking. Understanding how these regulators mediate EV cargo trafficking will provide insight into how neurons regulate Rab11-mediated EV cargo trafficking.

B230/P0808

Rab11a mediated regulation of multivesicular body trafficking in pancreatic beta-cells - implications for type-2 diabetes

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Exosomes, secreted via multivesicular bodies (MVBs) are emerging as critical mediators of intercellular communication and functioning of pancreatic-β-cells. A reduction in the density of MVBs at the plasma membrane (PM) has been observed in β-cells from type 2 diabetes (T2D) donors suggesting impaired MVB secretion, that may compromise exosome-mediated protective functions and contribute to β-cell dysfunction. MVB trafficking and exosome release in β-cells remain poorly understood, although trafficking of insulin secretory granules (ISGs) in β-cells has been well studied. As both content and secretion of exosomes are altered in T2D, understanding the molecular machinery regulating MVB trafficking is critical to uncover how these pathways are altered in T2D contributing to its progression. Few studies in non-secretory cells, suggest Rab11, a small GTPase as one of the regulators of MVB trafficking. To investigate the role of Rab11a and its effector Sec15, an exocyst complex component, in MVB trafficking of β-cells, we performed live-cell imaging of CD63-labeled MVBs. Using confocal and TIRF microscopy, we compared CD63+ MVBs with NPY-labeled ISGs. We found that MVBs are less in number, exhibit distinct spatial distribution, and have slower fusion kinetics compared to ISGs. Interestingly, CD63+ MVBs showed partial colocalization with Rab11a, implicating its role in MVB trafficking. Expression of a dominant-negative Rab11a mutant (S25N) lead to perinuclear accumulation of CD63+ MVBs and significantly reduced their density near the PM, indicating impaired trafficking. To assess the fusion of MVBs, we used CD63-pHluorin to visualize MVB-PM fusion events in live cells. Rab11a S25N expression resulted in reduction of fusion events and delayed release, revealing impaired exosome secretion. Further, we identified Sec15, as a key downstream mediator in MVB trafficking. CD63 partially colocalized with Sec15, and co-expression with wild-type Rab11a enhanced Sec15 puncta formation. In contrast, under Rab11a S25N expression, Sec15 was diffusely cytoplasmic, suggesting disrupted Rab11a-Sec15 interaction compromises MVB trafficking. Our study establishes a critical role of Rab11a and its effector Sec15 in regulating MVB trafficking and exosome release in pancreatic β-cells. This may help us understand the protective mechanisms against β-cell stress and death during T2D.

B227/P0805

Random mutagenesis screen to identify gain-of-function alleles in the exocyst

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Polarized exocytosis is a trafficking process in eukaryotes that establishes cell polarity by directing vesicle fusion to specific plasma membrane domains. The exocyst is an essential hetero-octameric tethering complex consisting of eight subunits that are all essential for its function. The exocyst undergoes allosteric activation through two input pathways during this process. One of the pathways is the Sro7 activation pathway which involves activating the exocyst through the Exo84 subunit. The outcome of activation is enhancing the exocyst's ability to tether vesicles to the plasma membrane by increasing affinity for the Rab GTPase. The other is the Rho pathway involving Cdc42, a Rho GTPase. Previous work has demonstrated that gain-of-function (GOF) mutations in exocyst subunits can mimic activation, revealing that the complex has multiple discrete activation mechanisms. We analyzed the functional roles of the internal subunits Sec5 and Sec10, whose contributions to exocyst activation remain unknown. Using error-prone PCR, we mutated the exocyst subunits

Sec5 and Sec10 before inserting them into gap-repair plasmids and transforming them into temperature sensitive yeast mutants sec2-41. A 5FOA retest was used to confirm candidate GOF alleles in both Sec5 and Sec10 that suppressed pathway defects. The GOF alleles demonstrate that such mutations can rescue pathway defects and further support a role for Sec5 and Sec10 in the allosteric regulation of the exocyst. From these steps, we developed seven mutant alleles in Sec5 and six mutant alleles in Sec10 with varying levels of suppression in the sec2-41 yeast strain. Each allele contained roughly 4 to 12 single point mutations. To continue this work, we tested these alleles for suppression in the temperature-sensitive yeast mutant cdc42-6 (defect in Rho pathway). We also tested the specific point mutations from the spontaneous GOF alleles to identify the individual point mutations that confer GOF activity.

B206/P0784

Redefining LH receptor signaling: Phosphoproteomic timeline reveals Rho GTPase-mediated endocytosis as a central mechanism from cholesterol trafficking to mitochondrial steroid biosynthesis

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Luteinizing hormone (LH) signaling through its G protein-coupled receptor is indispensable for steroid hormone production in male and female reproduction. Classical models emphasize Gs-cAMP-PKA–dependent phosphorylation events as the key initiating mechanism. Here, using global phosphoproteomics in MA-10 cells, we identified 4,056 phosphoproteins and mapped their dynamics at 15, 45, and 90 minutes following LH stimulation. Strikingly, canonical PKA substrate phosphorylation was not detected, and contrary to prevailing models, STAR/STARD1 activity is not regulated through direct PKA phosphorylation. Instead, pathway analysis highlighted the Rho GTPase cycle as the top signaling module at all time points, implicating cytoskeletal and endosomal regulation as the primary drivers of signaling coupled with cholesterol mobilization. Early phosphorylation of RhoD and FCHO1 marked activation of endosomal trafficking and nucleation of clathrin-coated pits, while continuous phosphorylation of ITSN2 and GGA2 supported vesicle maturation and cargo sorting. SCYL2 and ARRB1 exhibited temporal cycles of phosphorylation, indicating adaptive regulation of clathrin adaptor recruitment. In parallel, phosphorylation of proteins involved in ER remodeling, autophagy, and mitochondrial fission revealed organelle-level restructuring to accommodate increased steroid output. Transcriptional priming was evident through early phosphorylation of TEAD3, followed by sustained regulation of CUX1, WWTR1, KDM3B, ELF1, TRRAP, ZHX2, and TAF6, reflecting a progressive transcriptional program to sustain steroidogenesis. Functional validation supported this signaling framework: doxycycline-induced expression of a constitutively active RhoA in MA-10 cells recapitulated all key features inducing steroidogenesis. Together, these findings delineate a signaling timeline in which LH receptor activation orchestrates Rho-dependent cholesterol trafficking, transcriptional regulation, and organelle remodeling to enable STAR-mediated mitochondrial cholesterol import and initiation steroid metabolism. These findings redefine LH receptor signaling, replacing the PKA-centered paradigm with a previously unrecognized Rho-driven signaling pathway as a key driver of steroid hormone biosynthesis.

B223/P0801

Site-specific O-GlcNAcylation Regulates COPII Trafficking by Shaping Sec24D's Interactome

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Glycosylation is a ubiquitous post-translation modification that regulates a variety of cellular mechanisms across most tissues. O-linked β-N-acetylglucosaminylation (O-GlcNAcylation), a type of intracellular glycosylation, has been repeatedly implicated in regulating protein transport by modifying coat protein complex II (COPII). Although the COPII pathway mediates all eukaryotic protein transit from the ER to the Golgi, little is known about how COPII is precisely regulated in response to the fluctuating intracellular environment. Here, we report that the site-specific O-GlcNAcylation of a key COPII component, Sec24D, dynamically regulates proper protein transit. Mass spectrometry revealed that Sec24D is O-GlcNAcylated at six distinct sites. CRISPR-based editing to separately mutate two of these glycosites showed differential interactions of Sec24D wild type versus glycosite mutants with hundreds of proteins. We further present functional studies of some of these novel Sec24D interactors in protein trafficking to shed light on the long-standing question of how COPII adapts to large cargo secretion, such as that of collagen. Ultimately, mapping the impact of O-GlcNAc on Sec24D's interactome will provide a more complete understanding on how distinct stimuli are integrated to modulate COPII activity and physiological outcomes. Future work may capitalize on the identification of potentially novel therapeutic targets from this study to address collagen pathophysiologies.

B210/P0788

SURF4 and TMED10 Define Functionally Distinct Endoplasmic Reticulum Exit Sites for Cargo-specific Export

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At endoplasmic reticulum exit sites (ERES), cargo receptors play a critical role in mediating packaging of nascent cargo proteins into transport vesicles and their entry into the subsequent secretory pathway. Despite advances in our understanding of cargo sorting and cargo receptor recognition mechanisms, the spatial and functional relationships between different receptors remain poorly characterized. In this study, we investigated two key cargo receptors, Surfeit locus protein 4 (SURF4) and Transmembrane emp24 domain-containing protein 10 (TMED10), both of which have been reported to facilitate the export of soluble cargo proteins from the endoplasmic reticulum (ER). We discovered that SURF4 and TMED10 localize to distinct subpopulations at ERES and demonstrate minimal colocalization with each other. Using a mitochondria-tethering assay, in which cargo receptors were fused to the streptavidin-binding peptide (SBP), we ectopically anchored specific cargo receptor to the outer mitochondrial membrane (MOM) and observed significant colocalization between the MOM and cargo receptor-enriched patterns. Through this assay, we observed that MOM-tethered SURF4 colocalized with its client protein, Sonic hedgehog, but not with TMED10. In contrast, MOM-tethered TMED10 colocalized with another p24 protein family member, TMED2, but not with SURF4. Electron microscopy analyses performed in HeLa cells revealed the formation of extended membrane-like structures enriched with Myc-tagged SURF4-SBP fusion protein around MOM. Biotin release experiments and an in vitro vesicle formation assay further indicated that these SURF4-enriched membrane structures are capable of detaching and returning to the ER, subsequently packaged into transport vesicles in a Secretion Associated Ras Related GTPase 1A (SAR1A)-dependent way. Additionally, we successfully affinity purified the vesicles enriched with SURF4 or TMED10. Quantitative mass spectrometry analyses indicate that SURF4-enriched vesicles and TMED10-enriched vesicles contain distinct group of cargo clients. Together, these results support a model in which ERES are heterogeneous: SURF4 and TMED10 segregate into distinct ERES populations to recruit different cargo cohorts for coat protein complex II (COPII) vesicle packaging.

B214/P0792

The C. elegans WASH complex supports transport and microtubule function.

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In C. elegans, only three Nucleation Promoting Factors (NPSs) that regulate branched actin through Arp2/3 have been identified, WAVE, WASP and most recently, WASH. We analyzed the biological functions of the orthologs of the C. elegans WASH complex. The WASH complex is similar to the WAVE complex, in that both complexes include 5 paralogous components. Surprisingly, only 4 of the 5 components had been identified in C. elegans, and the role of WASH in transport had not been described. We used existing mutations, RNAi and CRISPR to determine the effects of loss of the WASH complex, focusing on two epithelial tissues: the adult intestine and the embryonic epidermis. We used two phenotypic assays to test the role of WASH components, including a candidate for the missing 5th WASH component, the FAM21/WSHC-2 proposed ortholog, CO5G5.2. (1) Using assays for protein transport, we showed that loss of any WASH component, using mutations or RNAi, resulted in the same phenotype: defective transport and accumulation of cargo on RAB-5 and PI(3)P-positive early endosomes. Other recycling endosomes were also significantly altered, and cargo was missorted towards late endosomes. These results suggests C. elegans WASH regulates sorting during endocytic recycling, through retrograde trafficking to the Golgi apparatus. (2) Using assays for embryonic development, we showed that loss of WASH components resulted in embryos that die during morphogenesis, and some that died during early cell divisions. Therefore, in contrast to loss of WASP or WAVE, which are needed during morphogenesis, loss of WASH resulted in some embryos that died before morphogenesis begins, due to cell cycle arrests. Both alpha and beta-tubulin::GFP expression was strongly diminished in these embryos. It is known that the WASH complex assembles at Centrosomes, so that Centrosomes support WASH. We therefore investigated if WASH feeds back on Centrosome function, to address the effect of WASH on microtubules (MTs). Thus, C. elegans has a conserved, 5-member WASH complex that appears to function, like its orthologs, in early endosome to Golgi retrograde transport. In addition, C. elegans provides a great system to investigate how the WASH complex coordinates MT-dependent events during early embryonic development.

B219/P0797

The Yeast Homolog of CTNS is a Golgi Protein and is Linked to Redox Homeostasis Independent of Cystine Transport

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Cystinosis is an inherited disorder caused by mutations in the highly conserved CTNS gene, which encodes the lysosomal cystine transporter cystinosin (CTNS). Our current understanding of CTNS function is limited to its role in lysosomal cystine transport. However, restoration of cystine export at the lysosome is insufficient to fully alleviate disease symptoms, suggesting that CTNS may have additional cellular functions. Here, we show using fluorescence microscopy and protein interactome analysis that Ers1, the budding yeast homologue of CTNS, is localized at the early Golgi instead of the vacuole membrane. Ers1's function at the Golgi is further supported by physical and genetic interactions with early Golgi recycling machinery components. Using metabolomics and electrophysiology assays, we find that Ers1 lacks cystine transport activity but exhibits strong genetic interaction with Golgi redox homeostasis genes. Furthermore, we show that a splicing variant of CTNS, CTNS-LKG, also localizes to the Golgi in mammalian cells. Together, our findings suggest a novel cystine-

independent role of Ers1/CTNS-LKG at the Golgi, with implications for understanding the full spectrum of cystinosis pathology.

B229/P0807

Unconventional secretion of the thrombopoietin receptor reveals distinct Rab 1A and Rab 6-dependent traffic in myeloproliferative neoplasm

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Thrombopoietin receptor (TpoR), which plays an indispensable role in the maintenance of hematopoietic stem cells and production of platelets, is known to be secreted through the Golgi-dependent pathway. However, TpoR has also been reported to localize in lysosomes, but whether the observed localization was indicative of degradation or a true secretion event is yet to be explored. To this end, we fused the pH-sensitive FRET pair of TOLLES-YPet with TpoR (SRAI-TpoR) that showed FRET quenching inside the lumen of lysosomes. Using this construct, we demonstrated the presence of quenched SRAI-TpoR on the cell surface, indicating that TpoR can indeed be secreted through low-pH compartments in the presence of JAK2 V617F. We also observed a decrease in the ratio of Endo H-resistant to sensitive cell surface TpoR, suggesting that TpoR trafficked through the autophagosome-lysosome pathway in the presence of JAK2 V617F. Interestingly, when the unconventional secretion pathway of TpoR was blocked using Bafilomycin A1 or ATG5 deficiency, the receptor followed the Golgi-dependent pathway. Furthermore, the traffic of TpoR in the presence of CALR mutants was seen to be strictly Golgi-dependent. Also, we observed a partial Golgi-independent traffic of TpoR W515L, where FRET-quenched puncta and Endo H-sensitive TpoR were both found on the surface. Next, we examined whether we could dissect the two secretion routes using specific Rabs. Here, Rab 1A appeared to be important for autophagosome-lysosome-mediated traffic of TpoR, while Rab 6 marked the Golgi-dependent secretion. Taken together, we showed that in the presence of JAK2 V617F, the Rab 1A-mediated unconventional traffic was preferred over the Rab 6-mediated Golgi-dependent secretion pathway. TpoR W515L utilized both Rab 1A and Rab 6-mediated traffic. Finally, the CALR mutations restricted the TpoR to the Golgi-dependent traffic marked by Rab 6. This data has been published recently (https://doi.org/10.1038/s41375-025-02676-6).

B224/P0802

Uncovering Cargo Clients and Accessory Factors of AP-1 and AP-4 through Vesicle Proteomics

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The trans-Golgi network (TGN) is a crucial sorting station in the secretory pathway, where adaptor protein (AP) complexes ensure selective cargo packaging into transport vesicles. However, the complete repertoire of cargoes and regulators associated with individual AP complexes remains poorly defined. Intriguingly, AP-4-mediated TGN export operates independently of clathrin, suggesting the involvement of uncharacterized accessory factors in vesicle biogenesis. To address these gaps, we developed an in vitro vesicle formation assay using wild-type HeLa cells or cells deficient in AP1γ1 or AP4ε, reconstituting their roles in packaging their known clients, Vangl2 and ATG9A, respectively. Coupling this assay with label-free quantitative mass spectrometry, we mapped distinct cargo profiles for AP-1 (which buds from the TGN and ARF1-positive endosomes) and AP-4, identifying the 45 kDa calcium-binding protein (CAB45) as an AP-1-dependent cargo and

the Type-1 angiotensin II receptor-associated protein (ATRAP) as an AP-4-dependent cargo. Additionally, we uncovered PRRC1 and WDR44 as novel cytosolic regulators essential for AP-4-mediated TGN export. Our study advances the mechanistic understanding of AP-1 and AP-4 in secretory trafficking and provides a robust strategy to systematically identify cargo clients and accessory factors for specific adaptor complexes.

B226/P0804

Using the C. elegans germline as a model for dissecting the regulation of lipid metabolism in complex tissue maintenance.

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The nuclear envelope is a part of a contiguous membrane system with the endoplasmic reticulum, the major site of lipid metabolism. Lipid precursors produced in the endoplasmic reticulum must be enzymatically modified to ensure proper membrane synthesis and storage. How cells can maintain the appropriate balance of membrane lipids and storage lipids remains to be fully investigated. To test this critical branching point, I am using the C. elegans germline, a complex tissue that forms as a syncytium containing 1000 nuclei. Using endogenous fluorescent tags, I found that key regulators of lipid synthesis and storage localize in a non-uniform pattern across germline nuclei, suggesting different requirements for their functions across the syncytial structure. In particular, the disease-associated lipid storage protein Seipin appears localized at the nuclear envelope in the proliferative zone of the germline, otherwise known as the stem cell niche. This region is also devoid of lipid droplets, indicating a potential local role for Seipin in the regulation of lipid synthesis or storage at the nuclear membrane, possibly to protect these specialized nuclei. Preliminary analysis indicates that Seipin, as well as other regulators of lipid storage, are required for the maturation and organization of the germline. Future work will determine how the distribution of lipid metabolism regulators is related to metabolic networks that specify and maintain cell states, as well as the overall morphology of this complex tissue, both in healthy and ‘stressed’ conditions, such as aging.

B218/P0796

Vps41 and Yck3 Facilitate the Disassembly of the Myo2-Vac17-Vac8 Complex to Release the Vacuole at its Correct Destination

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Intracellular trafficking is tightly regulated to ensure correct delivery of cargoes and other molecules to various cellular locations. The transport of some cargoes is provided by myosin V, an actin-associated motor protein. In Saccharomyces cerevisiae, Myo2 is the myosin V motor responsible for transporting vacuoles, secretory vesicles, mitochondria, peroxisomes, and lipid droplets to the bud during polarized growth. For vacuole transport, Myo2 forms a complex with Vac17, an adaptor protein, and Vac8, a vacuole membrane protein. The initiation and termination of vacuole transport are coordinated by proteins acting on Vac17 to promote its incorporation into the Myo2-Vac17-Vac8 complex or its extraction from the complex and degradation. For initiation, Cdk1 phosphorylates Vac17, promoting its association with Myo2, while Kin4 and Frk1 prevents premature degradation of Vac17 in the mother cell. During termination, Cla4 at the bud cortex phosphorylates Vac17, which activates the E3 ligase Dma1 to ubiquitylate Vac17 for degradation. Additionally, Vps41 and Yck3

are necessary for the termination of vacuole transport and likely act in parallel with Dma1 and Cla4. Cells without Vps41 or Yck3 are defective in the release of the vacuole from the Myo2-Vac17-Vac8 complex and in the degradation of Vac17. However, how Vps41 and Yck3 facilitate the release of Vac17 from the Myo2-Vac17-Vac8 complex is unknown. Vps41 is best characterized as a subunit of the HOPS tethering complex, but other HOPS subunits are not required for the release of Vac17 from the Myo2-Vac17-Vac8 complex. To gain insights into Vps41's role in vacuole release, we generated a series of truncation mutants targeting functional domains of Vps41 and found that the C-terminus of Vps41 was necessary for both HOPS functions and vacuole release. These findings suggest that Vps41 outside of HOPS is regulated to use the C-terminus to facilitate disassembly of the Myo2-Vac17-Vac8 complex.

B212/P0790
VTIA regulates protein targeting to the vacuole, vacuole morphology and polarized growth
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SNARE proteins (soluble N-ethylmaleimide-sensitive attachment protein receptors) are critical for membrane fusion. V-SNARES travel with the vesicle and interact with T-SNARES on the target membrane, enabling fusion of the vesicle with a specific target membrane. VTI (VPS (Vacuole Protein Sorting) 10-interacting) SNARES have been implicated in endolysosomal trafficking. Trafficking to the vacuole, the lysosome of plants, is complex given that distinct classes of vacuoles - lytic and storage - coexist in the same cell. In the model plant, Arabidopsis thaliana, the VTI family (VTI11, VTI12 and VTI13) is involved with nonredundant processes like shoot growth, autophagy and root hair development. However, the extent to which these functions are conserved in other plant lineages is unclear. Here, we have taken advantage of the moss Physcomitrium patens, whose tissues are only a single cell layer thick, allowing us to easily visualize the endomembrane system. We used CRISPR/Cas9 to knock out the four VTI genes in a background with a fluorescent vacuole membrane marker. We found that Δvtia plants grow significantly slower than wild-type and have abnormal vacuolar morphology. Instead of the elongated tubular vacuoles found in wild-type plants, Δvtia mutants have large spherical vacuoles that are often very close to the tip of the cell. The Δvtia mutant also had increased levels of the vacuole marker, indicating more of the protein was accumulating on the vacuole membrane. In contrast to Δvtia, the Δvtib, Δvtic and Δvtid mutants grew similar to wild-type and had normal vacuole morphology. Endogenous tagging of VTIA revealed that VTIA localizes to both small puncta near vacuoles and the vacuolar membrane, as well as to larger puncta distributed throughout the cell. Given that VTI11 and VTI13 in Arabidopsis have been found on multivesicular bodies (MVBs), or prevacuolar compartments, we tested whether VTIA localized to MVBs. However, treatment with Wortmannin, which is known to cause MVBs to rapidly fuse into donut-shaped structures, had no effect on VTIA localization, suggesting that VTIA does not reside on MVBs. Based on these findings, VTIA is a key regulator of vacuole morphology, protein targeting to the vacuole and polarized cell growth in P. patens.

Neuronal Cell Biology - Glial cell biology

B179/P1305
Catechin hydrate promotes GFAP expression and elongates the branches of astrocytes in rat neural stem cells
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Catechin hydrate (CH) is a flavonoid family and exists in green tea. In the current study, the effect of CH on NSC fate control has been explored . CH enhanced not only mRNA but also protein expression of glial fibrillary acidic protein during NSC differentiation. However, the number of astrocytes was not significantly altered when determined by immunostaining. CH induced leukemia inhibitory factor, and bone morphogenetic protein mRNA expression and up-regulated phosphorylation of STAT3 and SMAD1/5/8 in differentiated cells. CH enhanced fibroblastic growth factor 2 mRNA expression and induced thin and elongated branches in astrocytes. Our results suggest that although CH does not increase the number of astrocytes, it regulates astrocytes to become longer during NSC differentiation.

B181/P1307

CLEC7A Deficiency affects Cerebellar Microglia development and Purkinje Cell Axon Myelination

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Proliferative region-associated microglia (PAM) are a subpopulation of microglia in the white matter region (WMR) of the brain during postnatal development. This type of microglia transiently and highly expresses a specific group of genes that includes Clec7a, Spp1, and Apoe. Expression of these genes is not found in healthy adult microglia. However, these genes are re-expressed in activated microglia associated with neurodegeneration. In the current project, we are studying CLEC7A deficient mice (Clec7a-/-) to investigate the role of this protein in the differentiation, maturation, and function of microglial cells during the postnatal development of the cerebellum. Our results showed that at postnatal day 7 (P7), when myelination is normally starting in the cerebellum, the number of WMR microglia was decreased along with the decreased proliferation (Ki67+ microglia) of these cells in Clec7a-/- mice compared to wild-type (WT) mice. The morphology of CLEC7A deficient microglia was not different from WT cells, however IBA1 immunoreactivity was higher in Clec7a-/- mice. To identify gene expression changes in cerebellar PAM cells from P7 CLEC7A deficient mice, probes for Spp1 and Apoe were used to perform RNAscope experiments. Interestingly, in the WT and Clec7a-/- mice, not all the PAM cells co-expressed Spp1 and Apoe. Furthermore, the majority of Spp1+ cells had lower levels of Apoe expression. We also found that the number of Spp1+ PAM cells and the fluorescence intensity of Spp1 and Apoe were significantly reduced in Clec7a-/- mice compared to controls. Importantly, myelination of Purkinje cells (PC) axons was significantly decreased in P10 Clec7a-/- mice. Since the refinement of Purkinje cell (PC) axonal collaterals occurs during early postnatal development (P7-P15) and is critical for proper myelination, we investigated whether this process is disrupted by the absence of CLEC7A in microglia. We observed a greater number of unmyelinated axons in Clec7a$^{-/-}$ mice compared to wild-type controls. Additionally, Clec7a$^{-/-}$ mice exhibited motor deficits in the ladder rung walking task, suggesting that microglial CLEC7A contributes to neuronal function. Ongoing studies are focused on analyzing microglial interactions with these axons to determine whether CLEC7A is involved in axonal refinement. Together, our findings indicate that CLEC7A deficiency in microglia impairs not only microglial maturation but also the myelination of PC axons.

B178/P1304

Dorsal Root Ganglia Neuron-Satellite Glia Interactions in Response to Physiological Levels of Shear Stress

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Dorsal root ganglia (DRG) contain cell bodies of neurons innervating skin, muscle and viscera that transduce and transmit peripheral pain, touch and proprioceptive sensory signals. These primary sensory neurons, and the satellite glial cells (SGCs) that envelope them, are subjected to diverse mechanical forces as a result of interstitial fluid flow, tissue deformation or injuries. We have applied intracellular Ca2+ imaging and electrophysiology methods to evaluate the mechanical responses of dissociated mouse DRG cultures and to investigate molecular mechanisms mediating this sensitivity. We found that both DRG neurons and SGCs exhibited detectable Ca^{2+} influx in response to physiologically low levels of shear stress (0.6 to 9.6 dynes/cm2). Concentration-dependent Ca2+ responses were also observed when DRG and SGCs were stimulated with Yoda1 (2.5 to 40 μM), a potent and selective agonist of the mechanosensitive channel Piezo1. Notably, shear-induced Ca^{2+} responses were markedly potentiated by Yoda1 (5 μM). These responses were further amplified by pharmacological co-stimulation with 4α-phorbol-12,13-didecanoate (PDD, 10 μM), a potent and selective TRPV4 Ca2+ channel agonist, indicating mechanistic interplay between Piezo1 and TRPV4. Electrophysiological studies revealed that Yoda1 (1 to 10 μM) decreased neuronal excitability, reducing action potential generation during current ramps while leaving membrane resistance unchanged. Our findings demonstrate that (1) DRG neurons and SGCs respond to mechanical forces imposed by interstitial fluid flow, and (2) Piezo1 and TRPV4 modulate the sensitivity of both DRG neurons and SGCs to shear stress. Due to the narrow space between neuron and SGCs, it is likely that interstitial fluid shear stress only directly impacts the SGCs. The high level of Piezo1 expression in SGCs and the mechanical insulation they provide to neurons suggest that these glial cells may serve as primary mediators of mechanosensory transduction within the DRG and could represent a novel cellular target to modulate abnormal sensory neuron excitability. Funding sources: NIH DK138832

B186/P1312

Glial Parkin Inhibits Developmental Activity-Dependent Neuron Pruning via Rac1 and Jun Kinase

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Parkinson's Disease (PD) is the second-most common and fastest-growing neurodegenerative disease globally. Genes linked to PD are expressed in both neuronal and glial cells of the nervous system; however, their molecular functions in glia remain understudied. Here, we report our discovery that PD-linked genes act in glia to regulate the conserved glial function of engulfing neuron-endings. Glial cells play critical roles in nervous system development and function and are increasingly implicated in neurodegeneration. The engulfment, or pruning, of neuron receptive endings (NRE) is a critical glial function. This contributes to the refinement of developmental neural circuits and learning & memory. While dysfunctions in glial engulfment are linked to Alzheimer’s neurodegeneration, the roles of glia or glial engulfment in PD remain unclear. We previously discovered glial engulfment in C. elegans, establishing this powerful genetic model as a platform to investigate engulfment with single-cell precision. Focusing on a single neuron-glia pair (AFD neuron - AMsh glia), we found that glia actively and dynamically modulate engulfment via glial CED-10/Rac1 GTPase. This impacts the AFD-NRE shape and associated animal behavior. From a genetic screen to identify additional regulators, we uncovered that the PD-associated gene PDR-1/PRKN regulates AMsh glial engulfment of AFD-NRE. Distinct from PRKN’s known neuronal role as an E3 ligase in PINK-1/PINK1/PARK6-mediated mitophagy, we identify that the ligase-deficient isoform PDR-1B localizes to a glial nanodomain adjacent to neuronal activity-driving ion channels. There, PDR-1B controls activity-dependent engulfment during the animal’s critical developmental period via CED-10/RAC1 and KGB-1/JNK. Finally, while PDR-1/PRKN regulates the developmental onset of engulfment at the juvenile-adult developmental transition, young adult animals exhibit behavioral defects, and aged adult animals also show AFD-NRE shape defects. We also identified the conservation of this glial function of PDR-1/PRKN in human glia and glia-like cells. Finally, we found multiple PD-linked genes that

regulate engulfment, including PINK-1/PINK, LRK-1/LRRK2, and DJR-1.2/DJ-1. All these genes are also expressed in mammalian glia, suggesting that analogous mechanisms may be conserved and drive the progression of human PD. Thus, our discovery that multiple PD genes regulate glial engulfment identifies this critical glial function as a core pathway in PD biology.

B180/P1306

Microtubule-caged phagosomes enable microglial size-selective clearance of large cellular targets

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How phagocytes overcome intrinsic size constraints to eliminate large and complex cellular substrates—a process fundamental to tissue homeostasis and disease modulation—remains poorly understood. Here, we identify a previously unrecognized, microtubule-mediated, size-selective macrophagocytosis in which microglia assemble distinctive cage-like microtubule architectures around phagosomes to engulf bulky targets, including stressed yet viable dopaminergic neurons in Parkinson's disease (PD) models. Integrated proteomic and single-cell transcriptomic analyses reveal that the microtubule-associated proteins CLIP1 and BIN1 coordinate cytoskeletal remodeling essential for the formation of large phagosomes. Strikingly, microglia-specific deletion of CLIP1 or BIN1 preserves vulnerable neuronal populations and improves motor function in PD models, while leaving the clearance of smaller α-synuclein aggregates unaffected. These findings uncover a structural mechanism that enables microglia to transcend intrinsic phagocytic size limits, providing a mechanistic basis for scale-adaptive phagocytosis and a conceptual framework for cytoskeletal remodeling during development, homeostasis, and neurodegeneration.

B182/P1308

PINK1/Parkin-dependent mitophagy mediates astrocytic inflammatory responses to mitochondrial damage

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Astrocytes directly influence neuronal survival and increasingly are understood to contribute to progression of neurodegenerative diseases, including Parkinson's disease (PD). Mitochondrial damage is a hallmark of PD pathology in both neurons and astrocytes. Damaged mitochondria are cleared by PINK1/Parkin-mediated mitophagy; loss-of-function mutations in either PINK1 or Parkin are sufficient to cause PD. Neuronal mitophagy is well-studied, but far less is known about the mechanisms by which astrocytes clear damaged mitochondria or how mitochondrial dysfunction in astrocytes affects neural health. To address these questions, we treated primary murine cortical astrocytes with oxidative phosphorylation (OXPHOS) inhibitors antimycin A (AA) and oligomycin A (OA). We observed PINK1-dependent accumulation of Parkin on damaged mitochondria, leading to phospho-ubiquitination of proteins in the outer mitochondrial membrane and the recruitment of the autophagy receptor p62/SQSTM1. To identify transcriptional changes caused by mitochondrial damage and the resulting activation of mitophagic machinery, we performed bulk RNA-sequencing on astrocytes isolated from WT, PINK1-/-, or Parkin-/- mice treated with AA/OA or a vehicle control. In WT astrocytes, TNF-α signaling via NF-κB was the most significantly upregulated pathway following OXPHOS inhibition. NF-κB can stimulate p62 production; in line with this, p62 levels increase following AA/OA treatment compared to the control, and NF-κB inhibition prevents this upregulation. This finding suggests NF-κB activation in astrocytes may enhance

clearance of damaged mitochondria. Astrocytic secretion of cytokines, including TNF-α, increased following mitochondrial damage; this secretion was decreased by NF-κB inhibition. Compared to WT astrocytes, PINK1-/- astrocytes showed a significant reduction in transcriptional signatures associated with TNF-α signaling following mitochondrial damage, while Parkin-/- astrocytes exhibited upregulation of both IFN-γ and IFN-α signaling. These findings indicate altered inflammatory responses to mitochondrial damage in the absence of functional PINK1 or Parkin. Finally, we analyzed scRNA-sequencing data for substantia nigral astrocytes harvested from human brain tissue from PD-positive or control samples. Distinct clusters comprised predominantly of PD-positive or control astrocytes emerged. Astrocytes in the PD-positive cluster were enriched for IFN-α and IFN-γ responses and NF-κB signaling, consistent with signaling observed in vitro post-OXPHOS inhibition. Together, these findings identify inflammatory signatures activated by mitochondrial damage in astrocytes, and establish this pathway as a potential contributor to neuroinflammation in PD.

B185/P1311

Role of Microglia on Dendritic Spine Density in Dentate Gyrus Granule Cells After Cerebral Ischemia

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Microglia contribute to the formation of functional neural circuits by pruning unnecessary synapses in an activity-dependent manner. The hippocampal dentate gyrus (DG) in our middle cerebral artery occlusion (MCAO) mice shows neuronal hyperactivity within a few hours after surgery. Thus, we evaluated whether microglia contribute to changes in synaptic density of DG granule cells after MCAO. DG granule cells were filled with Lucifer Yellow to visualize dendritic spines that receive excitatory synaptic inputs. In MCAO mice, dendritic spine density was lower compared to Sham at postoperative hour (POH) 6. However, microglial depletion via administration of PLX3397 prevented the MCAO-induced loss of dendritic spines. The density of microglia in the molecular layer of the DG did not differ between MCAO and Sham mice. MCAO mice exhibited an increase in the volume of CD68-positive lysosomes within microglia compared to Sham, suggesting an upregulation of dendritic spine engulfment. However, the number of PSD95-positive dendritic spines within CD68 of microglia did not differ between MCAO and Sham mice. Cultured microglia were challenged with DiO-labeled synaptosomes isolated from hippocampus at POH6. Microglia preferentially phagocytosed synaptosomes from Sham mice compared to MCAO. Synaptosomes obtained from POH6 MCAO mice were derived from synapses that had escaped elimination and exhibited resistance to microglial phagocytosis. By POH6 in vivo, microglia had likely already phagocytosed synapses that were susceptible to elimination. Nevertheless, the number of PSD95-positive dendritic spines engulfed by microglia did not differ between Sham and MCAO mice. Previous studies have indicated that the existence of PSD95-negative dendritic spines. We propose that microglia preferentially phagocytose PSD95-negative dendritic spines after MCAO, thereby contributing to the overall reduction in the number of dendritic spines.

B183/P1309

Roles of Microglia Derived from Tissue Stem Cells in the Infarct Area

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Cerebral ischemia causes tissue necrosis, resulting in the formation of an infarct area. Tissue regeneration within the infarct area is limited because of the restricted capacity for stem cell induction in the central nervous system. However, we have demonstrated that ischemia-induced multipotent stem cells (iSCs) are generated within the infarct area, and that these iSCs differentiate into microglia. In this study, we investigated the roles of iSC-derived microglia in the infarct area using a permanent middle cerebral artery occlusion (MCAO) mouse model. To characterize iSC-derived microglia, conventional microglia and iSC-derived microglia were magnetically isolated, and their transcriptomes were compared. iSC-derived microglia exhibited higher expression of vascular development–related genes than other types of microglia. Histological analysis revealed increased numbers of CD31- and Ki67-positive proliferating endothelial cells, as well as increased vascular volume, within the infarct area. By contrast, depletion of iSC-derived microglia using PLX3397, a colony-stimulating factor 1 receptor inhibitor, suppressed MCAO-induced vascular changes in the infarct area. To further investigate vascular function, fluorescently labeled dextran was injected into the infarct area. We confirmed its translocation into the vascular wall and lumen. Mass spectrometry imaging revealed that lipids in the infarct area decreased over time, whereas PLX3397 treatment inhibited this lipid reduction. Notably, infarct size at 7 days after MCAO was larger in PLX3397-treated mice than in control mice, despite no difference at 3 days after MCAO. These findings suggest that iSC-derived microglia promote vascular remodeling within the infarct area, thereby contributing to efficient clearance of infarct tissue via the vasculature.

B184/P1310

The Effect of Arcadlin on Dendritic Spine Phagocytosis by Microglia After Cerebral Ischemia

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Dendritic spines receive excitatory inputs. Microglia selectively phagocytose dendritic spines by recognizing “eat me” signals presented on their surface. We have identified that microglia reduce the number of dendritic spines in the hippocampal dentate gyrus (DG) after middle cerebral artery occlusion (MCAO), although the underlying mechanism remains unclear. Arcadlin (Acad), also known as protocadherin-8, is a type I transmembrane protein and a regulator of dendritic spine number. Furthermore, Acad is upregulated in the DG after MCAO. We therefore hypothesized that Acad contributes to the dendritic spine elimination mediated by microglial phagocytosis. In this study, we investigated the role of Acad in microglial dendritic spine phagocytosis after MCAO using wild-type (WT) and Acad knockout (Acad KO) mice. Acad protein expression in DG synaptosomes peaked 6 hours after MCAO, prompting us to examine the DG at this time point. The degree of MCAO-induced dendritic spine loss was comparable between WT and Acad KO mice. However, the proportion of large spines was increased in the MCAO group of Acad KO mice. These results suggest that the types of dendritic spines phagocytosed by microglia may differ between WT and Acad KO mice. We then examined microglial dendritic spine phagocytosis in WT and Acad KO mice. PSD95-positive signals located in CD68-positive lysosomes within Iba1-positive microglia were counted as dendritic spines phagocytosed by microglia. The number of PSD95 signals engulfed by microglia was significantly increased by MCAO in Acad KO mice, but not in WT mice. These results suggest that Acad inhibited the microglial phagocytosis of PSD95-positive dendritic spines.

Neuronal Cell Biology - Intracellular trafficking and organelle dynamics

B190/P1316

A novel role for Presenilin-2/γ-secretase in neuronal homeostasis: link to Alzheimer's pathogenesis

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Mutations in the gene encoding Presenilin-2 (PSEN2), a catalytic component of γ-secretase, cause familial Alzheimer's disease (FAD); yet the molecular mechanisms underlying this pathology remain poorly understood. Endolysosomal and mitochondrial dysfunctions are well-established early features of Alzheimer's disease (AD), preceding β-amyloid (Aβ) deposition and synapse loss. However, these two processes have largely been studied in isolation. We showed previously that PSEN2/γ-secretase has a restricted localization to late endosomes/lysosomes (LE/Lys), in contrast to PSEN1/γ-secretase that resides at the cell surface and in endosomes. In this study, we identify a novel role for PSEN2 in regulating organelle homeostasis in neurons, and which involves the more recently described function of LE/Lys as hitchhiking platforms for RNA granules and localized protein synthesis in axons. To investigate the role of PSEN2 in this context, we generated new iPSC-derived induced human neurons (iNeurons) that are either PSEN2 deficient (PSEN2KO) or express an FAD-linked mutant variant (FADPSEN2) and cultured them in microfluidic devices. We found that PSEN2 disruption impairs LE/Lys trafficking, increases their contact time with mitochondria, and triggers mitochondrial morphological abnormalities, as observed by electronic microscopy. These defects were recapitulated in primary hippocampal neurons underscoring conserved underlying mechanisms. Transcriptomic analysis of isolated axons and somadendritic compartments revealed a selective upregulation of over 500 transcripts in axons of PSEN2KO and FADPSEN2 human neurons compared to wild-type; herein, mitochondrial-related processes were among the top enriched gene ontology terms. We further demonstrate elevated local protein synthesis, consistent with the transcriptomic profile. Notably, herein, some of these translation events colocalized with LE/Lys–mitochondria contact sites. Further, we observed a reduction in axonal RNA granules in both PSEN2KO and FADPSEN2 neurons. Therefore the lower association of RNA with granules could potentially provide more free mRNA accessible to ribosomes, aligning with the increased translation we observed. Altogether, our results position PSEN2 as a critical link between endolysosomal dynamics and mitochondrial function, through regulating local translation. Altered expression of PSEN2 affects lysosomal delivery and motility, and as such local translation required for mitochondrial health. Our findings provide a mechanistic explanation for the convergence of lysosomal and mitochondrial defects, offering new insights into Alzheimer's disease pathogenesis.

B198/P1324

An Extracellular Vesicle-based Strategy for Targeted Fluorescent Protein Delivery in Neurons

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Synaptobrevin-2 (Syb2) is a SNARE protein that mediates neurotransmitter release in the nervous system. Our lab found that Syb2 is also exchanged among neurons via extracellular vesicles (EVs). Host neurons can rapidly incorporate exogenous Syb2 into their synaptic vesicle cycle to support neurotransmitter release. Here, we use

a fusion of Syb2 with the pH-sensitive GFP (Syb2-pHluorin) to track the incorporation of Syb2-containing EVs into neurons and the trafficking of exogenous Syb2 to synaptic vesicles at synapses. We determined that Syb2-containing EVs are endocytosed via dynamin- and clathrin-independent pathways. After endocytosis, exogenous Syb2 is transported to axons and synapses, and incorporates into functional synaptic vesicles. These Syb2-pHluorin positive synaptic vesicles are endocytosed with either ultrafast (<1s) or fast (~1-3s) kinetics during synaptic transmission, suggesting limited diffusion and high fidelity in the fast retrieval of synaptic vesicle molecules immediately after fusion. This work introduces a novel application of EVs as vehicles to deliver fluorescent molecules in a neuron-specific, targeted manner to investigate protein transport mechanisms without overexpression artifacts. Our findings expand our understanding of the mechanisms EVs use to enter neurons; future work will be aimed at elucidating the specific surface signals that mediate EV binding and recognition of target neurons.

B193/P1319
Analysis of IRBIT as a Regulator of Phosphoinositide Metabolism
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Changes in the levels of phosphoinositides in neurons are associated with a wide variety of cellular responses such as alterations of action potential firing patterns and neuronal plasticity. One critical component of this signaling system is the release of calcium ions from the endoplasmic reticulum through inositol 1,4,5-trisphosphate (IP3) receptors. Over the last couple of years, inositol 1,4,5-trisphosphate receptor-binding protein released with inositol 1,4,5-trisphosphate (IRBIT) has emerged as a regulator of calcium release through IP3-receptors. While IRBIT is expressed in a wide range of different tissues, its highest expression levels have been detected in the brain, indicating an important role for IRBIT in controlling neuronal activity. In order to characterize how IRBIT influences cellular activity, we ablated IRBIT in a human embryonic kidney cell line and performed a whole-genome expression analysis via RNA-Seq to identify genes that might be regulated through IRBIT-controlled calcium signaling. Our comparison of data obtained from wild-type and IRBIT-knockout human embryonic kidney cells revealed some of the highest changes in expression levels in genes encoding for proteins involved in phosphoinositide metabolism. Analysis of phosphoinositide levels via lipid extraction followed by mass spectrometry analysis showed altered ratios of phosphatidylinositol to phosphatidylinositol-bisphosphate species in IRBIT-knockout cells as well as changes in phosphatidylinositol-trisphosphate amounts. Our findings indicate an important role for IRBIT in controlling phosphosinositide metabolism. Research reported in this project was supported by an award from the National Institute of General Medical Sciences of the National Institutes of Health under grant number 1R15GM150377-01.

B199/P1325
Annexin A11 Co-Localizes with ALS/FTD-Associated RNA Binding Proteins in Healthy Neurons and Co-Aggregates with Transport Proteins in ALS/FTD Post-Mortem Brains
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Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia with TDP-43-immunoreactive pathology (FTLD-TDP) are fatal neurodegenerative disorders characterized by TDP-43 aggregation. Recently, our lab

discovered that a subset of patients with ALS and FTLD-TDP have co-aggregation of Annexin A11 (ANXA11) with TDP-43. ANXA11 is a protein that tethers RNA granules to lysosomes, allowing RNA granules to hitchhike on lysosomes undergoing microtubule-based transport. This transport maintains RNA distribution along axons and allows for regulated local translation, which is vital for synaptic function and mitochondrial health. It is still unknown which subtype of RNA granules ANXA11 transports and whether this mechanism contributes to aggregate formation in ALS/FTLD-TDP. To identify RNA binding proteins that interact with ANXA11 in an unbiased manner, I employed APEX-based proximity biotinylation in rat hippocampal neurons expressing N-terminally-tagged ANXA11 followed by mass spectrometry. Compared to a cytoplasmic APEX control, pull down from neurons expressing APEX-ANXA11 enriched for the ALS/FTD-linked RNA binding proteins TDP-43 and MATR3, suggesting biologically relevant interactions with ANXA11. I will validate these hits in rat hippocampal neurons and human iPSC-derived neurons using proximity ligation assays. To determine whether similar RNA binding proteins can be found in ANXA11 aggregates in human patients, I am using proximity biotinylation by antibody recognition (BAR) on post-mortem brain tissue from patients with ANXA11 aggregates. Initial experiments indicate enrichment for ANXA11 and proteins involved in vesicle-mediated transport and cytoplasmic translation. We anticipate that further definition of the mechanisms mediating ANXA11-mediated RNA transport and how this transport is affected in disease may shed light on the impact of RNA transport dysregulation in ALS/FTLD-TDP.

B196/P1322

Displacement of ER Luminal Proteins to the cytosol in Drosophila Atlastin mutant synapses

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The endoplasmic reticulum (ER) is a continuous organelle that extends throughout neurons and regulates many neuronal functions, including neurotransmission and synaptic plasticity. Mutations in proteins controlling ER shaping and organelle contacts cause Hereditary Spastic Paraplegia (HSP), a neurodegenerative disorder, highlighting the critical role of ER structure in neuronal function. However, the ultrastructure and dynamics of the neuronal ER remain under-investigated, particularly at presynaptic terminals. We used live super-resolution Airyscan microscopy in larval Drosophila melanogaster motor neurons to investigate presynaptic functions coupled to ER structure and dynamics. We examined null mutants of the HSP gene Atlastin (Atl), an ER-shaping protein that regulates ER tubule fusion and tethering. Using ER membrane markers, we found that the ER in Atl mutants forms robust networks with only mild dynamics defects, contradicting previous attributions of extensive ER fragmentation. However, luminal ER markers are displaced from the ER to the cytosol, representing a novel form of ER stress not previously described in neurons. The endogenous ER chaperone BiP is also found in the cytosol in Atl mutants, raising the possibility that displaced proteins could acquire cytosol-specific functions. Remarkably, this phenotype is synapse-specific and has not been observed in Atl loss-of-function studies in non-neuronal cells, emphasizing the importance of conducting experiments in bona fide neurons to understand upper motor neuron dysfunction in HSP. To explore functional consequences of ER dysfunction, we examined ER-endosome interactions, which are controlled by other HSP-linked genes and regulate endosome maturation and cargo recycling. Loss of Atl at presynaptic terminals altered the abundance and axonal trafficking of specific neuronal cargoes. We hypothesize that ER stress at presynaptic terminals may chronically disrupt endosomal traffic, potentially contributing to neuronal degeneration in HSP patients.

B187/P1313

Enhanced recruitment of lysosomal repair pathways in response to lysosomal damage in astrocytes versus neurons

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Lysosomal damage impairs proteostasis and contributes to many neurodegenerative diseases. In response to lysosomal membrane damage, cells can employ several mechanisms to restore lysosomal integrity. Recent proteomic profiling has revealed that lysosomal protein composition varies across cell types, including those in the brain, suggesting that lysosomes may have specialized properties depending on the cell type. However, cell-type-specific properties of lysosomes, particularly in their resilience to membrane damage, in neurons versus astrocytes is poorly understood. Here, we compare the response of neurons versus astrocytes to lysosomal injury induced by a lysosomotropic methyl ester (e.g., LLOMe), with a focus on three key pathways: ESCRT-mediated membrane repair, TBC1D15-mediated membrane reformation, and the PITT pathway to replenish lipids. To elucidate cell-type-specific responses, we use a neuron-astrocyte coculture system that recapitulates several morphological, proteomic, and functional signatures of neuron-astrocyte interactions in vivo. We first established functional evidence that LLOMe effectively induces lysosome damage in both neurons and astrocytes based on galectin-3 recruitment to luminal lysosomal b-galactosides, elevated lysosomal pH, and engagement of lysophagy receptors TAX1BP1 and p62. Next, we investigated the ESCRT-mediated repair pathway which utilizes membrane scission to repair small perforations in the lysosome membrane. Despite lysosomal damage occurring in both cell types, astrocytes showed a preferential recruitment of ESCRT repair machinery to damaged lysosomes, as compared to neurons. This enhanced ESCRT recruitment in astrocytes was observed with multiple components of the ESCRT pathway (e.g., CHMP2B, CHMP2A, ALIX and IST1). Moreover, enhanced ESCRT recruitment was conserved in astrocytes isolated from different brain regions (e.g., the hippocampus or cortex). Additionally, the lysosomal membrane reformation pathway regulated by the RAB7-GAP, TBC1D15, was also more robustly activated in astrocytes as compared to neurons. By contrast, the PITT pathway, detected with PI4K2A and ORP9, which mediates lipid shuttling between the endoplasmic reticulum and damaged lysosomes, was engaged in both cell types. Our data reveal a divergence in how neurons and astrocytes mobilize repair pathways to manage lysosomal damage. These data may reflect differences in lysosomal resilience between astrocytes and neurons and inform therapeutic strategies to correct lysosomal dysfunction in neurodegenerative diseases.

B195/P1321

Mapping the MIRO1-TRAK1 interaction that regulates mitochondrial trafficking in neurons

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Mitochondria are actively transported along the microtubule cytoskeleton by the molecular motors kinesin-1 and cytoplasmic dynein. Kinesin and dynein-dynactin are linked to mitochondria by TRAK proteins, which bind MIRO on the outer mitochondrial membrane (OMM). In neurons, mitochondrial transport throughout axons and dendrites is required to meet local energy demands. Defective mitochondrial trafficking is implicated in neurodegenerative and developmental disorders, underscoring the importance of proper mitochondrial distribution for neuronal function. Here, we map the MIRO1-TRAK1 interaction to two distinct sites within TRAK1 using biochemical, cellular, and structural approaches. We localized Site-1 to TRAK1425-428. Targeted

mutagenesis of 425IPGS428 demonstrates this motif is sufficient for MIRO1 binding in vitro and MIRO1-mediated recruitment of TRAK11-440 to mitochondria in cells. AlphaFold3 predicts Site-1 residues 425IPGS428 form a loop that inserts into a pocket between MIRO1's cGTPase and ELM2 domains. Mutation of MIRO1 D334K, a key residue in this pocket, disrupts the interaction. We mapped a second interaction site to TRAK1569-623 (Site-2) and determined a cryo-EM structure of a Site-2 peptide-MIRO1 co-complex. Two highly conserved residues within TRAK1, W589 and L597, insert into hydrophobic pockets within MIRO1's nGTPase and ELM1 domains, respectively. Mutation of either residue ablates MIRO1 binding to Site-2 in vitro, while compound mutations significantly reduce mitochondrial localization of TRAK11-640 in cells. Mutation of Site-1 within the context of a longer TRAK11-640 construct did not affect mitochondrial recruitment, suggesting Site-2 serves as the primary driver of the interaction. Of note, both Site-1 and Site-2 interactions are independent of MIRO1's nucleotide- and calcium-binding states. Overall, these findings provide a molecular model of the MIRO1-TRAK1 interaction at the OMM: MIRO1 forms a dimer facilitated by TRAK1, which binds MIRO1 at two distinct sites. These sites differentially contribute to TRAK1's mitochondrial localization and likely influence mitochondrial trafficking in neurons, a possibility we are now exploring.

B197/P1323

Modeling and Rescue of KIF1A-Dependent Axonal Transport Defects in C. elegans.

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Mutations in the kinesin motor protein KIF1A cause severe neurodevelopmental and neurodegenerative disorders, largely due to impaired axonal transport. However, the genetic pathways that regulate or compensate for these defects remain poorly understood.To identify genetic modifiers of KIF1A-dependent axonal transport, we performed a forward genetic screen in Caenorhabditis elegans. We discovered a mutation that alleviates axonal transport defects and partially restores locomotor function in a worm model of KIF1A-related neurological disease. Further analysis suggests that this genetic modifier may act through pathways involving other motor proteins.Our findings highlight a previously unrecognized genetic interaction that can improve axonal transport defects caused by KIF1A dysfunction. This work provides a foundation for dissecting compensatory mechanisms and may inform therapeutic strategies for KIF1A-related disorders.

B192/P1318

Modulating WIPI2 and DIRAS1 to rescue neurodegeneration in Caenorhabditis elegans dopaminergic neurons

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Aging is accompanied by a decline in neuronal function which can lead to neurodegeneration. This is associated with neurodegenerative diseases (NDDs), such as Parkinson's and Alzheimer's diseases. Studies have shown that autophagy, a degradative pathway involved in maintaining cellular proteostasis, declines with age and becomes dysregulated in NDDs. Neurons are post-mitotic and long-lived cells that rely heavily on autophagy. Although autophagy declines with age in neurons, little is known about autophagy's contribution to the onset of neurodegeneration. In this study, we used C. elegans to examine how neuronal autophagy contributes to the neurodegeneration of dopaminergic Cephalic (CEP) neurons during aging. C. elegans CEP neurons naturally degenerate during aging. We hypothesized that manipulating two early components of the autophagy pathway could postpone the age-associated neurodegeneration of CEP. First, we examined WIPI2, a

critical component for autophagosome formation. The Stavoe lab previously found that overexpression of a phosphodead mutant of WIPI2 restores the age-related decline of autophagy in murine neurons. Additionally, preliminary data indicate that phosphodead WIPI2 mutant worms have significantly longer lifespans than wild type worms. Thus, we hypothesized that phosphodead WIPI2 could rescue CEP degeneration. We find that the phosphodead WIPI2mutant rescues CEP degeneration during late aging. Second, we examined DIRAS1, a putative positive regulator of neuronal autophagy. We hypothesized that the overexpression of DIRAS1 would also rescue CEP degeneration. Surprisingly, we find that overexpression of DIRAS1 in CEP neurons did not show a clear protection of CEP degeneration. Unexpectedly, in young worms overexpressing DIRAS1 in dopaminergic neurons, there are fewer CEP neurons than in young wild type animals. In contrast, by late aging, worms overexpressing DIRAS1 displayed more CEP neurons than wild type animals, pointing to a rescue of CEP degeneration. DIRAS1 levels decrease with age in mouse and human brain. It is plausible that the overexpression of DIRAS1 restores levels in older worms to that of younger worms, thus limiting CEP degeneration during late aging. Our ongoing research will test a phosphomimetic WIPI2mutant to evaluate neurodegeneration in CEP, examine why there are fewer CEP neurons at a young age in DIRAS1 overexpression worms, and investigate whether overexpressing DIRAS1 only during late aging is sufficient to prevent CEP degeneration.

B203/P1329

Most axonal mitochondria in neurons of the mammalian central nervous system lack mitochondrial DNA and consume ATP

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In neurons of the mammalian central nervous system (CNS), axonal mitochondria are thought to be indispensable for supplying ATP during energy-consuming processes such as neurotransmitter release. Surprisingly, we find that in cortical pyramidal neurons (CPNs), two subtypes of cortical inhibitory interneurons and dopaminergic neurons of the substantia nigra, the majority of axonal, but not dendritic, mitochondria lack mitochondrial DNA (mtDNA) both in vitro and in vivo. Additionally, live imaging of tagged mitochondrial nucleoid proteins and a novel, genetically encoded marker of mitochondrial DNA (mt-HI-NESS) reveals that new mitochondria entering neuronal processes are largely devoid of mtDNA arguing for a compartment specific seeding model. Finally, using dynamic, optical imaging analysis of genetically encoded sensors for mitochondrial matrix ATP and pH, we demonstrate that in axons of CPNs, but not in their dendrites, mitochondrial F1F0-ATP synthase (Complex V) functions in a reverse way, hydrolyzing ATP and extruding H+ out of the matrix to maintain mitochondrial membrane potential. Our results demonstrate that in mammalian CNS neurons, axonal mitochondria are unlikely to play a major role in ATP generation, despite playing other functions critical to regulating neurotransmission such as Ca2+ buffering.

B191/P1317

Neurofilament transport kinetics can be explained by the activity of small numbers of kinesin and dynein motors

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Neurofilaments, which are the intermediate filaments of nerve cells, are long flexible protein polymers that are transported bidirectionally along microtubule tracks in axons. Disruption of either kinesin-1 or dynein impairs neurofilament movement, implicating these motors in neurofilament transport. Kymograph analysis of neurofilament movement has revealed that the filaments exhibit short bouts of anterograde and retrograde motion with average run lengths of ~1µm interrupted by pauses and occasional or repeated reversals. This suggests that the bidirectional movement of neurofilaments may be the result of a competition between motors of opposing directionality. Here, we use computational modeling to explore whether such a model can explain the experimentally observed transport kinetics and to predict the number of motors involved. We used stochastic models of kinesin and dynein motors to simulate neurofilament movement in axons by teams of motors. Our motor models account for the load-dependence of the kinetic rates and interact mechanically in that each motor experiences the forces generated by all other motors acting on the same filament. Since the number of engaged motors influences the run length, run time and pause duration of a cargo, these kinetic properties can be used to infer motor number. We used an automated filtering process to tune the number of kinesin and dynein motors in the transport model to match the distributions of run velocities, run lengths, run times and pause times for neurofilaments in a large experimental dataset (~37,000 runs and pauses acquired by streaming acquisition of the movement of fluorescently tagged neurofilaments with 30.3 millisecond resolution). We found that the movement can be explained by the activity of a small number of kinesin and dynein motors. Most of the time, the model predicts that only a single motor is engaged, with recruitment of additional motors of the same directionality leading to longer runs. Simultaneous engagement of multiple motors is relatively rare, thus minimizing energy expenditure, but when those motors are opposing, it often results in a reversal, suggesting that reversals may arise from a transient tug-of-war. Experimental confirmation of these predictions will require direct measurement of the forces acting on moving neurofilaments, which is technically challenging.

B188/P1314
Neuronal Aß generation is elevated by Swedish APP bypassing a lysosomal trafficking pathway that degrades wild-type APP
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Cleavage of amyloid precursor protein (APPwt) by BACE-1 is the determinative step for generating amyloid β (Aβ), the primary component of amyloid plaques in Alzheimer's disease (AD). BACE-1-mediated APP cleavage is thought to occur mostly in neuronal endosomes. The Swedish variant of APP has two point mutations (KN670/671ML, APPSwe) that cause early-onset AD in patients and up to ten-fold more Aβ production than wild type. This difference in the rate of Aβ formation has been primarily attributed to the efficiency with which BACE-1 cleaves APP. It is unclear if APP mutations associated with increased Aβ formation change the neuronal trafficking of APP and increase its exposure to BACE-1. We used live-cell microscopy to characterize APPwt and APPSwe trafficking and their targeting to endosomes, the compartment where BACE-1-mediated cleavage and Aβ generation are thought to occur. In non-neuronal cells, APPwt and APPSwe were both trafficked to endosomes with high efficiency. Trafficking of APPwt in cultured hippocampal neurons differed substantially from non-neuronal trafficking: only a small fraction of the protein localized to dendritic endosomes. Of the APPwt that was endocytosed in dendrites, most was targeted to lysosomes for degradation. Such degradation would prevent Aβ formation and may be neuroprotective. APPSwe differed in its neuronal trafficking from APPwt. APPSwe was targeted to neuronal endosomes more frequently than APPwt. The trafficking of

endocytosed APPSwe to dendritic lysosomes was less efficient, potentially enhancing exposure to BACE-1 in endosomes. Specific staining for Aβ revealed that APPSwe expression resulted in more vesicles containing Aβ than expression of APPwt. Surprisingly, a large number of Aβ-positive vesicles were not endosomes. These vesicles were Golgi-derived and contained Aβ, likely derived from APP that had not trafficked to the plasma membrane. This study shows that APP trafficking is a key determinant in Aβ generation and that trafficking differences between APPwt and APPSwe contribute to the rate of Aβ formation. The trafficking of endocytosed APP to lysosomes is a potential neuroprotective mechanism that avoids Aβ formation. The ability of APPSwe to avoid lysosomal degradation may contribute to the increase in Aβ formation associated with this mutation. Finally, the differences in APP trafficking between neurons and non-neuronal cells emphasize that neuron-specific studies are essential to understand the function and processing of APP in the nervous system.

B194/P1320
Neuronal polarity requires regulation of Ral1A and exocyst-mediated vesicle tethering
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Neurons are polarized cells with multiple dendrites and one axon. Polarized delivery of membrane proteins is mediated by selective vesicle trafficking. Previous studies identified some of the mechanisms that regulate polarized cargo sorting and the kinesin-mediated long-range transport of polarized vesicles. However, it is unclear if polarized cargo delivery—the fusion of selectively transported vesicles in axons or dendrites—also depends on specific regulatory machinery. We found that polarized cargo delivery may be tightly regulated: axon-selective vesicles readily move in dendrites but do not fuse; dendrite-selective vesicles fuse only in the somatodendritic domain. Despite the likely importance of this process to neuronal health, the specific machinery that targets polarized delivery is unknown. To identify potential regulators of polarized delivery, we performed live-cell imaging in cultured hippocampal neurons. We found that two GTPases—Ral1A and Rab11—labeled dendrite-selective vesicles visualized with transferrin receptor. Neither GTPase was present on axon-selective vesicles visualized with NgCAM. Because Ral1A and Rab11 are effectors of the exocyst tethering complex, we performed live TIRF microscopy to determine the localization of exocyst tethering at the plasma membrane. We found exocyst structures appeared at the somatodendritic plasma membrane for tens of seconds prior to vesicle fusion, consistent with tethering behavior. These exocyst sites colocalized with dendrite-selective vesicles during fusion with the plasma membrane, indicating that exocyst participates in dendritically polarized exocytosis. Because Ral1A is the primary regulator of exocyst activity, we asked if Ral1A regulation was required for proper polarized delivery. Expression of constitutively active Ral1A enhanced dendritic fusion of dendrite-selective vesicles. Ral1A hyper-activation also resulted in dendritic fusion of the axonally polarized cargo NgCAM and the loss of neuronal polarity. These data suggest Ral1A-mediated exocyst activation is a vital step in dendritically polarized vesicle fusion. Furthermore, Ral1A/exocyst regulation is crucial for preventing dendritic fusion of axon-selective vesicles and the maintenance of neuronal polarity.

B200/P1326
Nutrient dependent spatiotemporal regulation of mTORC1
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Neurons rely on the biosynthesis and uptake of metabolites to support functions such as dendrite development and synapse formation. Proper timing and amounts of these processes is essential for neuronal

function and health. Of these metabolites, cholesterol has emerged as a regulator of these basic neuronal processes, but the mechanism underlying these contributions remain unclear. In addition to its role as a membrane building block, cholesterol is a potent stimulator of metabolic signaling through the mechanistic target of rapamycin complex 1 (mTORC1). mTORC1 is a lysosomal multi-protein kinase that senses amino acids, cholesterol and growth factors to promote anabolic processes such as translation, lipid and nucleotide biosynthesis. The nutrient sensing ability of mTORC1 is afforded by a network of dedicated nutrient sensors and regulatory proteins that promote its translocation to the lysosomal limiting membrane. Over the years much of the work aimed at understanding the regulation of mTORC1 has been done in cells which lack functional compartmentalization. This limitation in the field has curtailed our understanding of how specific nutrient inputs like cholesterol might promote the precise timing and amount of mTORC1 activity in compartmentalized cells such as neurons. Using iPSC-derived neurons (iNeurons), we sought to better understand the nutrient dependent spatiotemporal regulation of mTORC1. We find that in iNeurons, mTORC1 can be acutely stimulated by exogenous amino acids and cholesterol. Furthermore, we observe that the dedicated cholesterol sensor of mTORC1 is upregulated upon iNeuron differentiation and is necessary for basal activation of mTORC1. Through immunofluorescence, we find that loss of this dedicated sensor primarily abolishes mTORC1 activity in dendrites. Conversely, we find that in an iNeuron model of a lysosomal storage disorder characterized by cholesterol accumulation, mTORC1 activity is elevated in dendrites. Altogether, this data sheds light on the compartmentalized regulation of mTORC1 by cholesterol and uncovers a previously unappreciated aspect of mTORC1 regulation in neurons.

B202/P1328

Pathogenic KIF1A variants differentially disrupt axonal trafficking but converge to a common deficit in synaptic function in human neurons

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The nervous system relies on billions of neurons connected through trillions of synapses to support a vast array of vital functions. Despite the critical importance of this synaptic network, the cellular mechanisms dictating synapse formation during human neurodevelopment remain unclear. Long-distance trafficking of synaptic components is critical for both synaptogenesis and the maintenance of synaptic function across lifespan. The microtubule motor KIF1A has a highly conserved role in driving the trafficking of synaptic vesicle precursors, while mutations in KIF1A are causal for the neurodevelopmental and neurodegenerative disease KIF1A-Associated Neurological Disorder (KAND). Here, we employ isogenic human induced pluripotent stem cells (iPSCs) gene-edited to express pathogenic KIF1A variants to assess how disparate mutations alter synaptic trafficking and function. We compared the effects of both loss-of-function and gain-of-function mutations in KIF1A motor activity. We found that both null (p.C92*) and hypoactive (p.P305L) mutations induce delayed neurite outgrowth, mislocalization of synaptic cargos, and decreased synapse density. Conversely, the hyperactive KIF1A mutation (p.R350G) supports neurite outgrowth but leads to aberrant motility of synaptic vesicle precursors along the axon. Further, live imaging reveals that hyperactive KIF1A induces deficits in the microtubule-dependent patterning of presynaptic components along the developing axon, suggesting a failure to respond to cytoskeletal cues directing cargo delivery. Remarkably, despite the distinct alterations in trafficking caused by loss- (p.C92*, p.P305L) and gain-of-function (p.R350G) KIF1A mutants, these distinct variants converge on a shared functional outcome, with decreased synaptic firing rates recorded via multi-electrode arrays. Together, these data provide insights detailing how pathogenic variants in KIF1A with distinct effects at the molecular level converge to a common downstream deficit in synaptic function.

B201/P1327

The signaling lipid phosphatidylinositol-3-phosphate (PI(3)P) coordinates parallel pathways for endocytic recycling at synapses

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The regulated trafficking of receptors from endosomes to the cell surface is a key mechanism to regulate synaptic function. However, mechanisms that regulate receptor recycling during enduring forms of synaptic plasticity such as long-term potentiation (LTP) remain unclear. Phosphatidylinositol-3-phosphate (PI(3)P) is an essential regulator of endosomal trafficking. Here, using primary hippocampal neurons, we find that PI(3)P is rapidly synthetized upon chemical induction of LTP (cLTP) and coordinates the recruitment of two distinct endosomal recycling pathways, the SNX17-Retriever and SNX27-Retromer pathways, to synaptic sites. We show that both pathways are necessary for the cLTP-dependent structural enlargement of dendritic spines, as well as cLTP dependent changes in synaptic strength. However, we find that the SNX17 and SNX27 pathways act in parallel and recycle distinct sets of cell surface proteins at synapses. Importantly, inhibition of PI(3)P synthesis blocks synaptic recruitment of SNX17 and SNX27, decreases cargo recycling, and prevents cLTP-dependent spine remodeling. These findings provide mechanistic insights into the regulation of endocytic recycling at synapses and define a novel role for PI(3)P in synaptic plasticity. Notably, low levels of PI(3)P have been proposed to underlie Alzheimer's disease, thus the importance of PI(3)P at post-synaptic sites may account in part for how defects in PI(3)P are linked to neurodegeneration.

B189/P1315

Tubulin autoregulation factors SCAPER and TTC5 recruit gamma-tubulin to non-centrosomal MTOCs for neuronal microtubule nucleation and axon regeneration

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Neuronal function and survival depend on highly stereotyped non-centrosomal microtubule (MT) arrays. How polymer abundance in these arrays is controlled remains poorly understood. We identified a role for SCAPER and TTC5, two factors previously implicated in tubulin mRNA autoregulation, in neuronal MT nucleation. In C. elegans neurons, loss of scpr-1, ttc-5 or both reduced polymer numbers to a similar degree as depletion of gamma-tubulin, the main MT nucleator. Using conditional single-cell degradation alleles and endogenous tagging we find that gamma-tubulin nucleates MTs in the neuronal cell body from late endosomes, and that scpr-1 and ttc-5 are required to recruit gamma-tubulin to these structures. SCPR-1 is also instructive, with its overexpression drastically increasing gamma-tubulin levels and increasing MT density. We propose that these mechanisms are conserved since human SCAPER rescues C. elegans mutants, and SCAPER knockdown in rat hippocampal neurons reduces γ-tubulin clustering at presynaptic sites and abolishes activity-dependent nucleation. Last, scpr-1, ttc-5 and gamma-tubulin are dispensable for developmental axon elongation but required for regeneration, where SCPR-1 recruits gamma-tubulin to the growth cone to promote regrowth after injury. These results uncover mechanisms that control neuronal cytoskeleton formation and function, and raise the possibility of cross-talk between tubulin autoregulation and MT nucleation.

Neuronal Cell Biology - Neuroimmunology

B250/P1374
Role of citrullinated vimentin in the pathogenesis and progression of multiple sclerosis
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Multiple sclerosis (MS) involves blood–brain barrier (BBB) dysfunction, leukocyte trafficking into the CNS, and ongoing neuroinflammation. Citrullination—the process of converting arginine to citrulline by peptidylarginine deiminases—produces neoepitopes that can intensify inflammation. Vimentin, a type III intermediate filament, appears outside cells during inflammation and is often citrullinated (CitVim). We hypothesized that CitVim acts as a damage-associated molecular pattern (DAMP) that weakens BBB integrity and promotes leukocyte extravasation. We measured CitVim levels in cerebrospinal fluid (CSF) and serum from MS patients and non-inflammatory neurological controls. To model barrier function, we used 2D and 3D human BBB platforms consisting of channels within extracellular matrices in which brain microvascular endothelial cells are interfaced with astrocytes. Barrier integrity was evaluated through fluorescent dextran permeability and tight-junction morphology, while neutrophil transmigration into the matrix was quantified during exposure to CitVim. Pharmacologic pathway analysis involved Toll-like receptor 4 (TLR4). CitVim levels were higher in MS CSF and serum compared to controls. Exogenous CitVim disrupted endothelial tight junctions, increased paracellular permeability, and boosted neutrophil extravasation into hydrogel matrices. CitVim is elevated in MS biofluids and alone can impair BBB function and promote leukocyte trafficking in a human 3D model. These results support CitVim as a mechanistic link between peripheral inflammatory signals and the entry of innate immune cells into the CNS in MS.

Neuronal Cell Biology - Neuronal development and differentiation

B174/P1300
3D human enteric neuron models derived from schwann cell precursors for disease modeling and regenerative applications
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Schwann cell precursors (SCPs) are multipotent progenitors capable of giving rise to diverse neural crest-derived lineages. While previous studies have suggested their potential to differentiate into enteric neurons (ENs), a reproducible and physiologically relevant demonstration has not been established. In this study, we successfully directed human SCPs toward an EN fate within a 3D culture system, generating a reproducible SCP-derived EN model. Compared with traditional 2D cultures, the 3D platform markedly enhanced neuronal purity, maturation, and functionality, thereby more closely recapitulating the physiological characteristics of the enteric nervous system. This model provides a unique opportunity to investigate mechanisms underlying congenital enteric neuropathies such as Hirschsprung disease, characterized by aganglionosis in the distal bowel. Furthermore, the system supports translational applications by offering a tractable platform for high-throughput screening of bioactive molecules, functional interrogation of disease-associated genes, and engineering of innervated gut tissue for regenerative medicine. Our findings demonstrate that SCP-derived ENs in a 3D context are not only a reproducible and physiologically relevant in vitro model but also a versatile resource that bridges basic neurodevelopmental biology and translational gastrointestinal research. By leveraging SCP plasticity, this approach expands the toolkit for studying enteric neuropathies and provides a

foundation for developing cell-based or secretome-based therapeutic strategies. Acknowledgements: This work was supported by the Korean Fund for Regenerative Medicine (23A0106L1), the KRIBB Research Initiative Program (KGM1232511), and the National Research Foundation of Korea (NRF) (RS-2023-00272798).

B171/P1297

Axon Pathfinding Regulation by Altering EphA4 Signaling

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Axon pathfinding is crucial for the formation of an organized neural network during development and for axonal regeneration after injury. While canonical axon guidance pathways have been identified, the regulation of signaling activity via receptor abundance, localization, and timing remains unclear. My research focuses on investigating the regulation of Ephrin type-A receptor 4 (EPHA4), an axon guidance receptor, by a newly identified membrane-tethered ubiquitinating complex in axon pathfinding. A surface proteomic screen revealed EPHA4 was one of only three receptors elevated more than twofold in Modulator of smoothened (MOSMO)-depleted, Multiple epidermal growth factor-like domains 8 (MEGF8)-depleted, and Mahogunin ring finger 1 (MGRN1)-depleted cells, suggesting that EPHA4 might be the target of the MOSMO/MEGF8/MGRN1 (MMM) complex, a module that has been reported to ubiquitinate G protein-coupled receptors (GPCRs) (Kong et al., 2021 & Parashara et al., 2025). Using protein quantification assays, I show that overexpression of MMM components resulted in less EPHA4, suggesting that the MMM complex downregulates EPHA4 protein levels. Ubiquitination assays further validate that EPHA4 is ubiquitinated in the presence of the MMM complex, indicating that the MMM complex initiates EPHA4 ubiquitination and degradation to control its surface availability. Notably, axon guidance defects are observed in the developing limbs of Mosmo and Megf8 knockout mouse embryos, which suggests that loss of MMM function disrupts EphA4 signaling activity to impair axonal growth and navigation. Collectively, this data suggests that modulations in the abundance of EPHA4, driven by MMM complex activity, affect axon pathfinding for neural network formation. Understanding the mechanism could provide insights into axon guidance regulation and identify potential therapeutic targets for diseases associated with axonal outgrowth.

B175/P1301

Differential proteomics of astrocytes at the cortex and the hippocampus of the mouse cerebrum by high-precision microscopy-guided photo-biotinylation

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Astrocytes are a subtype of glial cells in the central nervous system. They play roles in neurogenesis, synaptogenesis, blood–brain barrier permeability, and extracellular homeostasis. Interestingly, different populations of astrocytes localized in specific regions of the brain area have unique morphological and functional characteristics, whereas the proteomic difference of these regional-specific astrocytes remains elusive. Here, we employed optoproteomics, an advanced technology integrates microscopy-guided automated photo-biotinylation, pulldown and mass spectrometry, to isolate and identify proteins localized at the mouse cortical and hippocampal astrocytes, respectively, to find proteins unique in the astrocytes of these two cerebral regions. Astrocytes in mouse brain cryo-sections were immunostained against the glial fibrillary

acidic protein (GFAP) and recognized as the target for two-photon illumination, which effectively activated proprietary photosensitive biotin-based probes in the media and triggered high-precision protein biotinylation. This cycle ran automatically across thousands of fields of view so that sufficient proteins were biotinylated for the subsequent pulldown and LC-MS/MS-based proteome identification. Known astrocyte markers including GFAP, LAMC3, PARK7 were found in the proteomes. Upon comparing differences in the two proteomes, several candidates that have not been reported in existing literature and were more enriched in either the cortex or the hippocampus were selected for immunofluorescence validation. We found that at least PLEKHB1 and SYBP3 were colocalized with GFAP in the cortical astrocytes but almost absent in the hippocampal astrocytes. In contrast, the hippocampal-enriched candidate MINK1 was positively validated in the hippocampal astrocytes but absent in the cortical astrocytes. Together, optoproteomics enables the discovery of novel region-specific astrocyte markers and generates testable hypotheses for further functional understanding of these regionally unique proteins.

B169/P1295

Examining de novo variants in BLTP2 to determine their pathogenicity in Drosophila melanogaster.

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Mutations in a variety of lipid transport genes have previously been associated with neurodevelopmental disorders (NDDs) and disease. Bridge-like transport protein 2 (BLTP2) is an ER-plasma membrane tethering protein that binds to lipids via its Apt1 domain that has yet to be implicated in NDDs. Recently, we identified a patient with a de novo heterozygous missense mutation in the Apt1 domain of BLTP2 who presented with developmental delay, facial dysmorphisms, muscular hypotonia, and many other symptoms. This patient's mutation in the BLTP2 gene occurs at a highly intolerant and conserved region in the gene, making it of key interest for our investigation into the neurodevelopmental role of BLTP2. Mutations in the fly ortholog of the BLTP2 gene, hobbit (hob), have been shown to be rescued by the expression of the human BLTP2 gene. Therefore, we chose to use drosophila melanogaster to model this potentially novel NDD. Our project establishes the phenotype of hob knock-down in flies and seeks to compare the rescue effectiveness of human BLTP2 and the patient gene mutation on these phenotypes. We have shown that RNAi knockdowns of hob are associated with a smaller brain size, motor deficits, and social behavior differences. We are collecting data on our rescue lines to determine if the patient mutation gene is responsible for NDD pathogenesis. Additionally, we plan to perform lipidomic analyses to establish the effect of hob on nervous system lipid homeostasis.

B167/P1293

Formation and remodeling of parallel fiber synaptic input to Purkinje cells in the mouse cerebellum

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Specific patterns of synaptic connectivity organizes individual neurons into neural circuits and networks. To uncover the developmental rules that underlie the formation and maturation of the connectivity patterns, we employed a connectomic approach in the mouse cerebellar cortex at five different stages during the first two weeks of postnatal life. Reconstruction of many Purkinje cells (20-50 per developmental stage), parallel fibers (300- several thousands) and the synapses between them (several thousand) at all the five stages showed significant changes taking place over postnatal development. The synaptic connectivity analysis revealed that

the number of parallel fiber inputs to Purkinje cells increased ~500 fold between birth and 14 days of age. Unexpectedly, at one week of age, Purkinje cells had generated thousands of parallel fiber-oriented filopodia. These dendritic filopodia are innervated by parallel fibers and thus may facilitate contacts between Purkinje cell dendritic spines and parallel fibers at later ages. Concurrent with the increase in parallel fiber synapses on individual Purkinje cells, was a concomitant increase in the number of Purkinje cells each parallel fiber innervated. Additional parallel fibers were invading the more superficial parts of Purkinje cell dendrites. Together, the increase in fan-out of individual parallel fibers to innervate greater numbers of Purkinje cells and the increase in the fan-in of additional parallel fibers that innervated each Purkinje cell gave rise to a rapid increase in the total number of synapses on Purkinje cells during the second postnatal week. Importantly there was no evidence of synapse elimination: parallel fibers add connectivity as development precedes but show no signs of pruning any of these connections with Purkinje cells in stark contrast to climbing fiber synapses. The number of synapses a parallel fiber establishes per Purkinje cell is constant across the developmental stages, usually (90%) one synapse per cell. Thus, the synaptic connectivity analysis suggests an accretive model of continuous synapse addition for the formation of parallel fiber to Purkinje cell circuit. Thus different strategies are employed by different excitatory inputs to the same postsynaptic cells. In addition, Our connectivity showed that the parallel fiber to Purkinje cell connectivity is highly selective and results in each Purkinje cell connecting to a distinct population of parallel fibers

B168/P1294

From Barcodes to Branches: PCDHγC3 Guides Astrocyte Morphology and Survival

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Self-recognition is a fundamental cellular process across evolution and forms the basis of neuronal self-avoidance. The self-avoidance mechanism in neurons is essential for brain development and neural circuit formation. This mechanism depends on precise cell–cell recognition mediated by the protocadherin (PCDH) family, which forms unique molecular barcodes enabling accurate self-versus non-self-discrimination. This recognition allows neurons to assemble large extracellular PCDH oligomers that facilitate self-recognition and subsequently activate intracellular signaling, driving dendritic repulsion upon self-contact. Although PCDH oligomerization is known to initiate dendritic repulsion, the precise downstream signaling pathways that transmit this signal in neurons remain unknown, representing a critical gap in our understanding of self-avoidance. A recent study showed that astrocytes provide a powerful model to study these mechanisms, as the protocadherin-γC3 (PCDHγC3) isoform is expressed deterministically and is essential for self-recognition in astrocytes development. Loss of PCDHγC3 disrupts this process, causing abnormal morphology and clumping of astrocytic processes. However, the signaling pathways activated downstream of PCDHγC3 also remain unclear. To address this gap, we established a knockdown system for PCDHγC3 in primary and immortalized astrocytes using viral particles carrying shRNA targeting a specific region of PCDHγC3. Our first preliminary results showed over 50% knockdown by qPCR. Strikingly, our second result revealed that PCDHγC3 knockdown not only induced morphological alterations in primary astrocytes but also included apoptotic markers such as cleaved caspase-3 and nuclear fragmentation, highlighting its essential role in astrocyte survival. This demonstrates the importance of PCDHγC3 for maintaining normal astrocyte morphology. In ongoing experiments, we are performing transcriptomic and proteomic analyses, including RNA-seq and proximity labeling, to map the signaling pathways and protein networks associated with PCDHγC3. Together, these findings demonstrate that PCDHγC3 is crucial for astrocyte morphology and survival and provide a foundation for identifying candidate signaling pathways underlying neuronal self-avoidance, offering insights into the molecular basis of neurodevelopmental disorders linked to clustered protocadherin dysregulation.

B170/P1296

Pharmacologic reprogramming of mitochondrial metabolism links human stem cell differentiation to neuroprotection in Retinal ganglion cells

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Metabolic collapse with mitochondrial damage presents the early pathology for retinal ganglion cell (RGC) degeneration in optic neuropathies such as glaucoma, yet no RGC-targeted therapies exist. Here, we identify a clinically tested small molecule that reprograms mitochondrial maturation and metabolic state to both promote differentiation and protect mature human RGCs. We performed a high-content live-cell mitochondrial screen in human pluripotent stem cell-derived RGCs (hRGCs), using the mitochondrial membrane potential reporter Mito-Tracker Deep Red (MTDR), to identify compounds that restore mitochondrial health. Healthy mitochondria maintain the membrane potential through active electron transport and proton motive force that is sensed by the MTDR probe to get inside the organelle. Hits were subsequently evaluated for neuroprotection by their ability to reduce cellular apoptosis in the differentiated hRGC culture. This led to the discovery of the 5-HT1A G protein-coupled receptor antagonist WAY-100635 (WAY). In wild-type hRGCs, WAY induced a reversible increase in cAMP via 5-HT1A engagement, followed by reversible activation of mitochondrial biogenesis through the master regulator PGC-1a. In hRGCs containing the glaucoma-associated Optineurin E50K mutation, which impair mitophagy, WAY improved mitochondrial health and conferred neuroprotection while shifting metabolism toward aerobic glycolysis, a metabolic reprogramming known to support neuronal survival in humans. Importantly, in mouse models of acute optic nerve injury and elevated intraocular pressure (a glaucoma risk factor), systemic WAY treatment robustly protected RGC axons, preserved cell bodies, and maintained functional circuitry between eye and brain. Strikingly, when applied at the progenitor stage, WAY accelerated mitochondrial maturation (increased cristae complexity) and promoted a metabolic switch toward oxidative phosphorylation (OXPHOS), thereby driving early cell-cycle exit and enhanced differentiation of RGCs. Together, our findings establish a first-in-class pharmacologic paradigm for simultaneously protecting and maturing RGCs via reversible metabolic and mitochondrial reprogramming, advantages that avoid the delivery challenges and constitutive effects of gene therapy. Given the central role of mitochondrial dysfunction across neurodegenerative disease, this approach may extend beyond glaucoma and applicable to Parkinson's disease and ALS, facilitating translation via repurposing of a drug with established safety.

B177/P1303

Proteomic and Functional Interrogation of Subcellular Signaling within Radial Glia Cells during Cortical Development

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Cortical development is a highly orchestrated process that relies on cellular crosstalk between diverse cell types and their local environment. Radial glial cells (RGCs), neural and glial progenitors of the developing cortex, exhibit a highly polarized morphology with a cell body adjacent to the ventricle and a long basal process that terminates in subcellular structures known as basal endfeet. RGC basal endfeet reside in a niche

composed of extracellular matrix, vasculature, pial fibroblasts, Cajal-Retzius neurons, and migrating interneurons. RGC endfeet are critical for preventing over-migration of neurons, but how they integrate and communicate signals within the local niche remains largely unknown. Here, we use unbiased proteomics, imaging-based approaches, and candidate analysis to address this question. Ultrastructural analyses of transmission and scanning electron micrographs reveal the presence of membrane and junctional structures between both RGC endfeet and with neighboring cell types, suggesting potential roles in signaling. To define the proteome landscape of the pial-endfeet niche, we employed an unbiased in vivo proximity-labeling method by targeting the biotin ligase TurboID extracellularly via a glycosylphosphatidylinositol anchor. We identified over 5,000 proteins, with approximately 500 proteins significantly enriched in RGC endfeet relative to the remainder of the cell. Gene ontology analyses reveal enrichment of proteins regulating extracellular matrix organization, vascular permeability, cell and focal adhesion, and cell-cell junctions. Many of these proteins have been implicated in neurodevelopmental disorders, such as lissencephaly, cortical malformations, and cerebellar dysplasia. To determine how RGC endfeet mediate cellular crosstalk with neighboring cells within the pial niche, we are currently functionally interrogating candidate junctional and receptor proteins in vivo. Altogether, this work will provide fundamental insights into subcellular mechanisms of cell-surface signaling during brain development.

B173/P1299

The Differentiated RGC-5 Cells vs. the Purified Retinal Ganglion Cells: Study on Their Morphological and Neuronal Marker Properties

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Objective：To differentiate retinal ganglion cell (RGC)-like cells(RGC-5) and compare them to purified RGCs in morphological, neuronal marker and excitotoxicity properties. Methods: RGC-5 cells were differentiated with Staurosporine with or without pre-incubation of Forskolin and IBMX, after which they were assayed and compared for morphology and specific marker antigen expression to immunopan-purified RGCs. Treated cells were also assayed for excitotoxic responsiveness. Results: Both treated or untreated RGC-5 cells expressed RGC marker protein Thy-1, similar to purified RGCs. However, unlike purified RGCs which displayed typical morphological features of neuronal cells with axons and dendrites, untreated RGC-5 cells were rather spindle-shaped with only few and short extensions. Treatment of RGC-5 cells with Staurosporine (100, 316 and1000nM) for 48 hours not only induced development of neurites and rounder cell bodies, but also increased expression of NMDA receptor 1 (NR1) and RGC-5 sensitivity to glutamate excitotoxicity. Even so, differentiated RGC-5's resistance to glutamate was almost 10 times greater than that of purified RGCs. Pre-incubation for 42 hours with Forskolin(10mM) and IBMX (0.1mM) followed by treatment with Staurosporine for only 6 hours enhanced expression of thy-1, NR1 and healthier cell morphology in culture. Conclusions: RGC-5 cells have been shown to be of mouse origin, and overtime the cell line has gradually lost RGC specific marker expression and glutamate-induced excitotoxicity. Our study demonstrated that cell differentiation treatment makes this cell line more similar to purified RGCs in terms of cell morphology, RGC specific marker expression, and glutamate-induced excitotoxicity, which suggests that RGC-5 should be used more cautiously and appropriately in related research.

B176/P1302

Tubulin autoregulation mediator TTC5 regulates neuronal morphology and migration

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The α/β-tubulin heterodimer is the building block of microtubules, dynamic self-assembling polymers that form the cytoskeleton of cells and tissues. Cells closely monitor soluble tubulin levels through a feedback process termed “tubulin autoregulation”, mediated by TTC5. When soluble tubulin is in excess, TTC5 binds to the β-tubulin N-terminus emerging from the ribosome which marks the associated transcript for decay. TTC5 mutations have been recently linked to intellectual disability, implicating the protein and tubulin autoregulation in neural development. Microtubules play critical roles at each stage of neurogenesis, from progenitor proliferation to cortical migration, and neurons rely on the dramatic upregulation of α/β-tubulin to drive rapid neurite extension. Using an iPSC-derived cortical-like neuron model, we show that tubulin autoregulation is activated across neuronal differentiation in a TTC5-dependent manner and is highly specific for tubulin isoforms. TTC5 knock-down causes defects in neuronal morphology including reduced axonal outgrowth and arborization accompanied by decreased microtubule dynamics in axonal growth cones. Moreover, TTC5 loss leads to a striking increase in neuronal motility, revealing an unanticipated intersection of TTC5 with cell motility pathways. CRISPR knock-in of a structure-guided TTC5 point mutation defective in autoregulation, and also of previously-uncharacterized patient disease mutations, recapitulate these phenotypes indicating that the role of TTC5 in microtubule homeostasis underlies both these cellular defects and disease. We extend our findings in vivo, demonstrating that TTC5 loss in mouse cerebral cortex projection neurons (CPNs) alters axon arborization and leads to hypermigration, the first such characterization of TTC5 in an animal model. Our study establishes the role of tubulin autoregulation in neuronal homeostasis and sheds light on disease mechanisms in patients with TTC5 mutations, with broader implications for neurodevelopmental and neurodegenerative diseases characterized by cytoskeletal dysfunction.

B172/P1298

Uncovering the effects of transmural pressure on pulmonary neuroendocrine cells in the developing lung

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Congenital diaphragmatic hernia (CDH) is a defect in which a weak or unclosed the diaphragm permits the herniation of abdominal organs into the thoracic cavity and thus the compression of the developing lungs. CDH leads to pulmonary hypoplasia characterized by small lungs with fewer branches and is a major cause of respiratory failure after birth. Branching of the airway epithelium is influenced by the transmural pressure across the epithelium and it is hypothesized that the transmural pressure is decreased in CDH. Pulmonary neuroendocrine cells (PNECs), which are the first epithelial cell to differentiate in the lung, are thought to serve as oxygen, chemical, and mechanical sensors, and the number of these cells increases in pathological conditions associated with altered pressure, including those related to premature birth. PNECs differentiate throughout the developing airway epithelium and then migrate to cluster into neuroepithelial bodies (NEBs) at branch points in the tree. Here, we combine a mouse model of CDH with experimental methods to alter transmural pressure to understand the role of pressure in the regulation of PNEC differentiation and NEB formation. RNA-sequencing analysis reveals that low-pressure conditions increase the expression of markers

for PNECs, suggesting that differentiation of these cells is inversely correlated with transmural pressure. Quantitative measurements show an inverse correlation of the number of PNECs and transmural pressure when varying the pressure using pharmacological treatments. Future work will define how physical and molecular mechanisms downstream of transmural pressure regulate the differentiation of PNECs.

Neuronal Cell Biology - Neuronal structure and function

B217/P1343
A hormone-driven growth spurt enhances dendritic complexity for the Drosophila Moonwalker Descending Neuron before metamorphosis
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The Moonwalker Descending Neuron (MDN) is part of a critical backwards locomotor circuit in Drosophila established during larval life, that persists through metamorphosis into adulthood. During pupation, MDN undergoes significant remodeling, including degradation of its existing neurites. However, careful observation of MDN over larval time revealed that these neurons experience a significant increase in dendritic complexity at 96h after larval hatching (ALH), despite the imminent remodeling event. This increase in complexity coincides with a switch in transcription factor expression: at 72h ALH MDN expresses Dbx, while at 96h ALH the cell expresses Pdm. To determine if this increase in complexity was due solely to cell-intrinsic mechanisms, or if there is a critical interaction with environmental signals at 96h ALH, we utilized neuronal cell culture. We found that application of mature larval extract, made from homogenized 96h ALH larvae, to MDN cultures at 60h ALH resulted in significantly higher levels of neurite complexity at both 72 and 96h ALH. We hypothesized that the insect hormone ecdysone may be responsible for the increased complexity we observed in vivo and recapitulated in vitro. This hypothesis was strengthened by observing expression of Ecdysone Receptor (EcR) expression at 96h ALH, but not 72h ALH in situ. Additionally, EcR expression was increased at both 72 and 96h ALH time-matched cultures reared with larval juice extract added at 60h ALH compared to those reared in media alone. Indeed, application of 20-hydroxy-ecdysone, the active form of ecdysone, caused a similar phenotype as mature larval extract. Future experiments for this project will include exploration of how transcription factor expression intersects with hormonal signals to generate neuronal phenotypes.

B211/P1337
A PKA-Anchoring-Dependent Switch Organizes Poly(ADP-ribose)-Containing Signaling Complexes at Neuronal ER-PM Junctions
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Neuronal somata contain abundant endoplasmic reticulum (ER)-plasma membrane (PM) junctions, including a subset that hosts micron-scale clusters of cAMP-dependent protein kinase (PKA). PKA recruitment to somatic ER-PM junctions is mediated by the regulatory subunit RI through its interaction with the RI-specific PKA anchoring protein SPHKAP. Previous work in heterologous cells showed that SPHKAP and RI co-expression generates large SPHKAP-RI assemblies resembling those in neurons, and that a peptide disruptor of AKAP binding (RIAD) prevents their formation. However, the molecular mechanism underlying RI-dependent SPHKAP clustering has remained unclear. Here, we used fluorescence microscopy, heterologous expression of SPHKAP mutants, and protein mass spectrometry to identify key structural domains in SPHKAP that mediate its RI-

triggered rearrangement. Surprisingly, we find that deletion of RI-anchoring sites in SPHKAP does not block clustering but instead yields constitutive SPHKAP assemblies, suggesting that these sites act as inhibitory modules normally masked by RI anchoring. In contrast, removal of the distal C-terminus abolishes RI-induced clustering. This region is structurally homologous to a poly(ADP-ribose) (PAR)-binding domain. PAR is an RNA-like polymer that can scaffold proteins and nucleate large multimolecular assemblies. Consistent with a PAR-mediated mechanism, SPHKAP-RI cluster size increases upon PARylation induced by hydrogen peroxide. Furthermore, we find that PARylated proteins co-purify with SPHKAP immunoprecipitated from mouse brain, including numerous RNA binding proteins. These proteins co-assemble with SPHKAP when expressed in heterologous cells, and PAR immunolabeling confirms its enrichment in SPHKAP-RI assemblies. Together, these results support a model in which RI anchoring functions as a molecular switch that relieves inhibitory constraints in SPHKAP, promoting PAR-mediated SPHKAP coalescence and organizing ER-associated signaling complexes enriched in RNA-binding proteins. These data suggest a previously unrecognized role for PAR in structuring local kinase signaling domains in neurons.

B205/P1331

Big Tau is Resistant to Toxic Aggregation in Living Neurons: Implications for Treatment of Tauopathies

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In neurons of the adult peripheral nervous system and certain neurons of the central nervous system such as brainstem and cerebellum, a high molecular weight (HMW) isoform of tau with double the size of the projection domain replaces the well-known low molecular weight (LMW) isoforms of tau. Unlike LMW isoforms of tau, this HMW isoform, termed Big tau, includes exon 4a, which encodes approximately 250 predominantly negatively charged hydrophilic amino acids. This holds true across species, despite low sequence conservation of exon 4a. Thirty years after its discovery, Big tau is rarely mentioned in the literature, perhaps because the functional significance of its larger size remains unknown. We have posited that it is the lack of transition to Big tau in most brain neurons that renders them especially vulnerable to tau pathology. Here, cultures of rat hippocampal neurons were transfected to express LMW human tau bearing two pathological mutations that result in rapid formation of toxic tau aggregates that cause degeneration of the neurons. When the mutant tau construct included exon 4a (thus making it Big tau), there was almost no such tau aggregation or toxicity. When the human sequence of exon 4a was swapped for zebra finch's exon 4a, which has almost no homology with the human sequence, the protection against aggregation and toxicity was intact. The protection was also intact when the first 1/3 of the human sequence was deleted but reduced when the last 1/3 was deleted, consistent with a less hydrophilic region toward the beginning of the 4a-encoded sequence and a more hydrophilic and negatively charged region toward its end. We conclude that neurons expressing Big tau are less prone to tau pathology because of the added length and biophysical properties provided by exon 4a. As to why most brain neurons do not avail themselves of the protective properties of Big tau, we found that microtubules associated with Big tau are severed by spastin less efficiently than microtubules associated with LMW tau, which is likely important for differences in average microtubule length that underlie differences in morphology between neurons of the brain and the periphery. Nevertheless, it is compelling to ponder that therapeutic expression of a small amount of Big tau in neurons that compromise the cognitive centers of the brain may prevent or reverse tauopathy-related degeneration.

B227/P1353

Constitutively Active Ras Drives Dendritic Hypertrophy in Drosophila melanogaster Larval Sensory Neurons

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The Ras signaling pathway is critical to a variety of cellular processes, including differentiation, growth, apoptosis, and gene expression. Some of these processes have been extensively studied as mutations in Ras pathway genes lead to many disorders and cancers. Some of these disorders, namely Rasopathies, manifest due to mutations that hyperactivate Ras signaling pathway through rendering the protein products of the mutated genes constitutively active (CA). These rare genetic disorders lead to growth abnormalities and intellectual disability phenotypes. Although there are therapeutics available to address symptoms and/or cancers driven by these diseases, they are only available for patients with specific mutations. Moreover, how the Rasopathy mutations affect nervous system development is incompletely understood. While previous in vitro studies have investigated the effects of CA Ras on neurite extension and spine development, there is a lack of consensus on how Ras affects dendritic development. Ras is a small GTPase that is active when bound to GTP, and inactive when bound to GDP. CA Ras is always active as once bound to GTP it cannot disassociate from it. To study the effects of mutated Ras on dendritic development, we use the Drosophila melanogaster (Dmel) larval multi-dendritic (md) neurons that have varying dendritic complexities, and are just below the translucent larval cuticle, which allows us to image these neurons in vivo using confocal microscopy. In Dmel there are two paralogs of Ras, Ras85D and Ras64B which are closely related to two different human Ras genes: classical and non-classical Ras. Expressing CA Ras85D and CA Ras64B leads to dendritic hypertrophy in Class IV md neurons. Although both paralogs lead to increases in total dendritic length and number of branches, only CA Ras85D expression increases the area covered by the neuron. Additionally, CA Ras85D leads to increases in microtubule levels. Collectively, Ras is a regulator of dendritic development that affects branching and branch lengths in neurons, and this effect is dependent on the paralog.

B236/P1362

Coro1A and TRIM67 collaborate in netrin-dependent neuronal morphogenesis

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Neuronal morphogenesis depends on extracellular guidance cues accurately instructing intracellular cytoskeletal remodeling. Here, we describe a novel role for the actin binding protein Coronin 1A (Coro1A) in neuronal morphogenesis, where it mediates responses to the axon guidance cue netrin-1. We found that Coro1A localizes to growth cones and filopodial structures and is required for netrin-dependent axon turning, branching, and corpus callosum development. We previously discovered that Coro1A interacts with TRIM67, a brain enriched E3 ubiquitin ligase that binds the netrin receptor DCC and is also required for netrin-mediated neuronal morphogenesis. Loss of Coro1A and loss of TRIM67 shared similar phenotypes, suggesting that they may function together in the same netrin pathway. A Coro1A mutant deficient in binding TRIM67 was unable to rescue loss of Coro1A phenotypes, indicating that the interaction between Coro1A and TRIM67 is required for netrin responses. Together, our findings reveal that Coro1A is required for proper neuronal morphogenesis, where it collaborates with TRIM67 downstream of netrin.

B213/P1339
crafting neural messages: the symphony of form, force, and function
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Neurons are known for their intricate cellular morphology. Axons in particular are exceptionally long (100-1000 mm) and ultrathin (100 nm). Their cable-like morphology is essential for conduction of electrical signals, or action potentials, throughout the brain and body. Thus, it has been long assumed that axons are tubular structures with occasional synaptic varicosities. However, our work has challenged this assumption. Using high-pressure freezing to preserve membrane morphology for electron microscopy or super-resolution imaging of live neurons, we performed ultrastructural analysis of axons in Caenorhabditis elegans motor neurons, mouse hippocampal neurons, and human cortical neurons. We discovered that axons are not simple tubes but rather exhibit a pearls-on-a-string morphology through their entire length, with the pearls being ~250 nm and the strings ~70 nm in diameter. This morphology is reminiscent of membrane tubes undergoing tension-driven instability. Consistent with this notion, the pearled area becomes smaller when hyperosmotic solution is applied and larger when hypoosmotic solution is applied. Interestingly, pharmacological perturbation of the cytoskeleton did not greatly alter axon morphology, suggesting that membrane mechanics drives axon morphology. In further support of this, increasing the membrane fluidity by cholesterol depletion from the plasma membrane led to a shrinking of the pearled membrane regions. Functionally, when axon morphology is altered with pharmacological cholesterol depletion, action potential velocity decreases. Similarly, neuronal stimulation that induces plasticity alters the pearled axon morphology. Our in silico modeling further supports our experimental data that membrane mechanics can cause pearled axon morphology and that pearled morphology greatly impacts action potential conductance. These data have revealed for the first time that axons are pearled not tubular, and that pearled axon morphology has an important functional role in neuronal activity and plasticity. I will discuss our initial discovery and current work.

B235/P1361
Cross-species dissection of alpha-spectrin function in neurons
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The cytoskeleton provides mechanical stability, organizes intracellular architecture, and supports cargo transport. These roles are especially critical in neurons, where extreme polarity, long axons, and compartmental specialization demand robust cytoskeletal organization. Disruptions in cytoskeletal components are increasingly recognized as drivers of neurological disease. Among these, more than sixty variants in the gene encoding non-erythroid α-spectrin (SPTAN1) have been reported in neurodevelopmental and neurodegenerative disorders. How different SPTAN1 mutations cause selective neuronal vulnerability and diverse clinical outcomes remains unclear. In Drosophila, the homolog of human α-spectrin is essential for nervous system development and synaptic maintenance, highlighting conserved spectrin-dependent mechanisms in neuronal function. Spectrin, together with actin, forms a conserved periodic lattice within the axonal submembranous cytoskeleton (SMC), revealed by super-resolution microscopy. This dynamic structure acts as a mechanical scaffold and organizes axonal proteins critical for signal conduction and adhesion. The core SMC unit is a heterodimer formed by binding between α- and β-spectrin. Two dimers associate to form $(\alpha\beta)_2$ tetramers, which anchor to actin via β-spectrin, creating the SMC scaffold. The central question is how

disease-linked SPTAN1 variants affect spectrin's structure, stability, and integration into higher-order assemblies, thereby driving neuronal dysfunction. To address this, we use a cross-species strategy combining Drosophila models, human iPSC-derived motor neurons, and patient fibroblasts to dissect α-spectrinopathy mechanisms across molecular, cellular, and organismal levels. Using Airyscan microscopy combined with quantitative image analysis, we examined neuronal compartment morphology as well as the abundance and organization of components of the spectrin scaffold in control and α-spectrinopathy neurons. SPTAN1 variants associated with hereditary spastic paraplegia (HSP) and hereditary motor neuropathy (HMN) differentially affect motor neuron biology by altering cluster formation, modulating α-spectrin abundance, and perturbing interactions with β-spectrin. Complementary Drosophila studies show that neuronal loss of α-spectrin and expression of an HSP-linked variant impair synaptic maintenance and disrupt axonal diameter and stability. Together, our findings demonstrate that spectrin disease-associated variants compromise fundamental aspects of neuronal development and maintenance.

B206/P1332

Cytoskeletal regulation of axonal homeostasis in human motor neurons

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease marked by selective motor neuron degeneration. Understanding what renders motor neurons especially susceptible, particularly their axons, remains a central question. Motor neurons possess exceptionally long axons, making them sensitive to disruptions in axonal homeostasis and highly dependent on mechanisms that maintain cytoskeletal integrity and efficient long-range transport. We hypothesize that dysregulation of cytoskeletal genes impairs axonal resilience and contributes to motor neuron vulnerability in ALS. First, we analyzed transcriptomic datasets for cytoskeletal gene signatures associated with motor neuron resilience versus vulnerability. We compared oculomotor neurons, which are resistant to ALS and remain intact until late-stage disease, to vulnerable spinal motor neurons. Of the significant differentially expressed genes from the GO term “microtubule cytoskeletal organization,” 25 of 31 (80%) were enriched in the resilient oculomotor neurons. In sporadic ALS, 144 of 225 (64%) of the significant differentially expressed genes from this microtubule GO term were downregulated. This suggests that the coordinated regulation of the cytoskeleton is essential for axonal resilience, and disruption of this regulation may contribute to ALS pathology. To probe the functional relevance of key cytoskeletal genes, we investigated the trafficking of the tubulin-binding protein Stathmin-2 (STMN2) and the effects of STMN2 depletion on motor neurons. Using human iPSC-derived neurons expressing endogenously tagged STMN2, we found that STMN2 is actively transported throughout axons at rates comparable to the fast kinesin motor KIF1A, underscoring its importance in distal axons. Importantly, STMN2 depletion using antisense oligonucleotides in motor neurons increased microtubule polymerization rates at baseline, but impaired microtubule recovery following transient nocodazole-induced depolymerization, consistent with a role in buffering soluble tubulin to enable cytoskeletal repair. In parallel, we are investigating MAP7 family members to determine whether similar disruptions in microtubule regulation occur. Together, these results highlight a cytoskeletal gene program critical for maintaining axonal resilience and suggest that its disruption may represent a core mechanism underlying motor neuron vulnerability in ALS.

B231/P1357

Developmentally Regulated Inhibition of Sensory Axon Extension in Response to Iron Chelation

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Gestational iron insufficiency results in altered nervous system development that is not restored upon subsequent iron supplementation. We assessed the effects of treatment with the iron chelators deferoxamine (DFO) and deferasirox (DFX) on axon extension by chicken embryonic neurons at embryonic day (E) 7 and E14 in vitro. In vivo, at E7 sensory axons are actively extending to their peripheral targets while at E14 the majority of axons have reached their targets. Treatment with either DFO or DFX disproportionally attenuated axon extension by E7 neurons relative to E14 neurons that were less impacted. Microfluidic experiments showed that the effects of iron chelation are mediated locally along the axon independent of the cell body. Using DFO as a model, since both DFO and DFX had the same effects on axon extension, DFO treatment simplified the growth cone morphology of E7 but not E14 axons. DFO also decreased the density of axonal mitochondria in a manner correlated with the onset of impaired axon extension. These results suggest that treatment with iron chelators during early pregnancy may retard development of sensory axons, and possibly other axons, and indicate a role for intra-axonal iron in the mechanism of early embryonic axon extension.

B222/P1348

Dipeptide repeats(DPR)-Mediated Regulation of hTERT Splicing and mRNA Stability in ALS

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Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig’s disease, is a fatal neurodegenerative disorder characterized by progressive motor neuron loss, muscle weakness, and paralysis. Several studies have reported increased telomere length in patients with ALS. Therefore, we examined TERT expression and splicing changes as potential contributors to altered telomerase activity in ALS. We established neuronal and glial disease models, including neuroblasts, astrocytes, and microglial cells, by stably expressing poly-GR50. Among these, U-87 MG exhibited the highest and most stable expression of poly GR-50, and thus were selected for further investigation of telomere dynamics and telomerase activity. DPR-Poly GR expressing U-87 MG showed elevated telomerase activity. We found an increase in both telomerase enzymatic activity and stability of certain TERT splice variants including both δEx2 and catalytic active form. Since both splicing variants and the full-length transcript showed increased expression in our results, it raises the issue that not only splicing but also mRNA stability should be evaluated simultaneously. This study aims to elucidate how poly-GR–mediated perturbation in regulation RNA stability contribute to changes in TERT regulation, potentially linking DPR toxicity to altered gene expression in ALS. Keywords : ALS, Lou Gehric’s disease, DPR, TERT, splicing variants

B207/P1333

Environmental Pollutants Bisphenol-A (BPA) and 4-Nonylphenol (4-NP) Disrupt Cytoskeletal Integrity and Gßγ Signaling in Neuronal Cells, Inducing Alzheimer's-Like Pathology

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Background: Alzheimer’s disease (AD) is a leading cause of dementia in the U.S. and is a major public health concern. Recent evidence suggests that environmental factors such as Bisphenol A (BPA) and 4-Nonylphenol (4-NP) could play a significant role in this process. 4-NP and BPA are synthetic organic compounds extensively used in industrial and household products, including plastics, pesticides, detergents, and rubber. Their

environmental persistence, estrogenic activity, and widespread contamination of air, water, and soil have made them significant public health concerns. Although epidemiological and experimental evidence suggests the involvement of 4-NP and BPA in AD, cellular mechanisms remain unclear. Goal: We investigated the pathological effects of BPA and 4-NP exposure on neuronal cells, focusing on microtubule (MT) integrity, Tau phosphorylation/localization, and disruption of G protein βγ (Gβγ) signaling, an essential pathway for neuronal differentiation and MT stability. Methods: This study utilized Human neuronal cells SHSY5Y. Both wild-type (WT) and Gb1g2 overexpressed SHSY5Y cells were used for this study. Stable conditional expression of GNB1 and GNG2 in SHSY5Y cells was developed using a lentiviral expression system (Tet-ON). Both WT and Gbg overexpressed cells were exposed to increasing concentrations of 4-NP or BPA (5mM and 10mM) and the cells were then analyzed using Confocal Microscopy to assess MT-disruption/neuronal damage and immunoblot analysis to test the effect of 4-NP and BPA on the expression/phosphorylation of tubulin/Tau. Results: Our data show that both BPA and 4-NP impair neurite formation, disrupt microtubule networks, and enhance Tau phosphorylation, a key hallmark of AD. Importantly, Gβγ overexpression was able to rescue MT assembly under normal conditions but failed to prevent cytoskeletal damage in cells exposed to BPA or 4-NP. We found that a downstream Gβγ signaling target, Gsk3β, had increased expression after treatment with 4NP and BPA. These findings suggest that BPA and 4-NP can override protective mechanisms by targeting multiple cellular pathways involved in cytoskeletal maintenance. Conclusion: These findings suggest that BPA and 4-NP may serve as environmental triggers for Alzheimer's-like pathology by impairing Gβγ-mediated signaling, destabilizing cytoskeletal structure, and enhancing Tau pathology.

B228/P1354

Epigenetic memory in development and distal microglial activation after demyelination revealed by spatial tri-omics

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Objective: To resolve, within intact tissue architecture, the chromatin, transcriptional, and proteomic programs that coordinate cortical development and neuroinflammation, and to test whether focal demyelination drives long-range microglial responses beyond the lesion rim. Methods: We built a spatiotemporal multiomic atlas using two spatial tri-omics assays: spatial ARP-seq (ATAC-RNA-protein) and spatial CTRP-seq (CUT&Tag-RNA-protein). We profiled sagittal mouse brain sections across postnatal days P0-P21 (cortex and corpus callosum) and an LPC demyelination/remyelination series, and benchmarked matched regions in developing human cortex. Cellular architectures were validated by CODEX multiplexed immunofluorescence and 3D cleared-tissue imaging (TRIC-DISCO). Data were integrated with single-cell references and modeled with generalized additive models to quantify spatial, temporal, and interaction effects; additional analyses parsed histone-repressive mark dynamics (H3K27me3) and motif activity. Results: In cortex, we identified temporal persistence and spatial spreading of chromatin accessibility at layer-defining transcription factors (e.g., CUX2, TBR1) despite declining RNA/protein, consistent with epigenetic memory that outlasts transcriptional output. In the corpus callosum, myelin-gene loci exhibited chromatin priming across lateral-to-medial subregions during the myelination window, and coupling analyses implicated layer-specific projection-neuron axonogenesis in coordinating oligodendrocyte maturation and myelination. In the LPC model, microglia were activated both at the lesion core and at distal white-matter sites aligned with long tracts; distal foci showed delayed induction of disease-associated microglia relative to the rim, alongside OPC proliferation and a transient CD11c+ microglial state, culminating in successful remyelination by day 21. Cross-context comparisons revealed shared modules connecting development and neuroinflammation, including neuron-oligodendroglia coupling and matrix remodeling; H3K27me3 redistribution paralleled transitions between inflammatory and repair states. Conclusion: Spatial tri-omics links chromatin state to cell-state trajectories in vivo, revealing (i) epigenetic

memory that stabilizes cortical arealization and (ii) tract-aligned propagation of neuroinflammation with delayed distal DAM kinetics after focal demyelination. These findings unify developmental and injury responses within a common spatial regulatory framework, suggest testable axon-to-glia signaling hypotheses, and provide a cross-species resource to interrogate multiple sclerosis pathobiology and guide circuit-level and epigenetic interventions.

B234/P1360
Exploring mechanisms of coordinated small and large EV biogenesis from a single neuron in C. elegans
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Animal cells produce diverse extracellular vesicles (EVs) that vary dramatically in size and biogenesis mechanisms — from small submicron-sized EVs to large cell-sized EVs. While these EV subtypes can carry distinct cargoes that either promote cellular fitness or drive pathology, the mechanisms controlling their biogenesis remain poorly understood. A critical unresolved question is how cells coordinately regulate the production of multiple EV subtypes within the same cellular context. We use the nematode Caenorhabditis elegans to investigate how small 100 nm ectosomes and large 3-4 um exophers are coordinately generated from an individual ciliated sensory neuron, providing insight into the cellular and molecular mechanisms that govern EV biogenesis and function in vivo. In C. elegans adult male animals, a set of 21 male-specific sensory neurons shed ectosome EVs from sensory cilia (Wang et al., 2014, 2020, 2021, 2024). These ciliary EVs are released into the environment and function in a signaling capacity. Using ciliary EV markers, we found that the male-specific neurons also release exophers, large EVs that bud from the cell bodies of the neurons. Exophers play neuroprotective functions in non-ciliated touch receptor neurons (Melentijevic et al., 2017). We are currently examining if the regulatory pathway of ciliary ectosome biogenesis affects exopher production and vice versa. The ciliary defective osm-5/IFT88 mutant that abolishes ciliary ectosome release also reduces exopher production in these male-specific neurons, suggesting overlap between EV biogenesis mechanisms of two EV subtypes. We are also determining whether EV cargo packaging is similar between ciliary EVs and exophers, by imaging our battery of fluorescently tagged cargo candidates. Finally, we are investigating how sensory cues such as mating partners affect EV cargo sorting and biogenesis of ciliary ectosomes and exophers. The long-term goal is to understand the mechanisms by which a single cell coordinates biogenesis of multiple EV subtypes in context-dependent manner.

B212/P1338
H3S10ph Changes are Linked to Poly PR Levels in C9 FTD/ALS
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Frontotemporal dementia (FTD) is a fatal, incurable neurodegenerative disease that affects the frontal and temporal lobes of the brain. Amyotrophic lateral sclerosis (ALS) is a motor neuron disease that displays significant overlap in its pathology with FTD. FTD and ALS are mostly sporadic; however, a small percentage of cases are linked to known genetic mutations. Expansions in the Chromosome 9 open reading frame 72 (C9orf72) are the most common mutation in FTD/ALS. These expansions result in the production of dipeptide repeat proteins (DPRs) which aggregate and cause neuronal death. Among these DPRs, Poly PR is most toxic. Epigenetics, heritable changes caused by gene expression with no change in the DNA sequence, might reveal novel pathological FTD/ALS mechanisms and showcase treatment strategies. One prominent epigenetic

mechanism involves the post translational modification (PTM) of histone proteins. These PTMs can impact gene expression. We have previously linked an increase in the phosphorylation of H3 at serine 10 (H3S10ph) with Poly-PR expression in FTD/ALS yeast overexpression models. H3S10ph is associated with actively transcribed genes. Here we will investigate Poly-PR abundance in C9orf72 patient derived fibroblasts to further link histone PTM changes to C9orf72 pathology. We find that C9 fibroblasts revealing increased H3S10ph will also reveal increased Poly-PR levels. Ultimately, these findings will uncover connections between epigenetic pathways and protein aggregation that will reveal novel openings for pharmaceutical therapy of FTD/ALS.

B210/P1336

Identifying the role of signaling protein Gbg in the mechanism of Alzheimer's disease using a Tet-On cell expression system.

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Background: Hyperhosphorylation of Tau is Thought to be associated with neurofibrillary tangle (NFT) formation, a hallmark of Alzheimer’s disease (AD). However, recent studies using brain biopsy specimens from AD and control cases, it has been demonstrated that NFTs are observed in both cognitively impaired and unimpaired individuals, challenging the assumption that tau phosphorylation is essential for AD progression. Another hallmark of AD is amyloid-β (Aβ) plaque deposition. In addition, microtubule (MT) disruption, neuronal loss, and reduced brain mass were consistently reported in AD brain. Our laboratory has identified that Gβγ, a critical component of the G protein–coupled receptor (GPCR) signaling pathway, interacts directly with tubulin/MTs, promotes MT assembly, and maintains neuronal cytoskeletal morphology. We previously demonstrated that pharmacological inhibition of Gβγ by Gallein inhibited neuronal differentiation, affected downstream PI3K/Akt/GSK3β pathway by altering GSK3β activity and inhibiting tau phosphorylation in human neuronal cells SHSY5Y. Goal: In this study, we utilized stable, conditional expression of GNB1 (Gb1) and GNG2 (Gg2) in SHSY5Y cells to investigate the roles of Gβ1γ2 in MT assembly, neurite outgrowth, and their mechanistic contributions to kinase pathway and amyloid-β (Aβ) deposition. Methods: Stable, conditional expression of GNB1 and GNG2 in SHSY5Y cells were created using a lentiviral expression system (Tet-ON). The Tet-On system regulates protein expression by inducing the conformational change of the regulating protein using drugs such as tetracycline or its derivative doxycycline (DOX). Results: Immunoblotting and confocal analysis revealed that DOX induction increased GNB1 and GNG2 expression in a dose-dependent manner, which correlated with enhanced neuronal differentiation, microtubule formation, and neurite growth. Gbg inhibitor Gallein or GSK3b inhibitor Tideglusib abolished neurite outgrowth, with cells appearing rounded. Notably, no-DOX controls exhibited high amyloid precursor protein (APP)/Aβ expression, whereas DOX-induced cells displayed minimal APP expression. APP expression was restored upon Gallein or Tideglusib treatment. Conclusion: These findings suggest that Gβγ expression promotes neurogenesis and may confer neuroprotective properties by regulating APP expression and neuronal differentiation.

B219/P1345

Investigating neuronal regeneration in the absence of a central nervous system in Clytia hemisphaerica.

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After injury, both tissue repair and reinnervation are critical for restoring function. While regeneration in the central and peripheral nervous systems has been extensively studied, far less is known about how systems

lacking a central nervous system respond to injury. Cnidarians provide a unique opportunity to address this gap, as they lack a centralized nervous system and instead rely on simple neuronal circuits. The hydrozoan Clytia hemisphaerica is an emerging model for studying nervous system regeneration. Like other cnidarians, Clytia medusae possess a diffuse “nerve net” embedded within the contractile subumbrella layer. Previous work has shown that neurons genetically ablated in Clytia can recover, but whether new neurons are generated remains unknown. Using immunofluorescence, we first established a tentative baseline count of neurons in the subumbrella layer. We then removed a gonad and allowed the medusae to heal for varying time intervals, comparing neuronal counts in wounded and unwounded regions. Future studies will focus on the cellular and molecular mechanisms underlying this regenerative process. Understanding the regenerative potential of this ancient nervous system may provide new insights into the evolution of neural repair.

B232/P1358
Measuring Microtubule Dynamics in Growing Neurons
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Neurons display a distinctive morphology with many branched dendritic processes and an axon which differ in subcellular organization. Microtubules play a critical role in the development of this complex cellular and subcellular structure and serve as tracks for the transport of materials essential for growth. However, the interplay between microtubule behavior and neuronal morphogenesis remains unclear. We are using automated microscopy and photoconversion experiments to quantitatively measure microtubule movements and turnover throughout entire processes in primary mouse hippocampal neurons developing in vitro. Our preliminary results indicate that microtubule behaviors depend not only on the identity of the process (i.e. axon or dendrite) but also on location within the process and developmental timing. We are also performing both large-scale fixed imaging and live time-lapse imaging to understand the statistics and dynamics of process growth. We are using these measurements to construct biophysical models that connect the underlying cytoskeletal dynamics of microtubules to neuronal process dynamics.

B225/P1351
Membrane-associated periodic skeleton regulates major forms of endocytosis in neurons through a signaling-driven positive feedback loop
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Endocytosis is an evolutionarily conserved process that enables neurons to internalize signaling receptors and membrane proteins, maintaining cellular homeostasis and supporting rapid responses to extracellular cues. The neuronal membrane-associated periodic skeleton (MPS), a lattice-like cytoskeletal structure composed of actin and spectrin, has been shown to restrict clathrin-mediated endocytosis (CME) at the axon initial segment of neurons, by gating clathrin-coated pit formation through membrane-localized “clearing” structures that are devoid of MPS. However, the extent to which the MPS regulates diverse forms of endocytosis across neuronal compartments, and how it is dynamically remodeled to permit trafficking on demand, remain unknown. While CME is relatively well characterized in neurons, the subcellular localization and physiological relevance of caveolin-mediated endocytosis, flotillin-mediated endocytosis, and fast endophilin-mediated endocytosis (FEME) have remained largely unclear. Here, we show that all four major endocytic pathways—CME, caveolin-, flotillin-, and FEME— are spatially gated by the MPS and occur specifically within MPS-free “clearing” zones

distributed across both axonal and somatodendritic compartments of mature neurons. These results, for the first time, map the spatial landscape of these lesser-understood pathways in neurons and reveal a unifying principle of cytoskeletal gating across endocytic mechanisms. Disruption of the MPS markedly enhances both basal and ligand-induced endocytosis across all four pathways, establishing its broad inhibitory role in pit initiation. We further discover that endocytosis can, in turn, remodel the MPS through a novel signaling-driven feedback loop: ligand-triggered endocytosis activates ERK signaling, which promotes calpain- and caspase-mediated spectrin cleavage. This targeted cytoskeletal degradation facilitates subsequent endocytosis, forming a self-reinforcing circuit that couples membrane trafficking with cortical architecture remodeling. Finally, we show that the MPS limits amyloid precursor protein endocytosis and thereby suppresses amyloid-β 1-42 production and neuronal apoptosis, implicating MPS integrity in the regulation of neurodegenerative processes such as Alzheimer's disease. Together, our findings establish the MPS as a dynamic, signal-responsive modulator of endocytosis and neuronal health. This work uncovers a general spatial gating mechanism that applies to diverse endocytic pathways, introduces a cytoskeleton-centered feedback loop for signal-dependent remodeling, and expands the functional significance of the MPS from passive structural support to active regulation of neuronal homeostasis and disease susceptibility.

B214/P1340

Mitochondria are absent from microglial processes performing surveillance, chemotaxis, and phagocytic engulfment

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Microglia are the resident phagocytes of the central nervous system (CNS). Microglia continually extend and retract their highly branched processes to perform tissue surveillance and respond to infection or injury. In the case of focal injury to the CNS, microglia processes immediately migrate (i.e. chemotax) to the site of the injury thus preventing further damage from spreading. Microglial phagocytosis along with coordination from other brain cells ultimately facilitates the return to homeostasis from this inflammatory injury. Tissue homeostasis is also linked to microglial metabolism as increased ROS production, mitochondrial DNA damage, and metabolic dysregulation are all prevalent in microglia from aged and neurodegenerative tissue. However, it is unclear how mitochondria are subcellularly partitioned in a healthy state as microglia perform their multifaceted functions of surveillance, chemotaxis, and phagocytic engulfment in the live intact brain. To study mitochondria in microglia we generated a triple transgenic mouse that has fluorescent reporters to visualize mitochondria specifically in microglia. Cranial window surgeries and intravital imaging were performed to study mitochondrial morphology and motility in the live animal thereby maintaining microglia in their native microenvironment. First, we found that mitochondria are not uniformly distributed in microglia processes with some processes containing many mitochondria while others have very few. Microtubules and hexokinase 2 mirror this uneven mitochondrial distribution indicating that these cytoskeletal and metabolic components are linked to mitochondrial organization in microglia. Surprisingly, most of the processes that engage in minute-to-minute surveillance do not have mitochondria. Moreover, mitochondrial motility is not altered by animal anesthesia even though microglial process motility is. Additionally, microglial processes that are responding to a focal lesion or engaging with a neuron undergoing programmed cell death do not contain mitochondria. Rather, the mitochondria have a delayed arrival into the microglia processes responding to these injuries. Together, these data reveal the heterogeneity in the mitochondrial subcellular distribution in microglia during surveillance, chemotaxis, and phagocytic responses.

B223/P1349
Neuropeptides regulate GABAergic neuronal remodeling in C. elegans
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Neuronal remodeling is essential for learning and behavioral modulation. Altered neuronal remodeling is implicated in neurodevelopmental disease, the underlying mechanisms of which are not fully understood. C. elegans is an ideal model system in which to study neuronal remodeling due to its small and well-characterized nervous system. Our lab investigates neuronal remodeling of a single GABAergic neuron called DVB. DVB modulates defecation behavior in both sexes, but in the male, DVB undergoes progressive neurite outgrowth that contributes to male mating behavior, including spicule protraction. DVB neurite morphology and spicule protraction behavior are altered under different conditions, such as mating experience and starvation. To identify the molecular mechanisms that regulate this neuronal plasticity, we compared the DVB neuron transcriptome of adult male and hermaphrodite C. elegans to identify genes that are differentially expressed - which could potentially function in male-specific DVB remodeling. Several neuropeptide-encoding genes are highly upregulated in the male DVB neuron compared to the hermaphrodite, so we hypothesized that neuropeptides (NPs) play a role in DVB neuronal remodeling. NPs are small molecule neurotransmitters that modulate neuronal activity and influence behaviors like locomotion, appetite/satiety, and sleep. To assess the influence of NPs on male DVB morphology and spicule protraction behavior, we first examined mutants with disruptions in NP synthesis and secretion pathways. A loss of function mutant in unc-31, which encodes a protein required for NP secretion, had longer neurites and more branches, as well as altered spicule protraction behavior. A loss of function mutation in egl-21, a gene encoding a conserved NP-processing enzyme, led to increased neurite branch length and number of branches. We are currently re-introducing the egl-21 gene in DVB alone and in all neurons to determine if NPs act cell- or neuron-autonomously, respectively, to modulate DVB remodeling. We also tested individual NP genes that are more highly expressed in the male DVB neuron to determine which NPs play a role in modulating DVB remodeling. Using endogenous transcriptional reporters, we confirmed the differential expression of two NPs in DVB, flp-28 and nlp-73, and found that flp-28 expression increases in DVB from day 1 to day 3 in males. Loss of function mutants in the NP genes for flp-28 and nlp-6 have altered DVB morphology and spicule protraction behavior. These findings provide strong evidence that NPs contribute to experience-dependent neuronal remodeling, offering insights into their broader roles in both healthy and diseased brains.

B221/P1347
Probe TDP-43 pathology and cryptic exon inclusions in human assembloid models for ALS
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Amyotrophic lateral sclerosis (ALS) is a fatal, adult-onset neurodegenerative disease characterized by progressive motor neuron degeneration, leading to muscle paralysis and eventually death. Many cases of ALS pathology involve TDP-43 nuclear depletion with cytoplasmic aggregation. Beyond aggregation, loss of TDP-43's RNA-binding function disrupts splicing control, driving inclusion of cryptic exons and premature polyadenylation that truncate key transcripts; a leading example is STMN2, whose depletion impairs axonal maintenance and regeneration. To examine how these events map onto upper (UMN) versus lower (LMN) motor-neuron vulnerability, we built a 3D human corticospinal "assembloid" by fusing human induced

pluripotent stem cell (hiPSC)-derived cortical organoids enriched in corticospinal neurons with spinal organoids containing LMNs, enabling interneuronal connectivity. We obtained ALS patient derived hiPSCs with C9ORF72 mutations with age-match controls for assembloid generation. Organoids were profiled at 30, 60, and 90 days for neuronal identity, viability, and inter-organoid axon projection, and we quantified TDP-43 mislocalization and cryptic-exon (CE)–containing STMN2 transcripts. We observed an age-dependent decline in motor-neuron counts by day 90, robust fusion with bidirectional axon growth that diminished over time in ALS lines, increased TDP-43 nuclear clearance with cytoplasmic accumulation versus controls, and a burden of CE-STMN2. These data support a model in which TDP-43 loss of function and STMN2 truncation contribute to corticospinal circuit failure. The platform provides a human system to dissect sequence and cell-type specificity of degeneration and to test STMN2-restoring or TDP-43–rescuing interventions.

B226/P1352
Regulation of cGAS-STING innate immune sensing and the herpes simplex type-1 latency program by heparanase
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The cyclic GMP-AMP synthase–stimulator of interferon genes (cGAS–STING) pathway is a central component of innate immunity, responsible for detecting cytosolic DNA and initiating antiviral responses. Herpes simplex virus type-1 (HSV-1), a neurotropic virus, establishes latency in sensory neurons and evades immune detection through poorly understood mechanisms. Our study investigates the role of heparanase (HPSE), an endoglycosidase known for remodeling the extracellular matrix, in regulating cGAS-STING activity during HSV-1 infection. Using in vitro and in vivo models, we report that HPSE suppresses the cGAS-STING pathway which contributes to viral reactivation. HPSE knockouts attenuate viral reactivation through an increased antiviral state and upregulated interferon signaling. These findings reveal a novel immune evasion strategy employed by HSV-1 and identify HPSE as a potential therapeutic target for modulating latency and reactivation.

B233/P1359
Septin-positive membrane curvatures are endoplasmic reticulum-plasma membrane contact sites in neuronal dendrites
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Septins are a family of GTP-binding proteins that preferentially associate with micrometer-scale domains of plasma membrane curvature. Previous studies have shown that septins can both inhibit and promote interactions between endoplasmic reticulum (ER) and plasma membrane (PM) store operated calcium channels STIM1 and Orai1 at ER-PM contact sites (ERPMCs). In neuronal dendrites, recent work indicates that the ER forms periodic junctions, which are essential for calcium signaling. In neuronal dendrites, septin complexes comprising of the Sept5/11/7 subunits localize to the base-neck regions of dendritic filopodia and spines, suggesting a potential role in organizing specialized membrane domains. Here, we investigated whether Sept7-positive sites of membrane curvature serve as docking sites for the endoplasmic reticulum. In cultured E18 rat hippocampal neurons, we performed immunostaining for septin 7 and septin 5 and analyzed their colocalization with tubular ER marker reticulon 4 (RTN4/Nogo), and the ERMC markers MAPPER and extended synaptotagmin using super-resolution SoRa spinning disk microscopy. We also used proximity ligation assays to assess molecular interactions at these sites. In parallel, we examined TAOK2, a kinase known to phosphorylate

septin7 and mediate ER linkage to microtubule plus ends. At the base of dendritic filopodia, ER tubules (RTN4/Nogo) make orthogonal contacts with Sept7-enriched domains of membrane curvature. Time-lapse imaging showed ER membranes (mCherry-Sec61) docking transiently onto Sept7-GFP domains of membrane curvature and entering filopodia. Preliminary quantifications show that 60-80% of septin (Sept5/7) positive membrane curvatures colocalize with MAPPER and extended synaptotagmin, and ~90% colocalize with TAOK2. Proximity ligation assays, which detect interactions between proteins within 40 nm distance, also resulted in positive signal between Sept5 and TAOK2 at dendritic branch points. Ongoing experiments aim to determine whether septin 7 enriched ERPMC sites act as signaling platforms that promote dendritic branch formation in coordination with TAOK2.

B224/P1350

Shatavarin IV imparts protection against LPS-induced inflammation and oxidative stress in SHSY5Y cell lines - implications in the TrkB-BDNF axis

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Shatavarin IV, a steroidal saponin produced in abundance, especially in the roots of Asparagus racemosus, is a traditionally recognized phytotherapeutic for the treatment of a plethora of human diseases. In the present work, the neuroprotective action of Shatavarin IV was demonstrated in SHSY5Y cell lines. Treatment with Asparagus racemosus (Shatavari) root extract (1 µg/ml) was able to successfully revert the inflammatory response and oxidative stress generated by lipopolysaccharide (LPS; 2.5 µg/ml) induction. In Shatavari treated cells, qPCR of cytokine transcripts demonstrated that the levels of the proinflammatory cytokines IL6 and TNF-α were reduced by 45% and 37% respectively whereas those of the anti-inflammatory cytokines IL-10 and TGF-β were upregulated by more than thrice the original values. Shatavari also resulted in significant reduction of relative reactive oxygen species (ROS) levels by 43% as compared to that assessed in LPS treated cells. These responses were significantly more prominent when pure Shatavarin IV (100 ng/ml) was used instead of the crude root extract confirming that the neuroprotective effects were actually mediated by Shatavarin IV. Addition of Brain Derived Neurotrophic Factor (BDNF), a small protein mediating neuronal growth and synaptic plasticity, resulted in further augmentation of Shatavarin IV mediated protection which led to the hypothesis that the phytotherapeutic exerted its protective effect by simulating the Tropomyosin Kinase (TrkB)-BDNF axis either alone or in conjunction with BDNF. Administration of Trk-B inhibitor K-252a dampened Shatavarin IV mediated protection which was in unison with our hypothesis. In silico docking of Shatavarin IV-BDNF complex with TrkB receptor tyrosine kinase resulted in a considerably strong binding energy of -10.3 kcal/mole whereas that of Shatavarin IV alone was slightly weaker at -9.9 kcal/mole employing a combination of Pi-alkyl bond, alkyl bond, hydrogen bond and Pi sigma bond in both cases. The interaction of Shatavarin IV with receptor tyrosine kinases (RTKs) was also corroborated indirectly by STITCH database which revealed strong interaction of Shatavarin IV with imatinib and dasatinib, two potent inhibitors of RTKs, The results cumulatively provided significant mechanistic insights to design Shatavarin IV or other structurally similar phytochemical-based drugs for augmentation of neuronal health and prevention of the onset of neurodegenerative disorders.

B209/P1335

SNX-RGS proteins are conserved phospholipid transporters which loss causes lysosome membrane lipid

disruption and neurodegeneration

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Recent advances in genomics, cell and structural biology suggest the involvement of inter-organelle lipid transport proteins (LTPs) in the pathogenesis of neurodegenerative disorders. LTPs bind and transport lipids between organelles influencing membrane integrity and aiding in membrane repair following damage. Such membrane repair is important for neurons, yet the mechanisms and machinery that maintain organelle membrane integrity remain elusive. Here, we reveal that members of the SNX-RGS protein family linked to spinocerebellar ataxia recessive 20 (SCAR20) encode a novel LT domain that binds to multiple phospholipids with a preference for phosphatidic acid (PA), a non-bilayer lipid which perturbs membranes when accumulated. In line with this, SNX-RGS protein depletion leads to pathological accumulation of PA and diacylglycerol on lysosomes, disrupting lysosome lipid composition. Following lysosome membrane perturbation, we find SNX-RGS proteins, SNX13 or SNX14 are recruited to damaged lysosomes, and their loss perturbs lysosome membrane damage repair in both HeLa cells and hPSC-derived neurons. Furthermore, our recent genetic studies have uncovered SNX13 mutations in a cohort of patients diagnosed with a novel SCAR20-like neurodegenerative disorder, supporting an overlapping role for SNX-RGS proteins in neuronal function and survival. These findings suggest that SNX-RGS proteins mediate multi-organelle lipid transport to act as neuronal lysosome quality control factors to safeguard lysosome membrane composition and aid membrane repair following lysosome membrane permeabilization (LMP). On-going work is focused on mechanistically defining how SNX-RGS proteins sense and repair LMP, including how their loss leads to neurodegeneration.

B215/P1341

Spastin as a therapeutic target for axonal degeneration and injury

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Axonal degeneration is a hallmark of many neurodegenerative disorders and a major obstacle to recovery after central nervous system injury. Spastin, mutated in Spastic Paraplegia type 4 (SPG4), is best known for remodeling microtubules (MTs), but its broader roles in axonal maintenance and repair have remained elusive. Spastin is produced as two isoforms from alternative translation initiation: M87, a cytosolic isoform with potent MT-severing activity essential to MT turnover, and M1, a membrane-associated isoform linked to ER morphology, organelle contacts, and calcium homeostasis, pathways potentially independent of severing. Here, we show that spastin is key for axonal regeneration and neuronal function in both rodent and human cortical neurons. Spastin suppression caused profound deficits in axonal regrowth, reduced synapse formation, and impaired neuronal activity, indicating a critical role in structural and functional repair. Remarkably, chemogenetic enhancement of neuronal activity via Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) restored regenerative growth in spastin-deficient neurons, establishing a mechanistic link between activity-dependent signaling and spastin function. Furthermore, restoring M1-spastin expression in spastin-deficient neurons rescued both axonal regeneration and neuronal activity, pointing to ER- and calcium-dependent signaling mechanisms unique to M1 that work in concert with the MT-severing activity of M87. Together, these findings position spastin as a master regulator of axonal integrity across contexts of both neurodegeneration and injury. By uncovering converging mechanisms that couple cytoskeletal remodeling (M87-driven) with ER-mediated calcium signaling (M1-driven), this work identifies spastin as a versatile, targetable node for intervention and a promising therapeutic avenue to restore axonal integrity where degeneration or trauma is the primary pathological driver.

B204/P1330

Tau Oligomers Destabilize the Nuclear Membrane and Drive Chromatin Reorganization in Alzheimer's Disease

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Nuclear membrane integrity is increasingly recognized as a potential regulator of neuronal health, yet its mechanistic role in pathogenesis of Alzheimer's disease (AD) remains unclear. Here, we identify a direct link between tau oligomerization and nuclear lamina disruption. Postmortem AD brain tissues exhibited severe nuclear lamina disorganization in neurons containing pathological tau. In PS19 tauopathy mice, oligomeric tau (oTau) was found to directly bind the Lamin B Receptor, leading to nuclear lamina disruption and aberrant nuclear invaginations. Transmission electron microscopy revealed these invaginations were accompanied by chromatin remodeling, suggesting downstream transcriptional dysregulation. To investigate the dynamics of this process, we employed a light-inducible OptoTau system (4R1N Tau::mCherry::Cry2Olig) in human iPSC-derived neurons, which result in a real-time recruitment of oTau to the nuclear membrane. Live-cell imaging, immunocytochemistry, and Western blotting demonstrated that optogenetically induced tau oligomerization rapidly triggered nuclear invagination and deformation. These findings indicate that nuclear membrane disruption is not merely a byproduct of tau pathology, but an early, possibly causative event in neurodegeneration. By establishing a mechanistic link between tau oligomerization, nuclear architecture collapse, and chromatin reorganization, this work provides new insight into nuclear vulnerability in AD and suggests that stabilizing the nuclear envelope may represent a novel therapeutic strategy.

B218/P1344

The Microglia Forebrain Assembloid Model Recapitulates Human Brain Development and Neuroimmune Biology

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Microglia represent innate immune cells in the central nervous system (CNS), with indispensable involvement in physiological and pathological processes to maintain neural homeostasis, networking, and synapse pruning; yet, their dysregulation drives neurodegenerative diseases, notably Alzheimer's disease and Multiple sclerosis. However, distinctions between murine and human microglia are a significant obstacle to comprehensive translational research. Research on human microglia has been limited by reliance on postmortem or post-surgery tissue and the accuracy of tissue culture models. We have developed a co-culture, "assembloid," combining human iPSC-derived brain organoids and microglia for a closer recapitulation of human tissue biology than any single culture model allows. In order to develop the assembloid model, we differentiated immune and neural cells in parallel along a defined course to optimally match human brain development at the stage of neuroimmune invasion. Microglia were differentiated from hematopoietic progenitor cells (HPC) and verified with TREM2, CD45 and CD11b expression. Simultaneously, forebrain organoids were differentiated through a 3D developmentally guided protocol. Microglia were then introduced to the forebrain organoid after primary neural proliferation, but prior to astrocyte development to replicate the normal co-development of these cell types. By recapitulating human brain and neuroimmune development, we saw a robust invasion of microglia into the forebrain organoids, with some localization near radial glia cells. Immunohistochemical staining for microglia-glial interactions showed earlier markers of maturity and more complex morphology compared to our control forebrain organoid culture. This suggests neuroimmune signaling accelerates

astrocytes' differentiation in the assembloid and supports tissue patterning in the developing brain. For a more systemic view of our model, we used metabolomic profiling. Mass spectroscopy (MS) detected ceramide, sphingolipids and lipid-mediated signaling that were unique to the assembloids. Lipid ontology analysis identified these as neuroimmune associated metabolites, normally present in the human brain, demonstrating a more comprehensive recapitulation of the human metabolome by the assembloid model. Continuing work includes deeper molecular profiling of neuro-immune and glial-immune co-patterning with histological markers and metabolomic comparisons to human brain tissue. The human forebrain assembloids developed here offer a model of human brain tissue patterning in development and homeostasis. Neuroimmune assembloid provides a more humanized platform for drug screening in neurodegenerative studies.

B230/P1356

The opsin homologue SRO-1 modulates light avoidance in a specific wavelength, by genetically interacting with the photoreceptor LITE-1 in C. elegans

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Opsins are light-sensitive G-protein–coupled receptors (GPCRs) widely used for photoreception across the animal kingdom. The nematode Caenorhabditis elegans encodes a single opsin-like protein, SRO-1, which is homologous to mammalian melanopsin expressed in intrinsically photosensitive retinal ganglion cells (ipRGCs). However, SRO-1 lacks the conserved retinal-binding lysine required for direct photon absorption. Although opsins are central to light detection in most animals, no functional opsin has been reported in C. elegans, raising the question of whether SRO-1 has a role in photoreception. C. elegans is known to have a photoreceptor named LITE-1 which functions to respond from short-wavelength light to blue light. Using lite-1, sro-1 and other photosensitive mutant animals, we performed several behavioral analyses and found that SRO-1 modulates avoidance behavior to UV/violet light through pathways associated with photoreception. The sro-1 null mutant animals displayed wavelength- and intensity-dependent defects in avoidance from UV/violet light, but not from blue light. Genetic epistasis analyses revealed that sro-1 functions in the same pathway with lite-1 in UV/violet light avoidance. Fluorescent reporter analyses showed SRO-1 localization to the plasma membrane in multiple sensory neurons, including ALM, PLM, and PHA where LITE-1 is known to mediate light avoidance, suggesting functional interaction between these two receptors. Ectopic expression experiments using muscle cells indicated that SRO-1 alone does not confer light sensitivity, however, co-expression of SRO-1 with LITE-1 enhanced UV-triggered responses at low light intensities compared to LITE-1 alone. These results suggest that SRO-1 either increases LITE-1's sensitivity at the low-intensity of UVA/violet light or facilitates downstream signaling in a context-dependent manner. Our results identify SRO-1 as a non-canonical opsin-like GPCR contributing to light avoidance in C. elegans and highlights a previously unrecognized mechanism for the detection of specific wavelengths under low-light conditions. This mechanism may be acquired for an adaptation to soil environments where UV penetration is naturally attenuated.

B229/P1355

The role of Centrocortin in neurodevelopment

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Modulation of the c-Myc oncogene by the paraneoplastic antigens CDR2 and CDR2L contributes to paraneoplastic cerebellar degeneration (PCD), an immune-mediated disorder marked by Purkinje neuron degeneration. We previously identified an embryonic role of Centrocortin (Cen), the sole Drosophila ortholog of CDR2/CDR2L in maintaining mitotic fidelity. However, the molecular mechanisms linking Cen to neuronal integrity and oncogenic signaling remain elusive. Here we report the neuronal function and molecular targets of Cen using custom Drosophila constructs and proteomics analyses. We demonstrate that depletion and neuron-wide over-expression of Cen showed significantly increased disruption in mushroom body, a neuronal structure analogous to the human hippocampus. We also observe locomotor defects in adult Drosophila, as measured by negative geotaxis assay (NGA). Moreover, expression of a Cen transgene rescues mushroom body defects in a cell-autonomous manner. We further find that Cen dysregulation alters Myc expression level in Drosophila larva brain extracts and reduces Myc within the proliferative neural stem cells (NSCs), progenitors of the neurons and glia. Ongoing proteomic analyses indicate that Cen interacts with factors involved in cytoskeletal organization, genome stability, and neuroanatomical development. Collectively, these findings establish Cen as a key regulator of neuronal structure and function. The interaction of Cen with cytoskeletal regulators supports its role in neurodevelopment and points to a mechanistic link between mitotic integrity, oncogenic signaling, and neurodegenerative processes. Our study highlights Cen as a valuable genetic model for PCD research.

B208/P1334

The Role oMAP4 in Organizing the Dendritic Microtubule Array

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Microtubule-associated proteins (MAPs) interact with microtubules to regulate their organization and properties. While tau is the best-known neuronal MAP, its closest relative that is expressed broadly across cell types is MAP4. Recent studies on muscle cells have revealed that the MAP4 gene undergoes alternative splicing to generate not only the uMAP4 protein that has been known for decades, but also a newly discovered isoform called oMAP4, which has an entirely different projection domain. oMAP4 is a strong microtubule crosslinker that immobilizes the microtubules that it crosslinks. Here we sought to determine whether oMAP4 might exist in neurons to help regulate microtubule mobility and organization. For this, we generated new antibodies to examine (with western blotting and immunocytochemistry) the expression pattern, tissue distribution, and subcellular distribution of oMAP4 in the nervous system. In rodent tissue from various ages, we found far greater levels of oMAP4 in some neurons (ex. hippocampus) than in others (ex. spinal cord). In cultured rat hippocampal neurons, we observed low levels of oMAP4 in immature neurites and axons, but significantly greater levels in dendrites, once they had developed. A recently reported feature of hippocampal dendrites is that minus-end-out microtubules form a central bundle within the dendrite while plus-end-out microtubules are dispersed around that bundle. This is a potential explanation for how dendrites manage organelle transport within a mixed-orientation microtubule environment. We found the localization of oMAP4 overlaps with these central bundles. We are exploring the possibility that oMAP4 plays a role in segregating microtubules of the two different orientations within the dendrite by crosslinking the minus-end-out microtubules into a relatively immobile central bundle, allowing for more efficient organelle transport by creating two lanes of traffic for motor proteins. Comparisons were also made to superior cervical ganglion neurons which do not express oMAP4 and do not have microtubule orientation segregation. Little is known about how corruption of the dendritic microtubule array contributes to the dendritic atrophy seen in neurodegenerative diseases, like Alzheimer's, where it is already known that MAP4 expression is altered. Corruption of oMAP4's role in dendrites may contribute to this atrophy.

B216/P1342
WNT Signaling Dysregulation in ALS: A New Lead in Uncovering the Molecular Basis of the Disease
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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by progressive loss of motor neurons, leading to muscle paralysis and ultimately respiratory failure. A clearly defined genetic background has been identified in fewer than 10% of cases, with one of the most frequent mutations being the point mutation P525L in the RNA-binding protein FUS. Our preliminary spatially resolved RNA sequencing data of patient-derived iPSCs carrying the P525L mutation revealed an upregulation of transcripts related to both canonical (β-catenin–dependent) and non-canonical (β-catenin–independent) WNT signaling pathways in the axons. These pathways play a central role in intercellular communication and regulate critical processes such as cell fate determination, polarity, cytoskeletal remodeling, proliferation, axon guidance as well as synaptic formation and maintenance. The observed upregulation of both canonical and non-canonical WNT components in patient-derived axons may provide new insights into the poorly understood molecular mechanisms underlying ALS. Furthermore, disturbances in WNT signaling are increasingly recognized as common features in other major neurodegenerative disorders, including Alzheimer's, Parkinson's, and Huntington's diseases. Using C. elegans we aim to detect and quantify changes in the localization of WNT signaling pathway proteins in the context of mutated FUS throughout adulthood and aging.

Neuronal Cell Biology - Synapse organization and function

B237/P1363
a-Synuclein and AP2 Localization in Different Nanodomains of the Synapse.
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α-Synuclein mutation and aggregation are associated with several neurodegenerative disorders, including Parkinson's disease, dementia with Lewy bodies and multiple system atrophy. α-Synuclein is expressed in the presynaptic compartment where it regulates clathrin mediated synaptic vesicle endocytosis. We have shown that α-synuclein regulates clathrin lattice size and curvature in vitro and identified a direct interaction between the heterotetrametric clathrin adaptor protein-2 (AP2) and α-Synuclein. α-Synuclein binds to isolated synaptic membranes in an ATP-dependent manner, similar to AP2 and the monomeric adaptor protein, 180 KDa (AP180), suggesting that α-Synuclein, AP2 and AP180 share a common synaptic membrane binding pathway. We also have shown that α-synuclein interaction with AP2 is necessary to maintain normal levels of AP2 on synaptic membranes. These findings demonstrate that α-Synuclein plays a critical role in stabilizing AP2 on synaptic membranes, an event which is required for initiation of clathrin-mediated synaptic vesicle endocytosis. While α-Synuclein link to synaptic vesicle recycling is clear, the precise location of α-synuclein at the nanoscale level in the various synapse domains remains unreported. The goal of this research is to examine the distribution of α-Synuclein and AP2 in the synapse under physiological conditions at a nanometer resolution. Here, we employ two synapse models, the <1µm diameter mouse synapse and the 1-2µm diameter lamprey giant synapse and improve its size and resolution through Magnify-expansion microscopy. Magnify-expansion microscopy physically enlarges the mouse and lamprey sample and improve the final image

resolution for confocal microscopy visualization. We have demonstrated a roughly three and six-fold expansion making the synapse reach approximately 3µm for the mouse and 12µm for the lamprey synapse, respectively. We observed that α-synuclein and AP2 can be found in several subdomains inside the synapse forming distinct nanodomains in the synaptic cluster, active zone, and periactive zone. In addition, we defined different areas using distinct synaptic markers to describe the localization of α-Synuclein and AP2 nanoclusters. The findings in this study provide novel and intriguing perspectives on the physiology of synapses and add a valuable tool for elucidating underlying pathogenesis mechanism and testing potential disease-modifying treatments.

B239/P1365
Developing an antibody panel for cell surface receptors at the synaptic cleft
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The Institute for Protein Innovation is a non-profit organization developing recombinant antibody panels targeting the human and mouse neuronal surfaceome. We focus on neurobiological pathways where there is a need for validated, reproducible affinity reagents. Using display technologies and machine learning, we systematically generate antibody panels that cover entire receptor families. This approach reduces antibody cross-reactivity and produces large, consistent sequence datasets that can be used for machine learning applications to develop further and optimize these antibodies. Our synaptic cleft antibody panel covers entire receptor–ligand families including neurexins/neuroligins, teneurins/latrophilins, ephrin receptors and (proto)cadherins. We are mapping cross-family interactions and identifying how they can be modulated using our antibodies. Each reagent is evaluated for binding kinetics, potency, specificity, and aggregation, and formulated as chimeric human/rabbit antibodies. These antibodies are distributed through Addgene following community validation. We are establishing a crowdsourced validation system with Addgene to build a reliable set of reagents supporting studies in neuronal migration and synaptogenesis. We also welcome input on additional targets that would benefit the field.

B243/P1369
ER-resident tethering protein, VAP, regulates presynaptic organelle calcium handling and synaptic transmission.
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Membrane contact sites (MCS) are a common structural feature in cell biology with both physiological and pathological implications. They exist in even the most polarized subcellular compartments, including the presynaptic terminal of neurons, where they play critical roles in intracellular communication. We recently demonstrated that dynamic uptake of calcium (Ca2+) into the presynaptic endoplasmic reticulum (ER) during electrical activity relies on MCS formed between the ER and plasma membrane formed by voltage-gated potassium channel, Kv2.1. Interestingly, this uptake is not a result of potassium conduction. Rather, it occurs through a noncanonical FFAT motif contained within Kv2.1's C-terminus. Loss of the Kv2.1 C-terminus impairs neurotransmission and ER Ca2+ uptake by more than 50%. Previous studies in heterologous cells suggest that Kv2.1 forms ER/PM MCSs through interaction with VAMP-associated proteins (VAPs) embedded in the ER membrane. To further investigate VAP's molecular function in the axon, we developed a CRISPR-based knockdown (KD) approach to acutely deplete both isoforms of VAP (VAPA and VAPB) in hippocampal neurons. We combined this knockdown approach with noninvasive optical indicators of synaptic vesicle exocytosis and subcellular Ca2+ in cultured primary rat hippocampal neurons. We demonstrate that loss of VAPA/B decreases

vesicle fusion (>50%), disrupts the formation of Kv2.1 clustering, and impairs Ca2+ influx during electrical activity within both mitochondria (>75%) and the endoplasmic reticulum (>50%). Impaired synaptic transmission by loss of VAP is caused by change in vesicle distribution between the recycling and reserve pools of synaptic vesicles suggesting a new presynaptic role for MCS mediated by VAP in synaptic transmission.

B240/P1366
Investigation of TACC's role in regulation of pre-and-post synaptic factors at the Drosophila neuromuscular junction
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Regulation of synaptic factors is essential for proper neuromuscular junction (NMJ) development. This specialized synapse is responsible for the translation of electrical stimulus to chemical work, branching the neuron to the muscle fiber, and facilitating muscle contraction. A multitude of pre and post synaptic proteins are responsible for maintaining structural changes, proper neurotransmitter release/collection, and overall function of the NMJ's active zones. Improper NMJ regulation can be associated with human neuromuscular disorders, therefore it is necessary to develop an understanding of the factors that may influence proper development and maintenance. Our target, dTACC is a protein coded from a family of genes known as transforming acidic coiled-coil (dTACC), and is localized at the pre-and-post synapse. Previous studies conclude that NMJ growth is negatively regulated by presynaptic dTACC. This finding inspired our investigation into how dTACC may regulate NMJ development postsynaptically. Using fluorescent microscopy; our experiments targeted the colocalization of different postsynaptic factors, and the accumulation of these factors when dTACC is expressed vs silenced. We find that all glutamate receptor subunits are closely associated with TACC (GluRII-A, B, and C); post synaptic proteins: FasII and Fmrp are moderately colocalized, and Ten-m, Dally-like, and Orb2 are minimally colocalized. Additionally, we report that TACC is required for GluRII(C) accumulation, and all other proteins trend towards an increase in accumulation when TACC is present, excluding Dally-like protein. TACCs role in regulation of these postsynaptic factors contributes to overall growth of the NMJ, adjusting complexity of the synapse, and therefore determining how the synapse will function.

B248/P1829
Live Super-Resolution Imaging Reveals Regulated Phase Separation of Postsynaptic Proteins
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Neuronal synapses are ordered on the nanoscale, and this order is essential for their function. Here, we demonstrate that nanoclusters of proteins critical for synaptic development and plasticity undergo dynamic association and segregation by liquid-liquid phase separation (LLPS) in living neurons. Live-cell super resolution STED imaging, FRAP, and chemical disruption of LLPS of endogenous post-synaptic proteins tagged with EGFP by CRISPR-Cas9 knock-in, demonstrate that PSD-95 and AMPARs, but not NMDARs, form dynamic LLPS conjugates in living neurons. The phase states of PSD-95 and AMPARs are dynamically regulated by neuronal development, extracellular calcium, and structural plasticity, linking phase separation to synaptic remodeling. These live-cell data of endogenous proteins suggest that LLPS forms distinct synaptic microenvironments that concentrate proteins critical for synaptic plasticity and development.

B246/P1372
Local translation of presynaptic proteins
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Presynaptic protein synthesis is essential for synaptic strength and plasticity. Protein turnover is an essential component of the synaptic vesicle cycle and actively cycling synaptic vesicles preferentially incorporate newly synthesized rather than aged proteins, indicating that maintaining a pool of newly translated proteins at the synapse is essential for appropriate synaptic function. Canonically, the bulk of presynaptic proteins were thought to be synthesized in the cell body and transported to synapses. However, given the millisecond timescale of synaptic vesicle exocytosis and endocytosis and the comparatively slow rate of transport down the axon, there is likely an alternative pathway to supply new proteins to synapses. Local translation supports the time-sensitive protein requirements of the presynapse without the need for lengthy trafficking of material from the cell body. Furthermore, it is likely that there is a subset of presynaptic proteins that would be especially amenable to support by local translation, for example, those with short half-lives or which are subject to frequent cycling. Many soluble proteins have been demonstrated to be locally translated in the presynaptic compartment. However, while several mRNA transcripts for integral synaptic vesicle proteins have been identified in the axon, their local translation in the presynaptic compartment has not been validated. Here we demonstrate axonal translation of a subset of synaptic vesicle proteins (Syt1, SV2a, and Vamp2) using puromycin proximity ligation assay to identify actively translating nascent proteins in situ. Based on this finding, we investigated real-time vesicle cycling dynamics using the pH-sensitive GFP derivative, pHluorin, in cells that were treated with the protein synthesis inhibitor, anisomycin. Interestingly, we observed a strong endocytic defect. To narrow down the mechanism of this defect, we performed zap-and-freeze electron microscopy to identify structural hallmarks of endocytosis in anisomycin treated and untreated hippocampal neurons. As a future direction, we will use mass spectrometry to analyze differences in presynaptic protein abundances between untreated and anisomycin treated samples to identify protein targets which may drive the endocytic defect. Additionally, we will determine whether other protein synthesis inhibitors cause similar defects. This work will identify the molecular and structural mechanisms by which decreased protein synthesis affects synaptic vesicle cycling and neurotransmission.

B245/P1371
Multi-faceted regulation of SYD-2 Liprin a abundance in the GABA motor neurons of C. elegans
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Neuronal regulation relies on a balance between excitatory (E) and inhibitory (I) signals; E:I imbalances occur in neurological and neurodegenerative disorders. Synaptic proteins and signaling molecules collaborate to maintain E:I balance; however, much remains to be elucidated about the pathways involved. The nematode Caenorhabditis elegans, with its simple, well-characterized nervous system, provides a useful model in which to study these mechanisms. The conserved synaptic scaffold protein, SYD-2 Liprin α, is essential in controlling the structure and function of the C. elegans neuromuscular junction (NMJ), but how SYD-2 is regulated is incompletely understood. We tested the hypothesis that the anaphase-promoting complex (APC) ubiquitin ligase and the calcium-dependent UNC-43 CaM Kinase II (CaMKII) control the synaptic abundance of SYD-2 Liprin α in inhibitory GABAergic motor neurons, where our previous work revealed genetic connections

between the APC and SYD-2 Liprin α. Like the APC, CaMKII negatively regulates Liprin α levels in mammalian cells, and unc-43 loss-of-function (lf) C. elegans have increased muscle contraction similar to apc(lf) mutants, also consistent with negative regulation of SYD-2 Liprin α. Our prior research showed that the APC promotes inhibitory signaling at the NMJ of C. elegans, as apc(lf) mutants exhibit reduced GABA release and increased muscle contraction, and synaptic levels of wild type SYD-2 Liprin α, but not levels of a GFP::SYD-2(**Δ**Dbox) mutant lacking APC recognition sites, are increased in APC-deficient worms. However, direct evidence of SYD-2 ubiquitination is lacking. Biochemical pulldown analyses of ubiquitinated proteins from worms expressing GFP::SYD-2 in GABAergic neurons indicate potential ubiquitination of GFP::SYD-2 in C. elegans; tests to confirm APC dependence are underway. Initial Western blots of total protein lysates from unc-43(lf) mutants suggest little effect of unc-43 on total GFP::SYD-2 levels; however, unc-43(lf) worms have increased numbers of GFP::SYD-2 clusters in the vicinity of cholinergic motor neuron presynapses compared to wildtype worms. Together, these data suggest UNC-43 may regulate synaptic localization rather than abundance of SYD-2 in GABA motor neurons. Future studies will explore both APC and UNC-43 regulatory relationships with SYD-2, connections between them, and regulation of SYD-2 by additional kinases. These studies will provide new insight into the regulation of synaptic protein abundance, how the APC, UNC-43, and SYD-2 contribute to inhibitory signaling at the NMJ, and how modulation of this pathway may ultimately aid development of therapeutics for neurological diseases involving synaptic signaling defects.

B244/P1370

Novel Molecular Factors Regulating Periactive Zone Proteins APA-2 and ITSN-1 in C. elegans Neurons

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The synapse is a vital unit of neuronal communication, and its dysfunction is implicated in disorders such as Alzheimer's and Parkinson's disease. Within the presynaptic terminal, the active zone (AZ) orchestrates neurotransmitter secretion while the neighboring periactive zone (PAZ) mediates synaptic vesicle reuptake. Although AZ dynamics and function have been intensively studied, the mechanisms that govern the delivery and localization of endocytic factors remain poorly characterized. Utilizing C. elegans as a model organism, we conducted candidate and forward genetic screens to unravel novel regulators supporting the delivery and subcellular localization of two essential endocytic factors: ITSN-1/Intersectin (a multi-domain scaffold protein) and APA-2 (the α subunit of the heterotetrameric adaptor protein complex AP-2). Our findings revealed that the kinesin-3 motor protein, KIF1A/UNC-104, is required for proper localization of both ITSN-1 and APA-2. This study also demonstrated that distinct isoforms of CLA-1 (the C. elegans ortholog of Piccolo and Bassoon) differentially regulate the positioning of APA-2, but not ITSN-1. We notably discovered that simultaneous disruption of UNC-104 and CLA-1 have synergistic effects on APA-2 mislocalization. Additionally, we have successfully isolated three mutant alleles that disrupt the localization of ITSN-1. In future studies, we will explore the mechanistic relationship between CLA-1 and UNC-104 in endocytic protein transport and further investigate the genes affected in the isolated mutants from the forward genetic screen using whole-genome sequencing. We will characterize their expression, function, and various sites of action.

B247/P1373

OSBP-Mediated Lipid Transfer Shapes Synaptic Function

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The mammalian brain's complex functionality depends on precisely orchestrated neuronal connectivity, mediated at synapses within nanometer- scale junctions. Synaptic activity relies on the regulated release of neurotransmitters from presynaptic neurons onto postsynaptic membranes. Cholesterol, a fundamental component of lipid rafts, ion channels, and exocytic proteins, is well recognized for its role in sustaining neurotransmitter release at synaptic clefts. However, how cholesterol is dynamically regulated within presynaptic nanodomains has remained elusive. Here, we uncover an unexpected role for the Oxysterol Binding Protein (OSBP)—classically known for regulating cholesterol exchange at Endoplasmic Reticulum (ER)–Trans Golgi Network (TGN) contact sites—in controlling presynaptic cholesterol dynamics. Using spatial proteomics, we mapped the OSBP interactome in the murine brain and, for the first time, revealed its proximity to multiple presynaptic proteins, including Synaptophysin. Super-resolution imaging confirmed OSBP enrichment near synaptic vesicles and demonstrated the presence of a complete cholesterol/PI4P regulatory machinery at presynaptic terminals, indicating that OSBP likely functions beyond canonical ER–TGN lipid trafficking, acting instead as a novel cholesterol-modulating factor at active synapses. We further showed that OSBP lipid transfer and Sac-mediated PI4P turnover modulate synaptic organization, while PI4Kβ dynamically relocates to synaptic vesicles upon depolarization, driving local PI4P production and OSBP recruitment. Disruption of OSBP activity or other components of the cholesterol/PI4P machinery impaired neurotransmitter release and vesicle fusion, as demonstrated by transfecting neurons with a GFP-tagged synaptic vesicle release indicator, Synapto-pHluorin, and a glutamate release sensor, iGluSnFR. Together, these findings establish OSBP as a central regulator of presynaptic cholesterol dynamics and neurotransmitter release, highlighting its potential as a therapeutic target in disorders of neuronal excitability.

B238/P1364

Role of tubulin (de)tyrosination in the microtubule-actin crosstalk in the dendritic spines

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Objective: To investigate how tubulin tyrosination/detyrosination influences dendritic spine invasion, actin dynamics, morphology, and function in mature neurons. Methods: Primary hippocampal neurons from Tubulin tyrosine ligase knockout (lacking tyrosinated tubulin) and Small vasohibin binding protein knockout (with reduced detyrosinated tubulin) mouse embryos were cultured until complete synaptic maturation, and analyzed using immunofluorescence and live imaging. Results: An imbalance in tubulin tyrosination altered multiple parameters of microtubule dynamics in both dendrites and spines. Increasing tubulin tyrosination enhanced overall microtubule dynamicity, whereas decreasing it reduced dynamicity. Notably, we show for the first time that tyrosinated microtubules invade dendritic spines and exhibit dynamic instability within them. Tubulin tyrosination positively correlated with the frequency of microtubule spine invasion as well as spine density, maturity, and volume. In both knockout models, actin enrichment in spines decreased; however, actin stability increased with elevated tubulin tyrosination. Future work will examine how tubulin-tyrosination imbalance affects spine morphology and function under synaptic activation. Statement of conclusion: Regulation of microtubule dynamics through tubulin tyrosination plays an important role in microtubule invasion into spines, actin organization, spine morphology, and spine density in neurons.

B241/P1367
UNC-43/CaMKII regulates the assembly of the presynaptic active zone in C. elegans
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Neurons communicate via a specialized interface known as the synapse comprised of pre- and postsynaptic specializations. At the presynaptic specialization, a group of conserved proteins form an electron dense region called the active zone (AZ), where AZ proteins regulate the recruitment and release of neurotransmitter-containing synaptic vesicles (SVs). While AZ proteins are well characterized, less is known about how they assemble into a functional synapse. Here we found that unc-43, the sole ortholog of calcium/calmodulin-dependent protein kinase II (CaMKII), is crucial for proper presynaptic assembly in Caenorhabditis elegans. Using airyscan microscopy, we examined the localization of endogenous AZ proteins labeled with split-fluorophores. We found that CLA-1/Piccolo-Bassoon, SYD-2/Liprin-α, and RIMB-1/RIM-BP localization are disrupted in unc-43 loss-of-function (lof) mutants, in which the number of the active zones labeled by these proteins is increased and mislocalized to outside of the presynaptic varicosity. These numerous active zone puncta are smaller and dimmer in fluorescence intensity. Interestingly, UNC-10/RIM localization appears to be unaffected while the signal intensity is reduced, suggesting that unc-43 is required for proper presynaptic assembly by differentially regulating the localization of many AZ proteins. On the other hand, gain-of-function (gof) mutants of unc-43 have “overdeveloped” synapses and exhibit increased clustering of the presynaptic proteins. FRAP experiments and timelapse videos uncovered higher mobility of active zone proteins, especially RIMB-1, in unc-43 mutant animals. Neuron-specific, but not muscle-specific knockdown of UNC-43/CaMKII using the auxin-inducible degron (AID) system suggests that UNC-43/CaMKII functions in the presynaptic neuron. Postembryonic degradation of UNC-43/CaMKII using the AID system resulted in disruption of CLA-1 localization, while postembryonic activation of CaMKII using a photo-activatable CaMKII rescued presynaptic localization of CLA-1, suggesting that UNC-43/CaMKII functions to maintain presynaptic structure. To test the functional conservation between human CaMKII and unc-43, we replaced the endogenous unc-43 locus with human CaMKIIA (hCaMKIIA). hCaMKIIA animals exhibit normal locomotion and presynaptic structure, suggesting the conservation between unc-43 and human CaMKIIA. We introduced a recessive, and a dominant mutation identified in the CaMKII gene in patients with intellectual disabilities. These mutations phenocopied presynaptic defects of unc-43(n498n1186) and unc-43(n498) mutants. Our work reveals a conserved role of CaMKII in regulating the proper assembly of presynaptic proteins.

Organelles - Endoplasmic Reticulum

B101/P0681
Elucidating the Structural and Functional Roles of Sarcalumenin, a Key Component of the Muscle Fiber Sarcoplasmic Reticulum
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Proper structural organization of muscle fibers is crucial for their function. This is particularly evident at the sarcomere level, where alternating bands of thin (actin) and thick (myosin) filaments mediate muscle contraction. A second example is the sarcoplasmic reticulum (SR), a muscle-specific subdomain of the endoplasmic reticulum (ER) and a critical mediator of the process by which neuronal excitation is translated

into muscle fiber contractility. Sarcalumenin (SRL), a relatively uncharacterized SR and muscle-specific protein, is recognized as an abundantly-expressed luminal Ca2+-buffering protein of the mammalian longitudinal SR. Intriguingly, SRL possesses a dynamin-like domain in addition to its Ca2+-binding domain, suggesting a parallel functional role in SR membrane remodeling. In fact, our evolutionary sequence analysis suggests that SRL proteins constitute an ancient and distinct branch of the dynamin protein family that localize within the ER/SR, and are present in organisms with muscles that contract synchronously and possess an elaborate SR. We have begun to characterize the single Drosophila homolog of SRL (CG9297), in order to complement and extend our ongoing studies of mouse SRL. Similar to its mammalian counterpart, Drosophila srl is expressed specifically in muscle fibers. We have generated a GFP-tagged variant of the Drosophila SRL protein, and find that it localizes, as expected, to the SR of larval body-wall muscles. Both homozygous disruption of the srl locus and muscle-specific RNAi-based knockdown result in larval lethality. Knockdown of srl at post-larval stages identifies a second essential phase of srl function during adult muscle formation. Intriguingly, srl is expressed and required in larval and adult tubular muscles, but appears to be dispensable for the function of the fibrillar indirect flight muscles, which power Drosophila flight, yet are SR-poor and contract in an asynchronous manner. Characterization of the srl mutant muscle fiber phenotype via 3D light and electron microscopy approaches reveals significant defects in SR organization, as well as impaired Ca2+ homeostasis in larval body-wall muscles, while overall sarcomeric morphology appears intact. All told, our findings imply a critical membrane remodeling activity of SRL in proper formation of the SR reticular network and in mediating muscle SR function, highlighting the unique ultrastructural requirements of the SR domain and the overlooked role of dynamin-like proteins in shaping membrane ultrastructure within the lumen of an organelle.

B097/P0677

ER-Shaping Proteins and Contact Site Organization in the Differentiated Sarcoplasmic Reticulum

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During development, the skeletal muscle cell endoplasmic reticulum transforms into the sarcoplasmic reticulum (SR), forming a highly organized structure with two main morphologies: longitudinal SR and junctional SR. The longitudinal SR consists of elongated tubules running parallel to muscle fibers and is primarily responsible for reabsorbing calcium following muscle contraction. In contrast, the junctional SR forms enlarged, sac-like terminal cisternae positioned at regular intervals, creating membrane contact sites with plasma membrane invaginations and specializing in the rapid storage and release of calcium during contraction. While the role of ER-shaping proteins (Climp63, Kinectin, p180, Atlastin3, Lunapark, Rtn2, Rtn3 and Rtn4) in establishing ER structure is well characterized in non-differentiated cells, their involvement in shaping the SR during development has not yet been explored. Furthermore, the relationship between SR morphology and its functional properties remains unclear. Here, using an in vitro skeletal muscle system, we performed a loss-of-function screen to identify ER-shaping proteins involved in the development of longitudinal and junctional SR subdomains. We discovered that ER shaping proteins are localized near the junctional SR. Additionally, loss of function of ER shaping proteins impacted the normal distribution of junctional SR proteins, which localized as elongated filaments instead of puncta. Notably, plasma membrane proteins that form membrane contact sites with the junctional SR were also mislocalized as filaments. Furthermore, we investigated the impact of ER shaping proteins on calcium dynamics and muscle contractility, linking SR structural integrity to its physiological function.

B103/P0683
Mechanical forces reshape endoplasmic reticulum luminal dynamics
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Cells are constantly exposed to mechanical forces such as compression, stretch, and matrix stiffness that shape their behavior. Over the past two decades, mechanobiology has shown that these cues regulate processes from stem cell differentiation to tissue remodeling, largely through cytosolic and nuclear pathways. Cytoskeletal remodeling and myosin contractility transmit external tension into intracellular signals, while lamin A/C and YAP/TAZ nuclear shuttling couple mechanics to gene regulation. Yet whether the endoplasmic reticulum (ER)—the largest organelle in the cell—can sense and respond to mechanical forces has remained unknown. Here we introduce ER-targeted genetically encoded multimeric nanoparticles (ER-GEMs) and apply single-particle tracking to probe luminal dynamics under compression. We show that mechanical compression markedly increases ER-GEM diffusivity, a response distinct from that of the cytosol. This mechanoresponse is mediated by cPLA2α and is further associated with agitation of the ER structural protein Climp63. Our findings establish the ER lumen as a mechanoresponsive compartment and uncover a previously unrecognized pathway through which mechanical forces may regulate secretion and enable cellular adaptation to physical stress.

B098/P0678
Nuclear envelope-endoplasmic reticulum junctions regulate the diffusional exchange of proteins to the nuclear envelope.
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The nuclear envelope (NE) is a specialized subdomain of the endoplasmic reticulum (ER), primarily responsible for the compartmentalization of the cytoplasm from the nucleoplasm. Key to its successful function is both the specific subset of enriched NE proteins, in addition to the shared pool of resident ER proteins, which collectively serve to maintain compartmental homeostasis. In theory, the direct continuity of the ER to the NE should provide unbiased access to these protein pools. In practice, topological constraints lead to unequal opportunities for molecular exchange between the two compartments. The ER and NE are connected through a limited number of poorly characterized NE-ER junctions, and any molecular exchange between the two regions will by definition be dependent on their distribution throughout the membrane. Additionally, the ER's complex and convoluted structural topology precludes uniform, omnidirectional molecular movement – protein diffusion can only happen within the trajectories defined by the organelle's membranes. While recent work has highlighted the capacity of such topologically constrained diffusion to asymmetrically alter protein accessibility at the peripheral ER, little is known as to how the ER's topological complexity and connectivity to the NE may influence the availability of proteins broadly at the NE. Borrowing from dynamic trafficking assays within the secretory system, we designed a chemically-inducible dimerization system that allows for the monitoring of protein exchange to the nuclear envelope. Using this system, we demonstrated the capacity of ER organization to influence the effectively available proteome at NE. We show that the NE has diffusionally-buffered regions, which, as a function of their sparse connectivity to the ER, are less readily able to access ER-resident proteins. Finally, we show as a proof of concept that this system allows for the microscopic identification of bonafide NE-ER junctions, where protein flux can be isolated to individual segments of membrane with high-speed live-cell microscopy.

B099/P0679

Polymerized protein response (PPR): A novel NF?B p50/p50 homodimeric signaling pathway triggered by pathogenic polymers in the endoplasmic reticulum

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Previous studies from our lab showed that accumulation of the polymerogenic variant α1-antitrypsin Z (ATZ) that causes the classical form of α1-antitrypsin deficiency (ATD) activates nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling pathway. ATD, which affects 1 in 3000-5000 live births causes liver disease by a gain-of-function mechanism, also referred to as proteotoxicity. The ATZ variant is poorly secreted, prone to misfolding and accumulates in the endoplasmic reticulum (ER) of the liver mostly as polymers. We observed more severe hepatic damage in the PiZ mouse model of ATD when it is bred onto a background deficient in NFκB activity, suggesting that NFκB pathway has a protective function against the proteotoxic effects of ATZ. NFκB, a family of transcription factors that are comprises homo- and heterodimers of five proteins (p50, p52, c-Rel, RelA/p65 and RelB) has broad biological effects including regulation of immunity, inflammation, cell growth, proliferation and survival. In this study, using the PiZ mouse model, we discovered that the NFκB response is characterized by NFκB p50/p50 homodimerization in complex with non-histone chromatin binding protein high mobility group AT-hook 1 (HMGA1). This is distinct from the NFκB p50/p65 heterodimerization induced by the canonical NFκB inflammatory response and the unfolded protein response (UPR), activated in response to the accumulation of misfolded or unfolded proteins in the ER. The NFκB DNA binding results were replicated in the HTO/Z cell line model, a HeLa based, Tet-Off inducible cell-line engineered for expression of ATZ upon withdrawal of doxycycline. NFκB p50/p50 homodimerization is also induced by other disease associated variants that aberrantly polymerize in the ER, such as neuroserpin G392E, arginine vasopressin (AVP)-Y2H and canine superoxide dismutase (SOD1)-E40K. However, disease-associated variants that do not form polymers, such AT-Hong Kong activate NFκB p50/p65 heterodimers and UPR. We are naming this novel and distinct signaling pathway the polymerized protein response (PPR). Our RNA-sequencing (RNA-Seq), assay for transposase-accessible chromatin (ATAC)-Seq and chromatin immunoprecipitation (ChIP)-Seq data show that PPR elicits a unique gene expression profile. We observed an upregulation of genes that transduce the signal from the ER to the cytoplasm, stabilize NFκB p50/p50 homodimers in the cytoplasm and promotes its translocation into the nucleus where they are stabilized by binding of HMGA1. We conclude that PPR is a proteostasis response pathway complementary to UPR and designed to protect the cell from proteotoxic effects of polymers that form aberrantly in the ER.

B102/P0682

Profilin 1 is important for endoplasmic reticulum homeostasis

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Profilin 1 (PFN1) is a ubiquitously expressed, essential actin-binding protein that promotes actin polymerization by facilitating the addition of monomers to growing filaments. In addition to regulating actin network assembly, PFN1 has been implicated in diverse cellular processes, including stress granule dynamics, nucleocytoplasmic transport, mitochondrial function, and microtubule regulation. A recent screen for organelle dysfunction identified a role for PFN1 in endoplasmic reticulum (ER) regulation. The ER is a dynamic

and interconnected network critical for protein and lipid synthesis, calcium balance, and the unfolded protein response. Here, we show that loss of PFN1 results in severe ER dysfunction, including major alterations in ER morphology, impaired protein folding, disrupted calcium homeostasis, and reduced formation of ER–mitochondria contact sites. Notably, further analyses suggest that PFN1 regulates ER integrity through mechanisms independent of its canonical actin polymerization function. These findings uncover a previously unrecognized role for PFN1 in ER homeostasis, with broad implications for its contribution to cellular physiology and disease.

B100/P0680

The C-terminal short helix of Sec11 in yeast signal peptidase complex is required for optimal cleavage of internal signal sequences

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In eukaryotes, signal peptidase complex (SPC) cleaves the signal peptide of endoplasmic reticulum (ER)-targeted proteins. Sec11 is a catalytic subunit of the yeast SPC, forming a complex with other auxiliary components in the ER membrane. Sec11 has a flexible C-terminal short helix (CTS) that is evolutionarily conserved in eukaryotes, however, questions on why Sec11 possesses such membrane-spanning domain at C-terminus and how it functions in the ER membrane remain elusive. To define the role of Sec11 CTS in yeast, sec11ΔCTS mutant was generated to conduct functional studies. Western blotting-based cleavage assay using model proteins with varying n-region length of signal sequences showed that cleavage efficiency of signal sequences with long n-region length (internal signal sequences) was impaired when Sec11 CTS was absent. On the other hand, cleavage of signal sequences with short n-region length in sec11 mutant cells was comparable to wild-type cells. These results indicate that the CTS of Sec11 is required for optimal cleavage of internal signal sequences.

B096/P0676

The endoplasmic reticulum autophagy receptor TEX264 drives epidermal differentiation and is dysregulated in Darier disease

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As keratinocytes differentiate and migrate upward within the epidermis, they must break down their organelles and nuclei to generate the compacted cornified cell layers that constitute the cutaneous barrier. This unique catabolic process is perturbed in various dermatologic disorders, but its molecular drivers remain poorly understood. Leveraging confocal imaging of live human organotypic skin cultures, we aimed to delineate the mechanism by which the large endoplasmic reticulum (ER) network is degraded during epidermal tissue maturation. Using the ER-localized tandem fluorophore GFP-RFP as a reticulophagy sensor, we visualized the ER undergoing progressive fragmentation and acidification in live differentiating keratinocytes. Moreover, we demonstrated that the ER remnants were routed into LC3-positive autophagosomes and were eventually engulfed by LAMP2-positive lysosomes. Coincident with ER breakdown in the uppermost epidermal layers, we found up-regulation of a selective reticulophagy receptor TEX264, which was induced by ER stress. Ectopic expression of TEX264 in undifferentiated keratinocytes was sufficient

to fragment their ER, while in highly differentiated keratinocytes, TEX264 over-expression accelerated ER elimination and induced nuclear shrinkage; these effects were abolished by mutating the LC3 interacting region of TEX264 required for its autophagic function. Further, we showed that knocking out TEX264 or inhibiting its activation by casein kinase 2 disrupted maturation of organotypic cultures, uncovering a critical role for reticulophagy in epidermal morphogenesis. Finally, in patient biopsies and an organotypic model of Darier disease, a genetic skin barrier disorder linked to ER stress, we found increased levels of TEX264 within epidermal lesions. In sum, our results identified TEX264 as a key driver of epidermal differentiation and led us to propose a novel model of skin barrier formation in which keratinocytes activate selective autophagy receptors in a programmed manner to orchestrate orderly organelle elimination.

Organelles - Endosomes and Lysosomes

B109/P0689
Activation and Regulation of PI4K2A in Response to Lysosomal Membrane Stress
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Cells employ various strategies to cope with lysosomal membrane stress and permeabilization. Recent studies and our own work identified PI4K2A-mediated phosphatidylinositol 4-phosphate (PI4P) production on lysosomes damaged by L-Leucyl-L-Leucine methyl ester (LLOMe). This promotes endoplasmic reticulum (ER)-lysosome contact site expansion and PI4P-cholesterol counter-transport between ER and lysosomes. In this study, we broaden the functional roles of PI4K2A on stressed lysosomes to encompass both enhanced membrane resilience and repair. We show that loss of PI4K2A or inhibiting OSBP-mediated counter-transport sensitizes lysosomes to membrane stressors. We then investigate PI4K2A activation and regulation. At steady state lysosomal PI4P levels are low but PI4K2A is present, implying that stress-related signals trigger its activation. We rule out calcium, lysosomal alkalinization and the ESCRT machinery as activators. Instead, we find that while LLOMe causes continuous PI4P accumulation, osmotic stressors induce only a transient response suggesting that PI4K2A may respond directly to changes in membrane order. Finally, we show that ubiquitination dampens PI4K2A activity providing another level of regulation for PI4P production in response to lysosomal damage.

B110/P0690
Characterizing Distinct Responses to Graded Lysosomal Membrane Damage
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Lysosomes constantly face environmental fluctuations that impose stress on their limiting membrane. Previous work by many groups, including our own, has identified restorative responses to lysosomal stress and damage. Among these are ESCRT machinery acting to repair limited damage and galectins responding to complete rupture. To more fully understand how cells respond to lysosomal disruption, we systematically monitored responses to lysosomal perturbation by the widely used compound L-Leucyl-L-Leucine methyl ester (LLOMe). In lysosomes, LLOMe is processed by cathepsin C (Cat C) into membrane-disrupting poly-leucine chains or accumulates as esterase-cleaved dileucines. By varying the dose and exposure time, we found that LLOMe induces dose-dependent alterations in lysosomal integrity—from gradual, small-molecule leakage with limited Gal3 recruitment at low concentrations to complete rupture with rapid and abundant Gal3 accumulation at high concentrations. ESCRT was robustly recruited at low doses where it contributes to stabilization and repair

and much less at high concentrations where ubiquitin and galectin help to drive lysophagy. Inhibition of CatC abolished leakage caused by low-dose LLOMe and corresponding responses including ESCRT (ALIX, CHMP4B) recruitment and PI4P accumulation. In contrast, effects of high-dose LLOMe resembled the osmotic rupture–induced effects triggered by Glycyl-L-phenylalanine 2-naphthylamide (GPN). These findings indicate that LLOMe induces lysosomal membrane stress through membrane permeabilization, GPN-like osmotic rupture, and potentially additional mechanisms, each causing specific, triggered recruitment of various cellular machinery. Together, our results establish LLOMe as a versatile tool for dissecting the continuum of lysosomal membrane damage and the corresponding range of cellular responses.

B107/P0687

Endolysosome pH Regulates Progranulin Processing and Granulin-Dependent Cell Survival

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Endolysosomes are cytoplasmic organelles that control cellular events necessary for cell growth and survival. The acidic lumen of endolysosomes is critical for the effective functioning of lysosomal enzymes including Cathepsin B and D that catalyze the production of granulin from its precursor progranulin (PGrn). PGrn is a multi-functional secreted growth factor ubiquitously expressed in multiple cell types including neurons and microglial in brain. PGrn deficiency has been linked to neurodegenerative diseases including frontotemporal dementia, Alzheimer's disease and Parkinson's disease, but the underlying mechanisms are not fully described. Others and we have reported that endolysosome de-acidification triggers endolysosome damage, impairs removal of cellular waste, and ultimately increases cell death. Because PGrn and granulins are vital for cell survival and granulins are produced in endolysosomes, we investigated the effects of endolysosome pH on granulin processing and cell death. Using SH-SY5Y human neuroblastoma and human embryonic kidney (HEK)-293 cells, we demonstrated that granulin promotes cell survival. In the SH-SY5Y cells, cell death was significantly reduced when cells were supplemented with human Progranulin (50 and 100 ng/ml) for 24 h. In PGrn-/- HEK 293 cells, cell death was significantly increased compared to HEK-293 wild-type and the effect was significantly reduced by hPGrn (100 ng/ml) treatment for 24 h. Levels of caspase 3, apoptosis inducing factor, and BAX were elevated in PGrn-/- cells; effects blocked by hPGrn supplementation. Deferoxamine, an endolysosome-specific iron chelator and MLSA-1, a TRPML1 agonist acidified endolysosomes, increased cathepsin B and D levels, and prevented increases in Galectin-3 - an endolysosome-damage-related protein. Bafilomycin-A1 de-acidified endolysosomes, accelerated endolysosome damage, decreased cathepsins B and D levels, and increased levels of PGrn. Taken together, our work demonstrates an important role of endolysosome pH in granulin processing. Further, our results suggest that the protective effects of PGrn and Grn can be enhanced by endolysosome-acidifying therapies and might find use in the treatment of neurodegenerative disorders.

B108/P0688

Exploring the Relationship among Rapid Responses to Acute Lysosomal Membrane Stress and Disruption

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Lysosomal membranes are susceptible to disruption by many agents, including trafficked cargoes and lysosomotropic compounds. Previous work has shown that Endosomal Sorting Complex Required for Transport (ESCRT) and Conjugation of ATG8 to Single Membranes (CASM) machineries are rapidly recruited to stressed and damaged lysosomes. ESCRTs promote resilience to rupture and repair membranes while the precise role of CASM is uncertain. Here, we sought to define the temporal and functional hierarchy among these and related responses. A leading contender for engaging ESCRTs has been the leakage of lysosomal calcium acting via the calcium sensor ALG-2. However we found that ALG-2 is dispensable for lysosomal repair. Given that ubiquitin is a well-established signal for ESCRT engagement in endosomal sorting, we used TAK-243, an E1 inhibitor, to abolish ubiquitination and observed that ESCRT is still recruited in response to damage. Since CASM is also a rapid response triggered by lysosomal alkalinization or exposed sphingomyelin, we asked if CASM might itself be a trigger for ESCRT recruitment. In cells lacking ATG8 conjugation machinery or treated with bafilomycin A1 to disrupt CASM, ESCRT recruitment to damaged lysosomes was strongly reduced. Yet using super-resolution fluorescence microscopy to observe damaged lysosomes, ESCRTs and ATG8s are spatially distinct on single organelles. Furthermore, CASM is often induced without recruiting ESCRTs. We propose that ESCRT-mediated resilience and repair is promoted by many factors but is not singularly dependent on calcium, ubiquitin, or CASM.

B106/P0686
LASER senses lysosomal damage and initiates ESCRT assembly for membrane repair
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Maintaining lysosomal membrane integrity is essential for cellular homeostasis, and its disruption contributes to neurodegenerative diseases. While the Endosomal Sorting Complex Required for Trafficking (ESCRT) machinery plays a key role in lysosomal repair, how ESCRTs first detect damage remains unclear. Through a genome-wide CRISPRi screen in damage-sensitized genetic background, we discovered a multimeric protein, renamed LC3-Assisted Sensor for ESCRT Recruitment (LASER), that bridges membrane damage sensing with repair. LASER, which normally resides at ER exit sites, binds to ATG8 (LC3/GABARAP) proteins conjugated to damaged lysosomal membranes. At the lysosome, ATG8-bound LASER forms multimeric assemblies that directly recruit the ESCRT-I subunit, Tumor Suppressor Gene 101 (TSG101), via its PSAP motif and avidity-driven interactions, triggering downstream ESCRT I-II-III polymerization to promote rapid membrane repair. Importantly, mutations in LASER, known to cause hereditary spastic paraplegia, disrupt its assembly and impair ESCRT recruitment, linking defective repair to TFG-associated neurodegeneration. Thus, LASER mediates spatiotemporal control of ESCRT polymerization and couples lysosomal damage to repair.

Organelles - Endosomes and lysosomes

B088/P0668
Mitochondria-Lysosome Coupling Contributes to Lysosome Acidification and Aging
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Nearly all cellular processes are pH dependent. The acidic pH inside the lysosome (vacuole in yeast) is essential for cellular content degradation, signaling, and autophagy. Defect in lysosome/vacuole acidification is a conserved hallmark of aging and age-related diseases. Traditionally, lysosome/vacuole is thought to import free protons (H^+) from the surrounding neutral cytosol. In this study, we uncovered a previously unrecognized,

conserved lysosome/vacuole acidification mechanism, involving lysosomal/vacuolar uptake of H+ from mitochondria through membrane contacts between mitochondria and lysosomes/vacuoles. Aging/senescence-associated disruption of mitochondria-lysosome/vacuole contacts causes lysosomal/vacuolar de-acidification, which can be reversed by expressing a engineered linker to connect these organelles and through an asymmetry-dependent rejuvenation process in daughter cells. Preserving lysosomal acidification in senescent human cells prevents the induction of major senescence-associated secretory phenotype factors and enhances autophagic flux. These findings reshape our current understanding of the mechanisms underlying lysosomal/vacuolar (de-)acidification in both young and aged/senescent cells.

Organelles - Golgi Apparatus

B105/P0685
Investigating the role of plastic pollutants in Golgi fragmentation and their impact on protein glycosylation
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Glycosylation, a critical post-translational modification occurring in the Golgi apparatus, regulates protein function and cellular homeostasis. Environmental stressors, including bisphenol A (BPA) and polystyrene microparticles, have been implicated in neurotoxicity, yet their effects on Golgi function remain poorly understood. While BPA-induced ER stress is documented, downstream consequences for Golgi integrity and glycan alteration are largely overlooked. Similarly, microplastic effects on organelle function are understudied. We hypothesize that both BPA and polystyrene microparticles disrupt Golgi structure and function, leading to altered glycosylation and cellular dysfunction in neuronal cells. Using human neuroblastoma cells exposed to BPA or polystyrene microparticles, we are examining Golgi morphology, stress markers, cellular viability, and glycosylation patterns. This comparative approach will determine whether diverse environmental toxicants converge on Golgi stress as a common mechanism of neurotoxicity. Understanding how environmental toxicants disrupt organelle-specific processes may reveal novel mechanisms underlying environmental neurotoxicity and identify potential therapeutic targets for neurodegenerative diseases such as Parkinson's disease.

B104/P0684
Yeast glycosylation enzymes recycle in three COPI-dependent intra-Golgi pathways
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A core function of the Golgi apparatus is the post-translational modification of secretory cargoes, primarily through N- and O-linked glycosylation. It is generally assumed that Golgi-localized glycosylation enzymes exhibit a polarized distribution that matches the order of oligosaccharide processing. We explored this idea in the context of Golgi cisternal maturation in yeast. The approach was to optimize the fluorescent tagging of yeast Golgi glycosylation enzymes and to characterize the kinetic signatures of enzymes involved in N-linked glycosylation. Functional tagging of yeast Golgi glycosylation enzymes has been challenging. Tagging the N-terminal cytosolic tails perturbs Golgi localization, while tagging the C-terminal luminal catalytic domains often results in missorting to the vacuole. We found that an internal deletion in the vacuolar hydrolase receptor Vps10 significantly reduces missorting of luminally-tagged glycosylation enzymes. The additional deletion of a Vps10 homolog further improves Golgi localization for some enzymes. Vps10 and its homologs likely participate in a quality control system that can be inactivated to allow for luminal tagging of Golgi proteins.

Kinetic analysis of glycosylation enzymes uncovered evidence for three membrane recycling pathways. One set of enzymes arrive at the same time as the early Golgi marker Vrg4. A second set of enzymes arrive at the Golgi after Vrg4 but before the late Golgi proteins that recycle with the aid of the AP-1 and Ent5 clathrin adaptors. Finally, a third set of enzymes arrive before Vrg4. Rapid inactivation of COPII quickly trapped certain Golgi proteins that cycle through the ER but did not trap any of the Golgi glycosylation enzymes. Based on these data, all glycosylation enzymes likely recycle within the Golgi in COPI vesicles. The earliest intra-Golgi recycling pathway operates prior to any previously described pathway of that type. Together, our results suggest that yeast glycosylation enzymes follow three sequential but kinetically overlapping intra-Golgi recycling pathways during an early stage of cisternal maturation. Surprisingly, the order of arrival of glycosylation enzymes does not strictly correlate with the order of their enzymatic actions. Compared to the traditional view of Golgi compartmentation, these findings provide a more nuanced view of glycosylation pathways.

Organelles - Mitochondria and Chloroplasts

B129/P0709
Age-Dependent Insulin Granule-Mitochondria Interactions Modulate Insulin Maturation
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Insulin is a peptide hormone secreted by pancreatic beta cells that is essential for maintaining normal glucose homeostasis. The intracellular maturation of insulin is known to be disrupted in diabetes; however, the molecular mechanisms and interorganelle communications that regulate this process remain poorly characterized. Recent studies have reported an increase in the proximity between mitochondria and insulin secretory granules (ISGs) following stimulation with insulin maturation–promoting agents. Interorganelle interactions are crucial for various cellular functions, including the transfer of lipids, ions, and metabolites. We hypothesize that the increased proximity between mitochondria and ISGs reflects the formation of membrane contact sites (MCSs) between the two organelles, which likely modulate proper insulin maturation. Because ISG maturation is a time-dependent process, we further propose that these contacts change dynamically as ISGs age. To test this hypothesis, we employed a genetically encoded SNAP-Tag system to label and track ISG–mitochondria contacts in distinct, age-defined ISG populations. Our findings demonstrate that mitochondria–ISG contacts are more frequent in younger ISG populations and decrease as ISGs age. Interestingly, very old ISGs again exhibit an increase in mitochondria–ISG contact sites. We also observed changes in the distribution of ISGs within the mitochondrial network as ISGs mature, suggesting a possible shift in subpopulations affected by insulin-promoting drugs depending on ISG age.

B132/P0712
Analyses of the impact of algal-symbiont on organelles in Hydra viridissima
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Understanding symbiosis is important for deciphering how organisms evolve and adapt to changing environments. In my studies of symbiosis, I chose Hydra viridissima, also called green Hydra, as an experimental model. Hydra viridissima has the ability to live with its algal symbionts residing inside Hydra cells. Under certain conditions, Hydra viridissima can also live without its algal symbionts. To understand this symbiotic relationship, I explored how mitochondria, the key organelle that provides energy and structural building blocks to eukaryotes, changes in both Hydra viridissima and its algal symbionts. For Hydra viridissima,

it is crucial to investigate the impact of the presence of oxygen producing algal symbionts on mitochondria. I have developed a protocol to dissociate hydra cells and conduct MitoTracker staining. As expected, I found that not all Hydra cells carry green algae. Interesting, Hydra cells with more algal symbionts exhibit lower mitochondrial activity and altered mitochondrial morphology as compared to cells without symbionts. I have also conducted LysoTracker staining that visualizes the lysosomes, another important organelle in eukaryotic cells. In contrast to the MitoTracker result, there is no significant difference between algae-hosting and non-hosting Hydra cells. These results reveal a previously unknown effect of the symbiotic algae on mitochondria.

B119/P0699
Assembly Mechanism of Human Optic Atrophy 1 Protein on Cardiolipin Membranes
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The mechano-chemical GTPase Optic Atrophy 1 (OPA1) is an essential biomolecular machine that regulates mitochondrial cristae remodeling and orchestrates inner mitochondrial membrane (IMM) fusion. Cryo-EM studies of cardiolipin (CL)-bound OPA1 have shown that it assembles into a flexible helical lattice through cooperative oligomerization, coordinated by interactions among its four domains: the GTPase, stalk, bundle signaling element (BSE), and paddle. However, the molecular mechanism by which these domains drive higher-order assembly remains elusive. Here, we systematically identified and functionally characterized interfacial residues that regulate OPA1 oligomerization and membrane remodeling. As an initial step, we generated a series of OPA1 domain truncations and evaluated their biochemical and functional properties, revealing that the GTPase domain is dispensable for oligomer formation. We then introduced site-directed mutations into candidate interfacial residues within the BSE, stalk, and paddle domains and tested these variants for lattice assembly and association with CL-enriched liposomes in vitro. We quantified lipid binding by co-sedimentation, while higher-order lattice formation on preformed lipid tubes and membrane remodeling by liposome tubulation were both visualized by transmission electron microscopy (TEM). Although all mutants retained lipid-binding activity, disruption of interfacial residues abrogated tetramer formation, blocked higher-order lattice assembly, and abolished membrane remodeling. Together, these findings delineate the assembly mechanism of human OPA1 and provide a molecular framework for understanding how pathogenic mutations destabilize cristae architecture in degenerative diseases.

B121/P0701
Cardiolipin dynamics promote membrane remodeling by mitochondrial OPA1
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Cardiolipin is a mitochondria-specific phospholipid that forms heterotypic interactions with membrane-shaping proteins and regulates the dynamic remodeling and function of mitochondria. However, the precise mechanisms through which cardiolipin influences mitochondrial morphology are not well understood. In this study, employing molecular dynamics simulations, we determined that cardiolipin molecules extensively engage with the paddle domain of mitochondrial fusion OPA1, which controls membrane-shaping mechanisms. Structure-function analysis confirmed the interactions between cardiolipin and two conserved motifs of OPA1 at the membrane-binding sites. We further developed a bromine-labeled cardiolipin probe to enhance cryoEM contrast and characterized the structure of OPA1 assemblies bound to the cardiolipin-brominated lipid bilayers. Our images provide direct evidence of cardiolipin enrichment within the OPA1-

binding leaflet. Last, we observed a decrease in membrane remodeling activity for OPA1 in lipid compositions with increasing concentrations of monolyso-cardiolipin. This suggests that the partial replacement of cardiolipin by monolyso-cardiolipin, as observed in Barth syndrome, alters the malleability of the membrane and compromises proper remodeling. Together, these data provide insights into how biological membranes regulate the mechanisms governing mitochondrial homeostasis.

B136/P0716
Exploration of vitamin B6 intracellular trafficking in Cryptosporidium parvum
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The obligate intracellular protozoan Cryptosporidium parvum (Cp) is an example of reductive evolution within Alveolata, having lost both its mitochondrial genome and many central metabolic pathways. It contains a scaled down mitochondrion, known as the mitosome, which lacks a conventional electron transport chain, the TCA cycle, and the capacity to generate ATP, yet retains essential pathways like ubiquinone and Fe-S cluster biosynthesis. Recent studies have demonstrated that treatment with the microbiota-derived gut metabolite pyridoxal (PL) inhibits growth of Cp in vitro. This finding is surprising because the phosphorylated form, pyridoxal phosphate (PLP), is the catalytically active form of the vitamin B6 cofactor, which is presumably essential for growth. Like its mammalian hosts, the protist lacks de novo B6 synthesis, but additionally the Cp genome lacks the enzyme pyridoxal kinase that is necessary to generate PLP that is required as a cofactor for many essential enzymes. To investigate B6 transport in Cp, we identified a homolog of the Saccharomyces cerevisiae mitochondrial carrier Mtm1 (cgd6_3880; CpMtm1), a member of one of two SLC25 families described to transport PLP across cellular membranes. CpMtm1 is predicted to localize to the parasite's mitosome, a degenerate mitochondrion that lacks many conventional systems. Its homology to other SLC25 family members and predicted localization to the mitosome suggests that CpMtm1 may transport PLP into the mitosome to support essential pathways. Here, we utilized CRISPR-Cas9 technology to generate CpMtm1-smHA expressing parasites in order to determine its subcellular localization using laser-scanning confocal microscopy on conventional and ultrastructure expansion microscopy samples. CpMtm1-smHA localized to discrete puncta, consistent with a mitosomal localization, which was confirmed via immuno-electron microscopy. Functional complementation studies revealed that CpMtm1 expression rescued growth of yeast Δhem25 cells, but not Δmtm1 cells, on respiratory medium. Additional experiments suggest that CpMtm1 is likely to transport PLP, with a similar substrate specificity to the human carrier SLC25A38. Ongoing work will test whether CpMtm1 expression can rescue PLP import in SLC25A38-KO K562 mammalian cells and to determine the transporter's essentiality for intracellular growth using conditional systems to knock down CpMtm1 expression in the parasite. These studies explore the functional plasticity of mitochondrial carriers an auxotrophic intracellular parasite with a streamlined mitosome.

B131/P0711
FMNL1 regulates late stage of mitochondrial division downstream of Drp1 and TGN vesicles
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Mitochondrial division is a tightly orchestrated process essential for regulating mitochondrial health, distribution, and overall cell function. Mitochondrial division is characterized by numerous molecular

determinants and interplay among organelles. The Endoplasmic Reticulum (ER) was first shown to be linked to sites of mitochondrial division providing a constrictive force. Years later, lysosome and trans-Golgi derived vesicles (TGN) were shown to impart important functional relevance to this process. Among molecular regulators, Drp1 plays a central role. Studies have shown Drp1 to be modulated by several post-translational modifications including phosphorylation and SUMOylation. Another important candidate that regulates mitochondrial division is the actin cytoskeleton. Actin filaments polymerized by the ER-bound inverted formin 2 (INF2) mediate division of both mitochondrial membranes. INF2-generated filaments promote ER–mitochondria contacts (ERMC), facilitate Ca+2 transfer, and drive inner mitochondrial membrane (IMM) division. These filaments also promote productive Drp1 recruitment at the division sites enabling outer mitochondrial membrane (OMM) division. Recent studies have shown that TGN vesicles power the final steps of OMM division through production of PI4P catalyzed by PI4KIIIβ, though the mechanism of final scission remains unclear. In this study, we identify the actin nucleator formin-like protein 1 (FMNL1) as a potential mediator of late-stage mitochondrial division downstream of Drp1 and TGN vesicles. FMNL family of formins are present in the Golgi apparatus and we hypothesized that FMNL1-derived actin delivered via TGN vesicles powers final OMM division. si-RNA mediated knockdown of FMNL1 in HeLa and U2OS cells showed an elongate mitochondrial morphology. Interestingly, FMNL2 KD in HeLa cells and FMNL3 KD in U2OS cells did not alter mitochondrial morphology indicating FMNL1 specificity. CRISPR-mediated KO of FMNL1 in HeLa cells also showed an elongated mitochondrial phenotype. Using live-cell assays we confirmed that FMNL1 depletion causes a significant reduction in the rate of unstimulated fission events in both HeLa and U2OS cells. FMNL1 depletion preserved INF2 activity, showed higher ERMC and an increase in both Drp1 and TGN recruitment on mitochondria suggesting FMNL1 acts downstream of these events. In fact, Drp1 overexpression in HeLa cells showed significant mitochondrial fragmentation not seen in FMNL1-depleted cells. FMNL1 emerges as a late-stage regulator of mitochondrial division. Finally, we located FMNL1 puncta at 80 % of fission sites in both HeLa and U2OS cells. Overall, our study identifies FMNL1 as a late-stage regulator likely controlling OMM scission downstream of Drp1 and TGN vesicles.

B137/P0717

Identifying Novel Mechanisms of Mitochondrial Quality Control in Cancer Cells

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Autophagy inhibition has been pursued as an anti-cancer target; however, it has shown limited efficacy in the clinic. There is an urgent need to develop more effective therapeutic strategies for non-responsive tumors. A key function of autophagy is to maintain mitochondrial health. Previously, we found that cancer cells can circumvent autophagy inhibition and still exhibit normal mitochondrial function, indicating that cells have autophagy-independent mitochondrial turnover, although the exact mechanisms have remained understudied. To model autophagy deficiency, we used cancer cells that have adapted to genetic knockout of the core autophagy protein, ATG7. We performed a genome-wide CRISPR screen in ATG7 KO cells using a pH-sensitive reporter of mitochondrial turnover via the lysosome and identified novel regulators of mitochondrial degradation. The top two hits were SET domain bifurcated histone lysine methyltransferase 1 (SETDB1) and Activating transcription factor 7-interacting protein 1 (ATF7IP), which are known to form a complex to regulate chromatin repression. We confirmed that depletion of ATF7IP in autophagy-deficient cells reduces mitochondrial turnover. Additionally, loss of ATF7IP reduces oxygen consumption rate and growth in galactose, indicating a loss of mitochondrial function specifically in autophagy-deficient cells. This indicates that autophagy-deficient cells employ a nuclear-encoded mechanism of mitochondrial degradation to maintain mitochondrial function. Previous work from our lab has shown that autophagy-deficient cells can use small mitochondrial-derived vesicles (MDVs) to degrade damaged mitochondria, bypassing canonical autophagy

machinery. Future studies will determine if SETDB-ATFIP regulates MDVs or constitutes a completely novel mechanism of autophagy-independent mitochondrial quality control. Additionally, ATAC-seq will be used to identify regions of chromatin accessibility regulated by the complex in wildtype and autophagy-deficient cells. Identifying autophagy-independent mechanisms of mitochondrial quality control could help inform therapeutic strategies to target cancer cells that can circumvent autophagy inhibition. Additionally, these mechanisms could be important in aging and neurodegenerative disease, which are known to involve dysregulated mitochondrial homeostasis.

B126/P0706

Investigating mitochondrial form and function function in radial glial cell endfeet during cortical development

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Cortical development relies on radial glial cells (RGCs), fundamental neural progenitors responsible for generating and organizing excitatory neurons and glia. RGCs are bipolar, consisting of a cell body lining the ventricle, and a basal process that terminates in basal endfeet. Disruptions to endfeet are associated with neurodevelopmental disorders such as human cobblestone lissencephaly. Despite their crucial role, remarkably little is known about how RGC endfoot morphology and function are established. Multiple lines of data from our lab point to a local role of mitochondria in endfeet, but this has never been explored. We hypothesize that mitochondria contribute to subcellular radial glia morphology and overall function. Electromicrographs demonstrate mitochondria present in endfeet. To comprehensively understand the mitochondrial network in endfeet, we are leveraging a MitoTag mouse model that GFP labels mitochondria upon exposure with Cre recombinase. Employing in utero electroporation (IUE) to express Cre and to sparsely label RGCs in embryonic brains, we are measuring mitochondria #, volume, and occupancy in RGCs. This will deepen our understanding of mitochondrial architecture and dynamics at the subcellular level in RGCs. To test the requirement of mitochondria in endfeet, we have broadly disrupted mitochondria via in utero injection of CCCP – a mitochondrial depolarization agent – into the embryonic cortex. CCCP treatment impacts basal endfeet organization, suggesting a potential functional role of mitochondria for proper endfeet morphology. Finally, we are investigating functions for a subcellularly localized mitochondrial transcript by depleting it in RGCs. Altogether, this project will advance an understanding of how subcellular function in RGCs dictate brain development.

B125/P0705

Long-lived mitochondrial proteins as pillars of mitochondrial ultrastructure

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Continuous replenishment of mitochondria is essential for maintaining high-quality organelles throughout the lifespan of post-mitotic neurons. Age-related impairments in mitochondrial renewal contribute to progressive neuronal dysfunction. The average half-life of mitochondrial proteins is estimated at less than 3 weeks (1); however, we found that a discrete subset of the mitochondrial proteome persists for at least 4 months in mammalian brains, hearts and oocytes (2). These mitochondrial long-lived proteins, or mt-LLPs are enriched at cristae invaginations and include OxPhos, MICOS, mt-DNA proteins, chaperones, and cytochrome C. We performed cross-linking MS-based quantitative analysis on native OxPhos complexes which revealed a

significant spatial restriction of mt-LLPS and co-preservation within the same cristae. Since cristae stability is intimately coupled to mitochondrial function, we propose that mt-LLPs could play a previously unrecognized role in the long-term stabilization of mitochondrial ultrastructure, which in turn might be imperative for mitochondrial fitness. Our ongoing research efforts are centered on (1) the investigation of molecular quality-control mechanisms governing the exceptional persistence of mt-LLPs, (2) the spatio-temporal distribution of mt-LLP within neuronal mitochondrial networks, and (3) their persistence in the context of cristae stability and remodeling. Insights from this work will provide a new understanding of the role of long-lived proteins in post-mitotic and long-lived cells, and could lead to novel targets for potential therapeutic interventions for neurological disorders. References (1) Fornasiero, E.F., Mandad, S., Wildhagen, H., Alevra, M., Rammner, B., Keihani, S., Opazo,F., Urban, I., Ischebeck, T., Sakib, M.S., et al.(2018). Precisely measured protein lifetimes in the mouse brain reveal differences across tissues and subcellular fractions. Nat Commun, 4230. (2) Bomba-Warczak E, Edassery SL, Hark TJ, Savas JN. Long-lived mitochondrial cristae proteins in mouse heart and brain. J Cell Biol. 2021;220(9). doi: 10.1083/jcb.202005193. PubMed PMID: 34259807

B135/P0715

Mitochondrial presequences harbor variable strengths to maintain organellar function

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Hundreds of mitochondrial-destined proteins rely on N-terminal presequences for organellar targeting and import. While generally described as positively charged amphipathic helices, presequences lack a consensus motif and thus likely promote the import of proteins into mitochondria with variable efficiencies. Indeed, the concept of presequence “strength” critically underlies biological models such as stress sensing, yet a quantitative analysis of what dictates “strong” versus “weak” presequences is lacking. Furthermore, the extent to which presequence strength affects mitochondrial function and cellular fitness remains unclear. Here, we capitalize on the high-throughput and kinetic nature of the MitoLuc mitochondrial protein import assay to quantify multiple aspects of presequence strength. We find that select presequences, including those that regulate the mitochondrial unfolded protein response (UPRmt), impart differential import efficiencies during mitochondrial uncoupling. Surprisingly, we find that presequences beyond those classically associated with stress signaling promote highly variable import efficiency in stressed and basal (i.e., non-stressed) conditions in vitro, suggesting that presequence strength may influence a broader array of processes than currently appreciated. We exploit this variability to demonstrate that only presequences that promote robust import in vitro can fully rescue defects in respiratory growth in Complex IV-deficient yeast, suggesting that presequence strength dictates metabolic potential. Collectively, our findings demonstrate that presequence strength can describe numerous metrics, such as total imported protein, maximal import velocity, or sensitivity to uncoupling, suggesting that the annotation of presequences as “weak” versus “strong” requires more nuanced characterization than is typically performed. Importantly, we find that such variability in presequence strength meaningfully affects cellular fitness in processes beyond stress signaling, suggesting that organisms may broadly exploit presequence strength to fine-tune mitochondrial import and thus organellar homeostasis.

B122/P0702

Mitochondrial size control in budding yeast

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Mitochondria are semiautonomous organelles whose biogenesis involves coordination of processes in the nucleus, cytoplasm, and in mitochondria themselves. Despite this complexity, cellular mitochondrial content is maintained within a limited range: cells do not continually lose or gain mitochondria in an uncontrolled manner. How this control is achieved remains poorly understood. We are developing and testing phenomenological models of mitochondrial size control, and investigating their molecular basis using live-cell microscopy and proteomics. We are examining the statistics of mitochondrial growth in single cells to differentiate between competing models of size control. We are measuring the abundance of proteins involved in production of nuclear-encoded and mitochondrial-encoded proteins, protein import into mitochondria, and lipid biosynthesis, and perturbing the rates of these processes to determine their impact on mitochondrial size control. Taken together, our results indicate that mitochondrial size control is decoupled from overall cell size control, and point towards a key regulatory role for mitochondrial protein import in mitochondrial size control.

B127/P0707
Molecular basis for the impaired mitochondrial dynamics in Barth Syndrome
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Barth syndrome (BTHS) is an important X-linked cardiomyopathic disease characterized by perturbations of cardiolipin (CL) metabolism in mitochondria. Loss of CL content or accumulation of monolyso-cardiolipin (MLCL) result in defects in cardiac and skeletal muscle cells, which are very energy dependent and particularly vulnerable to mitochondrial dysfunction. CL interacts with the core components of mitochondrial fusion and division machines and plays a central role in a plethora of metabolic and regulatory processes that determine cell function and fate. However, the precise mechanisms through which CL influences mitochondrial morphology are not well understood. In this study, employing molecular dynamics (MD) simulations, we determined that CL molecules extensively engage with the mitochondrial fusion protein Optic Atrophy 1 (OPA1), which controls membrane-shaping mechanisms. Structure-function analysis confirmed the interactions between CL and two conserved motifs of OPA1 at the membrane-binding sites. We further developed a bromine-labeled CL probe to enhance electron cryo-microscopy (cryo-EM) contrast and characterized the structure of OPA1 assemblies bound to the CL-brominated lipid bilayers. Our images provide direct evidence of CL enrichment within the OPA1-binding leaflet. Last, we observed a decrease in membrane remodeling activity for OPA1 in lipid compositions with increasing concentrations of MLCL. This suggests that the partial replacement of CL by MLCL, as observed in BTHS, alters the malleability of the membrane and compromises proper remodeling. Together, our findings will provide a framework for understanding the role of mitochondrial dynamics in BTHS and highlight an important step toward exploring the pathophysiology of BTHS and related clinical therapeutics.

B130/P0710
Multiple pathways prevent bi-parental mitochondria transmission in C. elegans
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Mitochondria are essential organelles in eukaryotic cells that provide cellular energy and are involved in numerous cellular pathways. They possess their own genome, the mtDNA, which encodes only a small fraction of a cell's genes, yet it remains crucial. Unlike the nuclear genome, which is inherited equally from both parents, the mitochondrial genome is passed down exclusively from the mother during sexual reproduction. The C. elegans model enabled the identification of several factors and mechanisms involved in sperm mitochondrial clearance. The discovery that the autophagy pathway and its adaptor ALLO-1 are involved marked significant progress. Additionally, studies in fish and flies revealed that CPS-6, a sperm mitochondrial endonuclease, plays a role in degrading C. elegans sperm-derived mtDNA. Sperm mitochondria-associated proteins FNDC-1 and PHB-2 were also found to play a role in the efficient removal of sperm-derived mitochondria. Despite these findings, the exact extent of sperm-mitochondria clearance, as well as the possibility of experimentally preventing it and inducing mtDNA transmission to progeny, remain unclear. To unravel the genetic interactions and assess the impact of simultaneous inactivation of known factors on the fate of sperm-derived mitochondria and their mtDNA, we employed quantitative cell biology approaches. Our findings revealed an additive effect of ALLO-1 and CPS-6 loss of functions. However, inactivation of known factors involved in sperm mitochondria clearance was insufficient to prevent sperm mitochondria degradation and enable paternal mtDNA inheritance across generations. Thus, unidentified mechanism(s) safeguarding maternal mitochondrial inheritance remain to be found. Likewise, the signals carried by sperm mitochondria that trigger their specific recognition and degradation in the embryo remain also elusive. To reveal both, we identified the proteins interacting with Atg8/LC3 in early embryos using in vivo TurboID labelling. Our results suggest that C. elegans employs at least three pathways to degrade paternal mitochondria. Using a proteomic approach, we have identified novel factors that regulate uniparental maternal mitochondrial inheritance. We are currently validating these factors and allocating them to the different pathways.

B123/P0703

Probing the evolutionary conservation of the ATAD3A ATPase domain

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ATPase family AAA domain containing protein 3, known as ATAD3, is an essential mitochondrial protein ubiquitous to nearly all eukaryotes. Despite extensive research in mammalian cell systems, the function of ATAD3 remains poorly understood. In humans, mutations in ATAD3 manifest as severe mitochondrial diseases and spontaneous abortion. Various cancers and aging have also been linked to ATAD3. ATAD3 is hypothesized to have a unique topology, with the C-terminal ATPase in the mitochondrial matrix and the N-terminus exposed to the cytosol, thereby spanning both the mitochondrial inner membrane (MIM) and the outer membrane (MOM). Given this topology, ATAD3 has been proposed to facilitate communication between mitochondria and other cellular compartments. It has also been shown to be involved in cristae organization and maintenance of mtDNA, an important regulator of mitochondrial morphology and immune system sensing. The complexity of this singular protein and the plethora of mitochondrial functions to which it is tied lends credence to further study in an experimentally amenable system. The C-terminal ATPase features highly conserved functional domains. Due to its evolutionary conservation, we hypothesize that the C-terminal ATPase from disparate lineages may rescue function in other organisms. Whereas most metazoans require a single ATAD3 copy for viability, seed plants, including Arabidopsis thaliana, have evolved the necessity for two—each from different groups. The basal land plant Physcomitrium patens has two ATAD3 genes, ATAD3A and ATAD3B, whose proteins are 85% similar. We isolated knockout plants lacking either ATAD3A or ATAD3B but were unable to generate plants lacking both, suggesting one copy is required for viability. While Δatad3b is mostly normal, Δatad3a is severely stunted with aberrant mitochondria and significantly reduced inner membrane. Since ATAD3A is sufficient for normal growth, manipulating the ATAD3A locus in the Δatad3b

background provides an excellent platform to assess conservation of ATPase function across lineages. We replaced the endogenous ATAD3A locus with ATAD3A cDNA in Δatad3b and recovered plants with a wild-type phenotype, suggesting that intron removal does not impair function. Using this line, we replaced the ATPase domain with sequences from A. thaliana, H. sapiens, and C. elegans. We successfully generated P. patens plants harboring the Arabidopsis ATPase domain. This demonstrates that despite over 400 million years of evolutionary divergence, the ATPase domain is functionally conserved. Ongoing work will test whether ATPase domains from H. sapiens and C. elegans can similarly rescue function.

B128/P0708

Specific adaptations of mitochondria during the egg-to-embryo transition

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In humans, an egg lies dormant for decades before it generates a new embryo. Given that mitochondria biogenesis is largely suppressed from late oogenesis until the blastocyst stage of embryonic development, the same mitochondria must be capable of both attenuated activity in the egg and rapid activation in the embryo. These stages have distinct metabolic and biophysical demands, but we still do not know how mitochondria support a seamless transition. Using Drosophila melanogaster, we performed an RNAi screen to assess how disrupting mitochondrial gene expression in oocytes impacts mitochondria in embryos. Immunofluorescence on embryos revealed a phenotypic class marked by heightened reactivity to antibodies targeting proteins in the inner mitochondrial membrane. Here, we present an example from this class of a mitochondrial inner membrane transporter whose loss results in a striking enlargement of mitochondria visible by super-resolution light microscopy. Using transmission electron microscopy, we find that these mitochondria maintained cristae organization and membrane integrity, suggesting that mitochondria function is preserved despite extensive swelling. To determine when in gametogenesis mitochondria enlargement first occurs, we examined the organelles in mature eggs and found them to be morphologically indistinguishable from wild-type eggs. This indicates that the swelling phenotype manifests specifically at the egg-to-embryo transition, a time when the egg rapidly rehydrates due to an influx of water. We propose that this transporter functions to tune mitochondrial osmolarity in response to changing cytoplasmic conditions accompanying egg activation. Together, these data provide insight into how mitochondria function contributes to the preservation of the germline and fertility across generations.

B134/P0714

Structural insights into GTP-coupled conformational changes in Mfn1 revealed by time-resolved transition metal ion FRET

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Regulation of mitochondrial shape, size, and cellular distribution is achieved through a balance of fusion, division, and microtubule-based transport. Outer mitochondrial membrane fusion is mediated by the mitofusin paralogs: Mfn1 and Mfn2. Dysregulation of mitochondrial fusion has been implicated in several neurological diseases including Charcot Marie Tooth Type 2A (CMT2A). CMT2A is caused by mutations in MFN2, which alter mitochondrial fusion and transport. The mitofusins are members of the dynamin

superfamily, large GTPases that couple conformational changes with the catalytic cycle to remodel cellular membranes. Molecular characterization of CMT2A variants indicate that the disease variants are impeded at different steps in the fusion mechanism. We predict that aberrant allostery is a major component of the diverse dysfunction in the CMT2A variants. Universally in the dynamin superfamily, allosteric regulation is critical to advance the process of membrane remodeling. To elucidate the conformational dynamics of Mfn1, we applied a novel transition metal fluorescence energy transfer system to a minimal construct called mini-Mfn1. We used time correlated single photon counting (TCSPC) to measure time-resolved FRET. This has the advantage of recording the distribution of FRET efficiencies of the donor-acceptor pair from an entire population of protein in solution. This allows calculation of the distance distributions of the protein sample and reveals structural heterogeneity. We performed our analysis after incubating the mini-Mfn1 with different nucleotides to represent the progressive steps in GTP hydrolysis. Past work has identified the structure of the same mini-Mfn1 when incubated with GDP and GDP•BeF3. This revealed a large conformational change when the free phosphate is released from the binding pocket. However, the conformation of mini-Mfn1 with GTP bound and nucleotide free Mfn1 remain unknown. Our data agrees with the known crystal structures when Mfn1 is bound to GDP and GDP•BeF3, demonstrating we can recapitulate structural data in solution. We identified the conformation of GTP-bound Mfn1, which is comparable to the GDP conformation. This indicates that Mfn1 undergoes two large conformational changes: first when hydrolysis occurs and then again when the free phosphate is released. Our data show that when GDP is released Mfn1 is in a novel conformation, which would be linked to the disassembly of the fusion complex. Our data reveal novel insights into the structure and energetics of GTP-driven conformational changes of Mfn1 and created a powerful system for future studies.

B138/P0718

TBC1D15 functions as an Arl4D GAP and promotes the mitochondrial translocation of Arl4D for organelle homeostasis

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ADP-ribosylation factor-like 4D (Arl4D), a Ras small GTPases superfamily member, was previously found to locate at the mitochondria and regulate mitochondrial functions at a GDP-bound status. However, it was unknown of the underlying mechanisms that governs the nucleotide-binding status and the resulting mitochondrial translocation of Arl4D. In this study, we uncover that TBC1D15, a well-known Rab7 GTPase-activating protein (GAP), serves as a novel GAP for Arl4D that mediates the mitochondrial translocation in serum-starved conditions. We found that TBC1D15 interacts with Arl4D through the TBC domain and promotes GTP hydrolysis of Arl4D. Furthermore, TBC1D15 depletion results in increased Arl4D activity and decreased Arl4D mitochondrial translocation under serum starvation. Altogether, these findings support TBC1D15 as an Arl4D GAP and reveal a new role for this GAP in modulating mitochondrial homeostasis.

B133/P0713

Testing Reversibility of Endosymbiotic Gene Transfer between Chloroplast and Nucleus

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Most organelle-targeted proteins are encoded in the nuclear genome, such that these genes are under the regulatory dominance of the nucleus. For the chloroplast, the essential organelle in photosynthetic eukaryotes, nuclear-encoded chloroplast proteins arise via two routes: First, genes of cyanobacterial origin were moved from the chloroplast genome to that of the nucleus through endosymbiotic gene transfer. Second, proteins of eukaryotic origin emerged to accompany the evolution of chloroplast function and structure. These proteins are reimported to the chloroplast with the help of transit peptides and the protein-import machinery. Expressing these nuclear-encoded proteins from the chloroplast genome and examining their functions should provide valuable insights into the regulatory process of organelle proteins and expand options for heterologous chloroplast engineering. In this study, we established a high-efficiency electroporation protocol to transform the chloroplast genome of the unicellular green alga Chlamydomonas reinhardtii and demonstrate stable expression from both mono- and bicistronic cassettes. Furthermore, we successfully expressed two nuclear-encoded chloroplast proteins in the chloroplast genome: FtsZ1 and EPYC1. FtsZ1 is a tubulin homolog of cyanobacterial origin and a core subunit of the chloroplast division FtsZ ring; EPYC1 is a multivalent Rubisco-linker of eukaryotic origin and is required for the formation of the pyrenoid, a sub-organelle essential for the CO2-concentrating mechanism. Regardless of the gene origin, both the chloroplast-encoded FtsZ1 (cp-FtsZ1) and EPYC1 (cp-EPYC1) localized correctly in the chloroplast, matching their nucleus-encoded counterparts. Unexpectedly, the cp-EYPC1 strains showed superior growth to that of transgenic nuclear EPYC1 when compared in the same epyc1 mutant background, suggesting that direct chloroplast-genome expression can enhance protein function and chloroplast performance (e.g., carbon fixation). Together, our study provides a robust and efficient protocol for chloroplast transformation in Chlamydomonas green algae and a platform for further understanding the regulation of organelle proteins under heterologous expression.

B124/P0704

Uncovering the relationship between MSTO1 and mitochondrial dynamics

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Mitochondria are constantly fusing, dividing, and moving on microtubules to maintain structural and functional integrity. Mitochondrial dynamics are regulated by cytosolic factors and post-translational modifications, providing critical integration into cellular physiology. In addition, maintenance of mitochondrial DNA (mtDNA) is essential for mitochondrial health, as the genome encodes essential oxidative phosphorylation proteins. Recently, mutations in MSTO1 have been linked to clinical disease phenotypes typical of mitochondrial dysfunction. MSTO1 patient-derived fibroblasts have short mitochondria and a striking loss of mtDNA. This suggests MSTO1 is a novel regulator of mitochondrial fusion and mtDNA maintenance. Outside of MSTO1 patient studies, nothing is known about the function of MSTO1 in vertebrates, highlighting a critical gap in our understanding of the molecular mechanisms underlying this disease. MSTO1 protein expression is almost undetectable in MSTO1 patients, indicating that amino acid substitutions destabilize the protein. To recapitulate MSTO1 patient phenotypes, I generated cell lines using the auxin-inducible degradation (AID) system to allow temporal control of MSTO1 protein stability. By four hours of auxin treatment MSTO1-FLAG-AID protein decreases to almost undetectable levels by Western Blot. MSTO1-AID cells treated with auxin for six days possessed shorter mitochondria than controls and MSTO1-AID cells treated with auxin for only three days. The extended length of time required for structural changes in the mitochondrial network suggests MSTO1 may indirectly regulate mitochondrial fusion. Given this, I considered additional functions. Research on the MSTO1 Drosophila melanogaster homolog, misato, describes a role in the stability of the TRiC chaperone. TRiC is a heterooligomer predicted to aid in the folding of about 10% of cytosolic proteins, with a critical role in folding tubulin and actin. Using my MSTO1-AID cells, I discovered that TRiC protein levels decrease as early as

48 hours after MSTO1 depletion. This raises the intriguing possibility that this conserved function of MSTO1 plays an important role in development or disease.

Organelles - Nucleus

B149/P0729
A conserved mechanism of membrane fusion in nuclear pore complex assembly
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The nuclear pore complex (NPC) is the central gateway between the nucleus and cytoplasm in all eukaryotes, and its biogenesis requires fusion of the inner and outer nuclear membranes. Although this membrane fusion step is essential for eukaryotic life, its molecular mechanism has long remained mysterious. In our work, we elucidate the mechanism by which two paralogous transmembrane proteins, Brl1 and Brr6, mediate membrane fusion in S. cerevisiae. Both proteins form ring-like multimeric complexes that remodel membranes and interact with one another across the nuclear envelope lumen through conserved hydrophobic loop motifs. Disrupting either multimerization or Brl1-Brr6 interaction blocks fusion and halts NPC assembly. Remarkably, phylogenetic analyses reveal that Brl1/Brr6 homologues are broadly distributed across eukaryotes, and functional experiments in human cells and D. melanogaster establish CLCC1 as an NPC fusogen in metazoans. Together, our results uncover a novel, conserved mechanism for membrane fusion in eukaryotes.

B156/P0736
A Sir2-dependent pathway regulates nuclear lipid droplet biogenesis
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The nuclear envelope (NE) is composed of two phospholipid bilayers termed the inner (INM) and outer (ONM) nuclear membrane. While the ONM is continuous with the endoplasmic reticulum, the INM maintains a distinct proteome and lipid composition. Recent studies in budding yeast have shown that the INM is enriched in diacylgycerol (DAG) and serves as a crucial site for nuclear lipid droplet synthesis. However, the mechanism regulating nuclear lipid droplet biogenesis remains to be further elucidated. Here we show that in S. cerevisiae the INM-localized protein Csa1 regulates the nuclear localization of the acyltransferase Lro1, which converts DAG to triacylglycerol to produce nuclear lipid droplets. We show that Csa1 contains an amphipathic helix which mediates its interaction with the INM. In response to nutrient depletion and at the onset of meiosis, both Csa1 and Lro1 concentrate to a subdomain of the INM adjacent to the nucleolus which we term the n-INM. In the absence of Csa1, Lro1 forms foci along the nuclear periphery. We identify the NAD+-dependent deacetylase Sir2 as a regulator of Csa1 and Lro1 at the n-INM. Disrupting the enzymatic activity of Sir2 causes both Csa1 and Lro1 to localize evenly along the nuclear periphery. Loss of Csa1 or Sir2 results in a decrease in lipid droplet formation. Our findings therefore reveal a novel Sir2-Csa1-Lro1 signaling pathway at the INM that regulates nuclear lipid droplet biogenesis and nuclear envelope remodeling.

B155/P0735

Determining the order of import of nuclear proteins post-mitosis required for the gradual activation of mRNA transcription, processing and export

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Gene expression must be re-established in the nucleus after every cell division, to reinstate transcription, RNA processing, and export. In addition, nuclear bodies, such as nuclear speckles that contain many mRNA-processing factors, reassemble. Prior studies have demonstrated the assembly of the nucleolus by the gradual return of rRNA transcription and nucleolar proteins following mitosis. However, it is unknown whether the timing of return of the many factors reactivating mRNA transcription, splicing, and export into the nucleus follows an ordered return or whether this is a random occurrence. Our study investigates the re-entry of nuclear proteins essential for re-initiating gene expression in conjunction with the formation of nuclear speckles during early interphase. The time-frame of this flux of molecules into the newly forming nucleus is short, making it challenging to follow temporally. We found that by targeting the nuclear pore complex it is possible to prolong the phase of nuclear entry of numerous RNA-binding proteins. Since many of these proteins concentrate in cytoplasmic granules that form during mitosis, we can use live-cell microscopy to follow their import dynamics after mitosis. We find that certain RBPs return in distinct waves and that their re-entry is tightly linked to nuclear speckle formation and the reactivation of gene expression. The absence of these processing factors in the nucleus led to impaired RNA splicing and export, which were restored upon the re-entry of these factors. We are determining which import mechanisms may be employed to regulate the timing of protein entry into the nucleus. Altogether, this study suggests a regulated hierarchy of nuclear import that is required for proper mRNA processing and export.

B147/P0727

Developing a chemical genetic approach to study VPS4/ESCRT-III functions in human cells

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The genome of eukaryotic cells is enclosed within the nuclear envelope, a double membrane whose integrity is required for the separation between nuclear and cytoplasmic compartments. Failure to maintain this compartmentalization leads to DNA damage and may result in chromosome fragmentation and rearrangements. The Endosomal Sorting Complex Required for Transport III (ESCRT-III) and VPS4, a AAA enzyme, mediate nuclear envelope sealing. However, the consequences of the misregulation of this dynamic process, completed within minutes, have remained unclear due to the lack of systems for acute inactivation. Moreover, VPS4, which is expressed in human cells as two paralogs, VPS4A and VPS4B, has recently been identified as a promising target for anticancer therapy. Yet, consequences of paralog-specific pharmacological inhibition of activity have not been analyzed. Here, we develop a chemical genetic approach that allows rapid VPS4 inactivation in human cells, thereby blocking ESCRT-III polymer disassembly. The approach is based on an engineered VPS4 protein variant that makes the enzyme susceptible to chemical inhibition in living cells. Using these VPS4 alleles in combination with chemical inhibitors, we probed the functions of VPS4/ESCRT-III in human cells. VPS4 inhibition led to rapid accumulation of ESCRT-III polymers on membranes and stalling of dependent cellular processes, such as autophagy and cell abscission during cytokinesis. In the nucleus, VPS4 inhibition caused the accumulation of membrane-associated CHMP7 polymers near centromeres, and persistent presence of CHMP7 resulted in DNA damage. These findings reveal a VPS4-dependent surveillance

mechanism that prevents toxic ESCRT-III polymer persistence in the nucleus and suggest a direct link between nuclear membrane remodeling and genome stability.

B152/P0732
ESCRT recruitment to meiotic nuclear envelope hole
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Meiotic nuclear division necessitates an underappreciated challenge for cells, where the homologous recombination of meiotic chromosomes under intense mechanical stress must be coordinated with rapid nuclear envelope (NE) remodeling. While work in the last decade has provided insight into the mechanism of mitotic NE remodeling, including the contribution of the endosomal sorting complexes required for transport (ESCRT) to membrane restriction and sealing, a mechanism of meiotic NE remodeling has been comparatively lacking. Though superficially similar, meiosis presents unique challenges to the cell. Notably, in the model yeast Schizosaccharomyces pombe, there is a transient loss the RanGTP gradient, without a full disassembly of the NE, in what has been termed a “virtual” NE breakdown. However, the full extent of nuclear compartmentalization loss remains unknown. Moreover, it is not clear how the current model of ESCRT recruitment to a NE hole, which relies on an intact NE permeability barrier, can be reconciled with the meiotic landscape. We have used live-cell fluorescence microscopy of GFP-ESCRT proteins to determine their role in NE remodeling during meiosis. We determine that GFP-ESCRTs are recruited to a meiotic NE following extrusion of the yeast microtubule organizing center, the spindle pole body (SPB), in both meiosis I and meiosis II. We find that Cmp7-GFP is recruited 78 seconds following SPB extrusion from a NE hole in meiosis I and 108 seconds following SPB extrusion in meiosis II. Unexpectedly, we find that ESCRT proteins persist asymmetrically at a single site of a meiotic NE hole from meiosis I and into meiosis II. Further, using a fluorescent reporter of passive diffusion in the nucleoplasm, MGM4, we determine the extent to passive diffusion is disrupted during “virtual” NE breakdown. Our results demonstrate the adaptability of the ESCRT machinery to respond to a NE hole in different cellular contexts and further elucidate the molecular mechanism of this protein complex.

B146/P0726
Exploring the role of the nuclear pore complex (NPC) in steroid-resistant nephrotic syndrome (SRNS) podocyte dysfunction
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Proper blood filtration through the kidneys is essential for human physiology and is carried out by specialized renal cells called podocytes. Mutations in components of the nuclear pore complex (NPC) have been linked to podocyte dysfunction and are causative for steroid-resistant nephrotic syndrome (SRNS), a disease that can lead to renal failure. To investigate this connection, we use both H9 human embryonic stem cells and conditionally immortalized human podocytes (CIHP-1) to study the connection between podocyte differentiation and NPC composition. Mass spectrometry (MS) reveals clear alterations in relative nucleoporin (Nup) abundance upon differentiation in both H9 and CIHP-1 cells. To probe Nup composition at the level of individual pores, we are using CRISPR/Cas9 gene editing to endogenously tag Nups with fluorescent markers, enabling live-cell imaging of NPC remodeling during differentiation. In parallel, we are developing a pipeline for

cryogenic electron tomography (cryo-ET) studies using both H9 and CIHP-1 cells. After understanding the impact of differentiation on NPC composition and morphology, we plan to generate cell lines that carry SNRS-associated Nup mutations to understand how disease-related mutations may impact NPC remodeling. In doing so, we aim to provide a detailed understanding of how NPC remodeling during podocyte differentiation is disrupted by disease-linked mutations, and how these changes contribute to the pathogenesis of SRNS.

B143/P0723

Fission yeast SPB proteins establish an alternative proteinaceous diffusion barrier at the nuclear envelope during mitosis

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The nuclear compartment is defined by the nuclear envelope and nuclear pore complexes (NPCs) that regulate traffic across this boundary by imposing a size-selective diffusion barrier. In our recent work studying the mitotic remodeling of the nuclear envelope, we uncovered a diffusion barrier that acts alongside NPCs to maintain the nuclear compartment. During the closed mitosis of Schizosaccharomyces pombe, the mitotic spindle is organized by the spindle pole body (SPB), which undergoes a cycle of insertion and extrusion in and out of the nuclear envelope. Despite the generation of a nuclear envelope fenestra to facilitate this SPB cycle, the nuclear compartment remains intact throughout cell division, even in contexts when this fenestra becomes enlarged, for example in late anaphase upon disruption of Cmp7, the nuclear envelope adapter for the endosomal sorting complexes required for transport (ESCRT) machinery that exerts a grommet-like function upon SPB extrusion. Thus, elements of the SPB likely form a remarkably plastic diffusion barrier that can expand to fill nuclear envelope fenestrations as large as 500 nm. Indeed, the integrity of the nuclear compartment is lost after conditional depletion of several SPB proteins, particularly the key structural components Pcp1 and Pcp89. Consistent with the ability of these proteins to respond to nuclear envelope hole size, they are both found at higher copy number at the SPB in cmp7Δ cells through a mechanism that we tie to the activity of polo like kinase (Plo1). This pool of Pcp1 occludes a fluorescent diffusion barrier reporter through a mechanism that is disrupted by treatment with the aliphatic alcohol, 1,6 hexanediol. Thus, we propose that mitotic nuclear envelope holes are plugged by the Plo1-dependent expansion of at least Pcp1, which may form a biomolecular condensate that can be tailored to nuclear envelope hole size and restricts the diffusion of large macromolecules.

B144/P0724

Genetic Variants in SMAD7 Enhance LMNA-associated Muscular Dystrophy Through Hyperactivation of TGFß-SMAD Signaling, Demonstrating Therapeutic Potential

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Mutations in LMNA, encoding A-type nuclear lamins, cause a collection of human diseases known as laminopathies, which include multiple types of muscular dystrophy (LMNA-MD). The symptoms of LMNA-MD are sensitive to genetic background, as individuals with the same LMNA mutation can have distinct diagnoses and/or variable disease severity. Here, we describe a family in which four siblings with the same LMNA mutation present with Emery-Dreifuss muscular dystrophy (EDMD) with highly variable severity. The objective

of our research is to identify genetic modifiers of LMNA-MD that drive variable disease presentations and use these modifiers to identify novel treatments for the disease. Using whole genome sequencing, we identified a DNA sequence variant that segregates with severe disease in this family. The variant resides in the coding region of the SMAD7 gene, which encodes a negative regulator of the SMAD signaling pathway. To functionally test the SMAD7 variant, we generated a Drosophila model containing both the LMNA mutation and SMAD7 variant in their respective Drosophila orthologs. We demonstrate that the SMAD7 variant has minimal effects on muscle function alone. However, in combination with the LMNA mutation, the SMAD7 variant enhances muscle defects, consistent with a genetic modifier. We found that mutant lamins alone activate SMAD signaling in Drosophila and human muscle. The SMAD7 variant enhances this activation in Drosophila, suggesting a mechanism for disease enhancement. To broaden our analysis, we sequenced the SMAD7 gene in a cohort of 45 unrelated individuals with LMNA-MD and identified six additional variants, two of which were modeled in Drosophila. These variants also enhance muscle defects through hyperactivation of SMAD signaling in muscle when expressed in combination with multiple LMNA mutations, supporting the broad applicability of SMAD7 as an LMNA-MD modifier gene. To translate these findings to potential treatments, we demonstrate that overexpression of wild-type SMAD7 and treatment with small molecule TGFβ-SMAD inhibitors rescue muscle defects in the Drosophila model, implicating the SMAD signaling pathway as a therapeutic target for the disease.

B151/P0731

GlcNAcylation of NPCs modulates the diffusion barrier in response to cell type and mechanical state

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Nuclear pore complexes (NPC) are embedded in the nuclear envelope and control molecular exchange between the cytoplasm and nucleoplasm. There is emerging evidence of plasticity in the molecular composition and dilation state of NPCs observed in different cell types and mechanical environments, but how such changes ultimately alter an NPC's selective sorting properties remains uncertain. In contrast, there is evidence that changing the levels of the post-translational O-linked N-acetylglucosamine (GlcNAc) modification of nucleoporins impacts NPC permeability. Here, we demonstrate that GlcNAcylation of NPCs is variable across cell types including in cultured neurons, which are depleted of GlcNAc and maintain a more stringent NPC diffusion barrier. These data suggest that cell-type specific NPC GlcNAcylation is a previously unappreciated mechanism that may tailor the selective permeability properties of NPCs to meet cell-type specific nuclear transport demands. Further, like NPC dilation, NPC GlcNAcylation is responsive to mechanical cues: we find that culturing cells on "stiff" or "soft" substrates either increases or decreases NPC GlcNAcylation, respectively, through a mechanism that is independent of altering the levels or localization of the enzymes that control global GlcNAcylation. Using pan-expansion microscopy and new conditional tools that specifically alter the GlcNAcylation of NPCs, we demonstrate that modifying NPC GlcNAcylation not only modulates the stringency of the diffusion barrier but is also sufficient to directly impact NPC diameter. These changes may also underlie our observation that nuclear volume equilibration after hyper-osmotic treatment is slower when NPC GlcNAcylation is inhibited. Together, our data support that the GlcNAcylation of nucleoporins is a driver of NPC dilation state that may help to explain changes to NPC permeability in different mechanical environments.

B140/P0720

Identifying Annulate Lamellae-Specific Factors to Uncover the Mechanism of Nuclear Pore Complex Assembly

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Nuclear Pore Complexes (NPCs) are essential for cellular function through their ability to maintain the nucleocytoplasmic barrier and facilitate selective molecular transport. They typically reside within the nuclear envelope (NE) and consist of multiple copies of around 30 evolutionarily conserved proteins termed nucleoporins (Nups). Due to their importance in the cell, impairment or aberrant assembly of Nups can lead to a range of tissue-specific diseases, including neurodevelopmental disorders such as Fragile-X syndrome as demonstrated in our laboratory. Two major pathways of NPC assembly exist in mammalian cells that occur either during mitotic exit or during interphase. However, a recent study has shown that during germ and embryonic cell development, NPCs are also able to form within parallel membrane sheets within the endoplasmic reticulum (ER), termed Annulate Lamellae (AL) and later be incorporated into the growing NE during G1. Our laboratory has since shown that beyond germ and embryonic cells, this structure and AL-NPCs are also present in normally proliferating somatic cells as well as in challenged or stressed cells. While investigating the biogenesis of the AL, our laboratory has identified the N-terminal portion of RanBP2 (Nup358) as being a crucial element of the AL biogenesis and AL-NPC formation. We have also shown that this AL-dependent assembly pathway can contribute approximately 30% of total newly formed nuclear pores, representing a complementary route to the canonical post-mitotic and interphase NPC assembly pathways. Our goal now is to uncover the exact mechanism of AL-NPC formation and to determine how these pre-formed NPCs are inserted into the NE. To achieve this, our laboratory has been working to identify AL specific factors. Through a proteomic study of RanBP2-interacting partners we have identified several promising candidates, including CLIMP63. In preliminary studies, we have found that this ER-resident transmembrane protein is required for proper insertion of AL-NPCs into the ER sheets and NE. Together, our findings support the theory of an AL-dependent pathway of NPC assembly, revealing a previously unrecognized mechanism of nuclear pore biogenesis and highlighting the essential role of AL in cellular homeostasis.

B141/P0721

Innate immune activation is not a generalized consequence of nuclear envelope rupture

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The nuclear envelope (NE) is a double membraned extension of the endoplasmic reticulum that surrounds the nucleus during interphase, compartmentalizing the nucleoplasm and cytoplasm of eukaryotic cells. It has become evident that the NE can be compromised during interphase in a process called NE rupture, typically resulting from mechanical forces acting on the nucleus and enhanced by defects in the structural components of the NE such as the lamina. These NE ruptures lead to the instantaneous loss of compartmentalization between the nucleus and cytoplasm yet are usually repaired quickly, utilizing mechanisms similar to those used for post-mitotic nuclear envelope reformation. Consequences of NE rupture have been reported to include DNA damage as well as activation of innate immune signaling, often via the cGAS-STING cytosolic DNA sensing pathway. However, there are reports that cGAS is inactivated by binding to chromatinized DNA present in the interphase nucleus and micronuclei as well as during mitosis. To directly investigate this discrepancy, we induced nuclear ruptures in non-cancerous and cancer-derived human cells and assessed the activation of the innate immune response by multiple complementary methods, including cGAS-STING-dependent and -independent signaling. We confirmed that the fundamental innate immune signaling pathways are active in these cells following introduction of exogenous cytosolic dsDNA or dsRNA. However, despite robust cGAS localization to nuclear DNA we saw no evidence of innate immune activation following induced spontaneous ruptures, nor in ruptures of non-cancerous cells with a repair defect caused by depletion of the critical NE

repair factor BAF. We also assessed the response of the cytosolic RNA sensors, RIG-1 and MDA5, following NE rupture and observed no evidence of their activation supporting that this pathway is not triggered by self RNA aberrantly exposed by NE rupture. In summary, we have found that innate immune activation is not a generalizable consequence of NE rupture.

B142/P0722

Interrogating the function of the nuclear lamina using deep mutational scanning in human iPSCs

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The nuclear lamina is a meshwork of intermediate filaments that scaffolds and encases the genome. Mutations to the lamin A protein cause a phenotypically diverse group of diseases known as laminopathies, which primarily affect musculoskeletal, adipose, skin, or vascular tissue, even though lamin A is broadly expressed. Disease-causing mutations can affect the turnover, abundance, and localization of lamin A in affected tissue. However, we do not understand how lamin mutations drive cell type-specific dysfunction. We have implemented deep mutational scanning (DMS) in human induced pluripotent stem cells (hiPSCs). With this approach, we can systematically profile the effects of lamin A sequence variation across pluripotent and differentiated cell types. Using serine integrase cassette exchange, we reconstituted lamin A knockout hiPSCs with a pooled library of ~18,000 untagged missense, insertion, deletion, and nonsense lamin A variants precisely integrated as a single variant per cell. By screening the effects of sequence variation on hiPSC fitness, we found that mutations to lamin A regions important for head-to-tail filament assembly are toxic to stem cells, even though lamin A is not required for hiPSC survival. We then analyzed the expression of the variant pool by immunostaining and flow sorting, which revealed that variants in same the head-to-tail interface are destabilized at the protein level. These toxic variants are rare in the human population and include known laminopathy mutations, highlighting the importance of head-to-tail assembly for the stability and function of the lamin meshwork. We are now screening lamin A abundance during differentiation into cardiac lineages, which are particularly affected in laminopathies. This DMS platform will allow us to explore cell type-specific mutational effects and to understand lamin A genotype-phenotype relationships in disease.

B139/P0719

Lamin and NEMP-1 function redundantly to maintain oocyte nuclear integrity

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The integrity of the oocyte nucleus is critical for fertility and reproductive success. During oocyte development, meiotic chromosome segregation and oocyte maturation depend on nuclear envelope (NE) proteins, and mutations in NE genes are linked to infertility or primary ovarian insufficiency. In C. elegans, depletion of LMN-1, the sole lamin protein, causes nuclear collapse in maturing oocytes and complete sterility. However, whether and how LMN-1 works with additional inner nuclear membrane (INM) proteins to safeguard oocyte nuclei remains unclear. Using genome editing, auxin-inducible degradation, genetic analysis, and high-resolution imaging, we discovered that LMN-1 and the conserved INM protein NEMP-1 function redundantly to prevent meiotic nuclear collapse. Simultaneous depletion of LMN-1 and NEMP-1 exacerbated nuclear defects compared to single depletions, demonstrating overlapping roles in maintaining meiotic nuclear integrity. Strikingly, co-depletion of the LINC complex component SUN-1 partially rescued these nuclear defects, implicating LINC-transmitted forces as drivers of nuclear destabilization when INM support is compromised.

Together, these findings identify redundant INM mechanisms that stabilize oocyte nuclei, offering new insight into how nuclear architecture safeguards reproductive success.

B150/P0730

Lamin-B1 maintains nuclear peripheral protein localization to promote proper genome organization and efficient cardiomyocyte differentiation during early development.

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The nuclear lamina consists of a meshwork of type V intermediate filaments called lamins. These filaments have roles in maintaining nuclear integrity and genome organization. In mammalian cells, there are four lamin isoforms that are encoded by three genes: Lmnb1 and Lmnb2 encode for lamin-B1 (LB1) and lamin-B2 (LB2), respectively, and Lmna encodes for lamin-A and the alternatively-spliced lamin-C (LA/C). Most lamin-associated cardiomyopathies involve mutations in LA/C, which have made them a focus of heart-related research. At a cellular level, loss of LA/C in cardiomyocytes results in reduced nuclear integrity, disrupted cytoskeleton, defects in genomic organization, and delays in differentiation. It is debated how much of the disease pathology is due to DNA and nuclear damage and how much is by impaired genome organization and aberrant transcription. In contrast to LA/C, the role of LB1 in cardiomyocytes is not well studied. However, since the heart starts developing and beating at E7.5, well before LA/C expression, LB1 may have an unrecognized role in early heart development. Using a cardiomyocyte-specific knockout of LB1, we had found the mice are viable and fertile but show mild cardiac defects compared to their heterozygous and wildtype counterparts. There is no significant increase in DNA damage or cell death in mutant animals from embryonic to postnatal development. We found that LB1 KO caused significant disruption of H3K9me3 foci and polarization of H3K9me2, that persists from embryonic timepoints into adulthood. Interestingly, although the LINC complexes and nuclear pore complexes (NPCs) also show asymmetric distribution to one side of the embryonic cardiomyocyte nuclei, these defects are rescued by 1 week of age. Whole chromosome painting shows large-scale and irreversible chromosome decompaction and stretching, which we attribute to chromatin interacting with polarized nuclear proteins being pulled away from the regions tethered to LA/C on the opposite side. These effects on genome organization are transcriptionally relevant, as RNA-seq of embryonic and postnatal hearts indicated dysregulation of genes associated with ion handling and sarcomere formation, suggesting impaired differentiation, signaling, and contractility. Collectively, these results show that LB1 is essential for genome organization, in part by maintaining nuclear periphery distribution, which supports timely cardiomyocyte differentiation and maturation. We further show that cardiomyocytes appear robust to alternative genome organizational states as these cells eventually differentiate and form a viable heart. These results may provide interesting insights to laminopathies or other pathologies where genome organization is disrupted.

B154/P0734

Nuclear speckles are regulatory hubs for viral and host mRNA expression during HSV-1 infection

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Herpes simplex virus type 1 (HSV-1) infection remodels the host nucleus, marginalizing chromatin and forming viral replication compartments (VRCs). Nuclear speckles, nuclear bodies enriched in RNA-processing factors,

reposition around VRCs and undergo structural changes. While viral mRNAs are transcribed in VRCs and host transcription is largely suppressed, the nuclear routes used by viral and upregulated host transcripts and their relationship with nuclear bodies, remain unclear. We show that immediate-early (IE) viral transcripts uniquely accumulate in nuclear speckles prior to export, unlike early or late transcripts, revealing a selective nuclear speckle-dependent pathway. Similarly, host mRNAs upregulated during infection traffic into nuclear speckles after transcription. Moreover, nuclear speckles are structurally remodeled, marked by lncRNA MALAT1 removal and increased dynamics of the nuclear speckle core protein SRRM2. Lastly, we found that blocking mRNA export causes IE transcripts to accumulate in nuclear speckles, and that nuclear speckle disassembly severely impairs IE mRNA export, preventing downstream viral gene expression. These findings establish nuclear speckles as dynamic regulatory hubs that selectively facilitate the processing and export of IE viral mRNAs during HSV-1 infection.

B148/P0728

Reconstitution of lamin assembly on nuclear pore complex-containing membranes

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Intermediate filaments called lamins line the metazoan nuclear envelope and organize the nucleus and genome. Unlike actin and microtubules, purified intermediate filament proteins assemble into non-physiological structures, making it difficult to connect lamin functions to their assembly and regulation. To overcome this challenge, we reconstituted lamin assembly without nuclear assembly using interphase Xenopus laevis egg extracts that recapitulate physiological context. Mimicking nucleoplasm conditions triggers dispersed lamin assembly in egg extracts. Such ectopic lamin assembly occurs on nuclear pore complex-containing membranes, but does not recruit known nuclear lamina components, demonstrating that lamin assembly is separable from the rest of the nuclear lamina and nucleus. This assembly assay in the physiological context of cellular components opens the door to mechanistically dissecting nuclear lamina function in nuclear organization.

B145/P0725

Vps4 is necessary for assembly of ESCRT-III at a mitotic nuclear envelope hole

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Vps4, a AAA-ATPase, is an integral component of the endosomal sorting complexes required for transport-III (ESCRT-III) machinery. These highly conserved membrane remodeling proteins are responsible for diverse cellular processes ranging from multivesicular body and exosome formation to nuclear envelope (NE) sealing. While Vps4 is known to provide the catalysis for ESCRT-III disassembly, it remains unclear the extent to which Vps4 contributes to assembly of the many ESCRT-III subunits required for NE sealing. In investigating the contribution of Vps4 to NE sealing, we performed C-terminal tagging in a model yeast, Schizosaccharomyces pombe. Unexpectedly, we show that that C-terminal tagging of Vps4 disrupts ESCRT-III subunit assembly, leading to Cmp7-GFP hyperaccumulation at the NE and a lack of accumulation of subsequent ESCRT-III subunits. We compared this loss-of-function mutant to well-documented catalytically dead and temperature-sensitive alleles of Vps4. In contrast, live-cell fluorescence microscopy reveals that N-terminal tagging

preserved Vps4 function at the NE during mitosis. Specifically, depletion of Vps4 N-terminally tagged with an auxin-induced degron (AID-Vps4) phenocopied C-terminal tagging of Vps4 in both Cmp7-GFP hyperaccumulation and absence of later subunits, suggesting C-terminal tagging of Vps4 leads to a loss of function. Importantly, ESCRT-III recruitment and dissociation was normal in cells expressing AID-Vps4 without depletion. Further, using correlative light and electron microscopy, we demonstrate Cmp7-GFP hyperaccumulates at sites of NE herniations. Having confirmed that N-terminal tagging retains functionality of Vps4, we went on to observe GFP-Vps4 localization throughout the NE sealing process. These results establish a necessity of Vps4 at a NE sealing site, not only for ESCRT-III disassembly, but assembly as well. Additionally, our results further demonstrate the challenges of tagging ESCRT-III proteins and the necessity of assaying functionality of tagged proteins.

Organelles - Organelle Biogenesis, Remodeling, and Degradation

B082/P0662
A Conserved Role for Endosulfine Alpha in Ciliogenesis
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Primary cilia are specialized cellular compartments that organize the molecular machinery of major signaling pathways and play critical roles during development and in adult tissue homeostasis. Defects in cilia morphogenesis or function result in genetic disorders called ciliopathies that are commonly associated with neurological deficits including retinal degeneration. A recent study identified loss-of-function (lf) mutations in the endosulfine alpha (ENSA) gene in patients with retinitis pigmentosa (RP) – a retinal ciliopathy; however, the mechanistic basis of ENSA(lf)–dependent RP remains unknown. ENSA and its paralog ARPP19 belong to a highly conserved family of cAMP-regulated phosphoproteins. We find that siRNA-mediated knockdown (KD) of ENSA but not ARPP19 impairs ciliogenesis in human RPE-1 cells. Moreover, in ENSA KD cells that retained cilia, the length of the cilium was significantly decreased. Together, these data indicate that ENSA is required for ciliogenesis in this cell type – a novel role for this gene. In line with our findings in RPE-1 cells, mutations in the C. elegans ENSA ortholog ensa-1 cause severe morphological defects in the specialized cilia of AWC olfactory neurons. Defects in AWC cilia morphology are accompanied by impaired AWC-mediated olfactory function in ensa-1(lf) animals, highlighting a key role for ENSA in cilia biology. ENSA function has been most extensively studied in mitosis. In this cellular context, activation of ENSA via phosphorylation by Greatwall kinase (Gwl) results in inhibition of serine/threonine phosphatase protein phosphatase 2A (PP2A). Using genetic epistasis analysis, we demonstrate that ENSA-1 functions in the same pathway with KIN-4, the C. elegans ortholog of Gwl, and SUR-6 (PP2A-B55) to shape AWC cilia morphology. We will report our ongoing progress toward mapping the downstream components of the ENSA-mediated ciliogenic pathway. Overall, our findings describe a novel role for ENSA in cilia biology and shed light on the potential mechanisms underlying RP.

B095/P0675
Ciliary protein TMEM138 regulates melanogenesis through intracellular trafficking and melanocortin-1 receptor signaling in mouse melanocytes
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Recent genome-wide association studies have identified intronic polymorphisms regulating the expression of the TMEM138 gene that correlate with skin pigmentation levels in native African populations. TMEM138 encodes a small transmembrane protein required for the formation of the primary cilium, an organelle found in most mammalian cells with roles in signal transduction and polarity but no known roles in pigmentation. Inactivating mutations in TMEM138 cause a form of Joubert Syndrome, a severe neurodevelopmental ciliopathy characterized by defects in multiple organs, and, anecdotally, dilute pigmentation in a few patients. To better understand how TMEM138 and the primary cilium function in melanogenesis, we knocked down or overexpressed TMEM138 in immortalized mouse melanocytes. While TMEM138overexpression had no significant effect, its knockdown resulted in a loss of pigmentation due to both reduced melanogenic gene expression and defective intracellular trafficking of several melanogenic cargoes. Using an shRNA screen targeting 30 additional ciliopathy genes in melanocytes, we found that the primary cilium itself is important for melanogenesis, and using cells knocked down for either of two additional ciliopathy genes we found that reduced pigmentation was caused by either a trafficking or a gene expression defect. We further showed that cells depleted of TMEM138 were not responsive to pro-melanogenic MC1R agonists, and that overexpression of MITF – the downstream target of MC1R – triggered a partial phenotypic rescue in these cells. Future work will determine whether the primary cilium is required for MC1R signaling, how this regulation is achieved, and the precise role of TMEM138 in intracellular trafficking. Identifying the mechanisms by which TMEM138 and other ciliopathy genes impact melanogenesis may provide unprecedented insights into melanocyte biology, ethnic disparities that drive disease susceptibilities and health outcomes, and the etiology of rare ciliopathies.

B090/P0670

Cristae shaped by stochastic mRNA localization and ATP synthase oligomerization

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Mitochondrial cristae form and remodel in concert with the lateral mobility and oligomeric state of ATP synthase, yet the spatial logic underlying this process remains unresolved. Here, we combine quantitative live-cell imaging with graph-theoretic network modeling to map the distribution of nuclear-encoded ATP synthase subunits within intact mitochondrial networks in live Saccharomyces cerevisiae. Components of the peripheral stalk, Atp3p (γ), Atp4p (b), and Atp5p (OSCP) preferentially accumulate at tube termini and branch points, producing periodic banding (“stripe-like” patterns) along tubules. Three-way junctions further enrich these proteins relative to edge centers, indicating position-dependent assembly cues rather than global accumulation in pre-existing cristae domains. Single-molecule tracking of the corresponding mRNAs using MS2–MCP labeling reveals a stochastic cytoplasmic distribution, resulting in protein delivery biased toward the tube termini, where diffusion-limited multimerization occurs. A stochastic assembly model incorporating network topology recapitulates the observed stripe-like patterns without positing dedicated nucleation sites. Together, the data support a self-organization mechanism in which freely diffusing ATP synthase protomers assemble into higher-order oligomers at geometrically privileged nodes to seed and sculpt cristae. This framework links mobility, local stoichiometry, and membrane geometry to the emergence of functional bioenergetic landscapes within mitochondria. Keywords: Mitochondria; Cristae; RNA localization; Network topology; Modeling.

B091/P0671

Isotropic ultrastructural imaging reveals novel membrane organization in ciliogenesis initiation

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The primary cilium is essential for development and tissue homeostasis, and its dysregulation is implicated in ciliopathies and cancer. However, the early membrane remodeling events that initiate ciliogenesis remain poorly defined. Using 3D volume electron microscopy and super-resolution imaging, we characterized the ultrastructural and molecular framework governing the initial stages of cilium formation. We show that the enlargement of small distal appendage-docked vesicles marks a critical transition point for ciliogenesis progression. This process is dependent on the distal appendage protein CEP164. These vesicles subsequently fuse to generate distinct tubular intermediates: a C-shaped ciliary vesicle (CCV) structure and a toroidal ciliary vesicle (TCV). These intermediates ultimately assemble into the mature ciliary vesicle that caps the mother centriole distal end. The formation and remodeling of these tubulovesicular structures require the membrane trafficking regulators EHD1 and RAB8, as well as the intraflagellar transport protein IFT88. Notably, EHD1 promotes ciliogenesis by driving membrane tubulation and facilitating removal of the CP110/CEP97 cap from the mother centriole, an essential step for axoneme initiation. We also find that these tubular intermediates coincide with the recruitment of transition zone proteins, suggesting their role in establishing the ciliary gate. Our findings redefine early ciliogenesis by revealing a novel sequence of membrane remodeling events and identifying key molecular players driving this process. This study underscores the power of isotropic 3D ultrastructural imaging and quantitative analysis for unraveling membrane trafficking and organelle biogenesis mechanisms.

B092/P0672

Lipid Droplet-Associated Opa1 Regulates Fatty Acid Release

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Mitochondria and lipid droplets (LD) are organelle alliances that dynamically coordinate fatty acid consumption and storage, respectively. Opa1, a mitochondrial protein that promotes inner membrane fusion, plays a key role in maintaining mitochondrial morphology, and thereby regulating multiple aspects of mitochondrial function. Opa1 has also been reported to regulate LD turnover, possibly via modulating mitochondrial fatty acid oxidation. Interestingly, Opa1 has been detected on LD in cultured adipocytes and adrenal carcinoma cells, as well as in LD proteomes, suggesting a potential direct role of Opa1 in regulating LD turnover. However, how Opa1 targets to and Opa1's functional impact on LD remains elusive. Here we identified a role of Opa1 in regulating fatty acids release from LD by directly localizing to the surface of them, and loss of Opa1 slowed down the breakdown of LD in U2OS cells. By a battery of imaging techniques and biochemical assays, we demonstrated a subpopulation of Opa1 resides on LD in multiple cell lines. Through domain mapping and AlphaFold prediction, we further identified that the exon4 coding sequence adapts an amphipathic helix and the hydrophobic grove within it is required for LD targeting. Intriguingly, a naturally occurring splicing variant lacking exon4 does not localize to LD, suggesting an intrinsic cellular mechanism regulating Opa1 targeting to LD. The localization of Opa1 to LD can also be regulated by the concentration of free fatty acids and pyruvate. Furthermore, we found Opa1 on LD can reduce the lipid peroxidation of LD depending on it GTPase activity. Collectively, these data reveal that Opa1 directly localizes to LD through exon4-dependent mechanisms, where it safeguards LD lipids from peroxidation and regulates fatty acid release.

B087/P0667
Loading TRAF signaling adapters to ciliary extracellular vesicles requires two types of channels.
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The polycystin family of cation channels, best known for roles in polycystic kidney disease, also plays key roles in embryonic development. Polycystin-2 (PKD2) and Polycystin-1-like 1 (PKD1L1) are required for left-right patterning during gastrulation; mutations in either cause randomized situs and congenital heart defects. Polycystin-1 (PKD1) and PKD2 also regulate connective tissue formation, with disruptions leading to craniofacial, axial, and long bone abnormalities. Polycystins function in cilia and extracellular vesicles (EVs), but the signaling cargo and mechanisms of polycystin EV signaling remain unknown. The C. elegans polycystins PKD-2 and LOV-1 are expressed in male-specific sensory neurons and localize to cilia that release polycystins into the environment in the form of EVs. We previously showed that environmentally released EVs signal between animals. The goal of our study was to discover the cargo of polycystin EVs in C. elegans to make inferences about polycystin EV signaling function. Our initial attempts at analyzing bulk EV preparations yielded ~3,000 proteins but lacked information about the protein composition of individual EV-subtypes, a common challenge of the EV field. To tailor our approach toward deciphering cargo composition of specific EV subtypes, we targeted proximity labeling enzyme TurboID to PKD-2::GFP EVs. The approach yielded fewer than 10 candidates, of which we validated the top eight as bona fide cargo of polycystin EVs using endogenous fluorescent reporters and super-resolution imaging. We discovered that neuronal polycystin EVs carry conserved TRAFs (tumor necrosis factor receptor-associated factors), TRF-1 and TRF-2. The process of TRAF loading to polycystin EVs required polycystin-1-like LOV-1 and auxiliary polycystin-associated channel-like PACL-1 and PACL-2 proteins, which are EV cargo and act as cation channels when expressed in Xenopus oocytes. Loss of either LOV-1 or PACLs resulted in the release of PKD-2 EVs lacking TRAFs, altering EV signaling potential. Our work discovers new mechanisms of regulated cargo loading to ciliary EVs and suggests EV-based mechanisms for signaling in health and disease. Mammalian TRAFs are implicated in skeletal development, and their disruption phenocopies conditional loss of polycystins (Pkd1 and Pkd2) in mice in the connective tissue, including shortening of long bones, tooth malformations, craniofacial abnormalities, and abnormal spinal curvature. The shared phenotypes between polycystin and TRAF deficiencies suggest that mammalian polycystins may regulate embryonic morphogenesis via TRAF-mediated pathways.

B081/P0661
Lysosomal expansion compartments mediate transition metal homeostasis in C. elegans
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This poster will focus on the relationship between heavy metal homeostasis of C. elegans and lysosomal expansion compartments. Previously, lysosomes were discovered to contain both an acidic and expansion compartment in excessive zinc conditions. Further experiments demonstrated that labile zinc is contained within the acidic compartment. CDF-2 (a zinc importer) was shown to locate to the expansion compartment membrane, highlighting lysosomes as a site of zinc homeostasis. Using Super-Resolution Microscopy, we have identified other heavy metals that extend the expansion compartment, primarily focused on copper. Using

transgenic worms with tagged CDF-2 and CUA-1.1 (zinc and copper transporters respectively) we can conclude both transporters localize to the same expansion compartment. Using a CF4 dye we can demonstrate that copper, like zinc, localizes to the acidic compartment. These results highlight lysosomes as a site of copper homeostasis.

B093/P0673
Mitophagy amplitude is regulated by an interplay between receptor protection and turnover
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Mitochondria are multifunctional organelles required for several critical cellular processes, including energy generation. Cells need to maintain mitochondrial health as accumulation of damaged mitochondria lead to energy failure, oxidative stress, and pathological diseases. Mitochondrial homeostasis is maintained by multiple quality control pathways, including mitophagy, which removes defective mitochondria from the mitochondrial network. However, a major outstanding question is how cells precisely control the timing, position, and duration of mitophagy episodes. During receptor-mediated mitophagy, the mitochondrial proteins BNIP3 and NIX directly recruit autophagy machinery to the mitochondrial surface, though their regulatory mechanism is still unclear. In recent years, two new BNIP3- and NIX-interacting proteins have been identified. PPTC7 targets BNIP3 and NIX for turnover by the proteasome to keep levels of the receptors low, whereas TMEM11 is proposed to spatially control mitophagy by forming a complex with BNIP3 and NIX at active mitophagy sites. However, it is unclear how each of these interactions are controlled and how they interplay with each other. Here, we identify a new negative regulator of mitophagy, ARMC1, which forms a complex with TMEM11 and modulates its association with BNIP3 and NIX. During mitophagy activation, TMEM11 dissociates from ARMC1, forming a complex with active BNIP3 and NIX. Importantly, in gain-of-function studies, we find that the presence of TMEM11 in a BNIP3/NIX complex impedes the ability of PPTC7 to target the receptors to the proteasome, causing BNIP3/NIX stabilization and prolonged mitophagy activation. Together, these findings support a two-stage mitophagy model: a pre-initiation step where mitophagy is inhibited by constitutive receptor turnover and an activation step where receptors are temporarily protected from degradation, allowing mitophagy to proceed freely.

B083/P0663
Modulation of lipid droplet formation by efflux pump Tpo1 alters ruthenium complex resistance in yeast
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In cancer, elevated lipid droplet (LD) abundance correlates with poor clinical outcomes. Consistent with models wherein LD's dynamically buffer drug toxicity, LD formation is induced by diverse chemotherapeutics, including the anticancer ruthenium complex indazolium trans-[tetrachlorobis(1H-indazole) ruthenate(III)], also known as KP1019. In an early clinical trial, KP1019 stabilized disease in five of six evaluable patients and showed no dose-limiting toxicity. In laboratory settings, KP1019 induces LD formation in both cervical cancer cells and the budding yeast Saccharomyces cerevisiae. In addition to LD formation, KP1019 treatment causes DNA damage, oxidative stress, and G2/M arrest in both cancer and yeast cells, suggesting S. cerevisiae is a suitable model for studying this drug. Genomic screens in yeast identified the promiscuous solute carrier Tpo1 (human ortholog: MFSD10) as a modulator of KP1019 tolerance. Paradoxically, given Tpo1's role in drug efflux, loss of this

transporter increases KP1019 resistance. However, one of Tpo1's substrates is medium chain fatty acids (MCFA's), so loss of tpo1 increases MCFA sensitivity. Here, we verify that sensitivity and show that tpo1 loss increases LD abundance. To determine whether LD homeostasis in general is a modulator of KP1019 tolerance, we supplemented the growth medium with MCFAs. The resulting increase in LD's correlated with antagonism of KP1019 bioactivity. Furthermore, the smaller LD's and lower cellular triglyceride levels caused by loss of the lysophosphatidic acid acyltransferase Loa1 correlated with sensitivity to KP1019, supporting a model wherein LD size and abundance contributes to KP1019 tolerance. In contrast to wild-type controls, KP1019 treatment of yeast lacking the medium chain reductase Etr1 (human ortholog: MECR) results in a dramatic decrease in Nile Red staining of LD's. Combined with the increased expression of Tpo1 following drug treatment, this result suggests that KP1019 mobilizes fatty acids from LD's and that timely restoration of LD lipid content depends on MCFA synthesis. Although future studies are needed to determine whether LD's contribute to KP1019 resistance by modulating oxidative stress, drug sequestration, or other modalities, our findings implicate LD and MCFA homeostasis as potentially druggable targets that modulate sensitivity to KP1019.

B080/P0660

Nucleolar dynamics during zebrafish oogenesis

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Oogenesis is the developmental process by which oocytes are formed, matured, and equipped to support fertilization and early embryonic development, making it essential for female fertility and species propagation. This process requires rapid cellular growth that in some species, such as the zebrafish Danio rerio, must be achieved in one week. This task requires a massive upregulation of protein synthesis, a process carried out by innumerable molecular machines, ribosomes. Biogenesis of these ribosomes takes place in the nucleolus, the membraneless organelle where ribosomal rRNA transcription, processing and subunit assembly take place. Despite its crucial role in oogenesis, the dynamics of the nucleolus within the developing oocyte is poorly understood. For this research, we use northern blot and hybridization chain reaction (HCR) to visualize rRNA transcription and nucleolar dynamics during zebrafish oogenesis. We discovered notable changes in rRNA synthesis, with early-stage oocytes actively synthesizing rRNAs, while late-stage oocytes shift toward rRNA processing. We also discovered significant changes in the volume, shape, localization and quantity of nucleoli in different stages of oogenesis. These findings suggest that the nucleolus is a highly dynamic organelle that undergoes developmentally programmed remodeling to support the biosynthetic demands of the growing oocyte.

B084/P0664

Organelle Signatures Determine Hepatocyte Identity and Capture Liver Remodeling Under Nutritional Stress

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Mitochondria, peroxisomes, and lipid droplets dynamically adapt their morphology, abundance, and spatial distribution to meet cellular metabolic demands. Yet, how these organelles are organized across tissues with heterogeneous microenvironments remains poorly understood. In particular, it is unclear whether organelle features encode information about tissue-level structure or function. This knowledge gap stems from the

absence of tools that enable organelle-to-tissue scale analysis with spatial resolution. To address this, we developed sOrganellomics, a pipeline that integrates confocal microscopy with Cellpose-based segmentation of cell boundaries and organelles. We applied this approach to mouse liver, a tissue characterized by nutrients, oxygen, and hormone gradients from the portal vein to the central vein. Using sOrganellomics, we quantified over 100 features of mitochondria, peroxisomes, and lipid droplets from thousands of hepatocytes in control, fasted, and Western diet–fed mice. Organelle features revealed distinct zonation patterns across the hepatic acinus. Feature-based clustering identified five hepatocyte classes, which were not linearly positioned from portal to central vein but distributed in varying proportions across zones, with the central vein region dominated by a single class. Nutritional perturbations reduced this diversity: fasted livers displayed four classes, whereas Western diet livers collapsed to two. Machine learning models trained on organelle features predicted hepatocyte class and nutritional state with >95% accuracy. Notably, our approach captured progressive organelle remodeling during hepatic steatosis in a high-fat diet–induced model, enabling quantitative tracking of disease progression over two months. In summary, sOrganellomics provides a spatially resolved framework for analyzing organelle architecture in tissue context. Our findings demonstrate that organelle features serve as functional signatures of hepatocyte identity and reveal how nutritional stress reshapes liver organization, offering a quantitative alternative to transcriptomic profiling.

B085/P0665

Seipin Transmembrane Domains are Necessary and Sufficient to Create Functional ER Sites of Lipid Droplet Biogenesis

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Aim and objectives of the study Lipid droplets (LDs) are endoplasmic reticulum (ER) derived fat storage organelles, born at seipin (Fld1/Sei1 in yeast) marked ER subdomains. The main aim of this study was to investigate and characterise the functional role of seipin transmembrane domains (TMDs) in LD biogenesis. Materials and methods: In our study we employed baker's yeast mutants lacking Fld1 in isolation or in combination with Ldb16, a Fld1 partner protein. Inducible yeast cells in which de novo LDs can be produced expressing either full length Fld1 or Fld1 lacking the luminal domain region (Fld1-ΔLR), or the human equivalent, hSeipin/hSeipin-ΔLR fused to mCherry were used for fluorescent microscopy experiments. Terbinafine drug sensitivity rescue experiments were performed to assess the functionality of the expressed constructs. Epitope tagged (HA/FLAG) Fld1/Fld1-ΔLR were generated for gel filtration experiments to determine oligomerisation. Site directed mutagenesis was performed to determine the key residues of Fld1-ΔLR. Thin Layer Chromatography (TLC) was used to perform neutral lipid analysis. LiveDrop probe was employed to visualize and characterize LD biogenesis sites in yeast Fld1 mutants expressing either WT Fld1-ΔLR or mutant version of Fld1-ΔLRP31G. Results: Yeast Fld1 mutants expressing either WT Fld1-mCherry or Fld1-ΔLR-mCherry displayed punctate distribution in the ER and restored LD morphology defect to similar extent of each other. Induction of de novo triacylglycerol (TAG) synthesis resulted in LDs being born at Fld1-ΔLR defined ER subdomains like WT Fld1. Fld1-ΔLR defined ER sites recruited various LD biogenesis factors including LiveDrop and also restored the defect of fld1Δ ldb16Δ double mutants to the extent of human seipin. Fld1-ΔLR expressing cells showed resistance to terbinafine drug sensitivity, indicating Fld1-ΔLR is functional in vivo, unlike mutant Fld1-ΔLRP31G. We demonstrate that Fld1-ΔLR assembles into homo-oligomers and the oligomerisation is independent of the partner protein Ldb16. We found that lack of luminal domain in Fld1-ΔLR resulted in increased steady state TAG levels, indicating crucial role of the luminal domain in TAG partitioning into LDs. Conclusion: Findings from the study demonstrates that the conserved TM domains are essential and sufficient to create functional LD biogenesis sites in the ER. Fld1-ΔLR marked ER subdomains are functional and

can recruit various LD biogenesis factors and restored the aberrant LD phenotype even in the absence of Ldb16. We provide evidence that Fld1-ΔLR can assemble into oligomeric complex which is crucial for the activity. We conclude that the Fld1-ΔLR is necessary and sufficient to establish functional ER sites of LD biogenesis.

B094/P0674

The Unkempt RNA-binding protein reveals a local translation program in centriole overduplication

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Excess centrosomes cause defects in mitosis, cell-signaling, and cell migration, and therefore their assembly is tightly regulated. The divergent Polo kinase, PLK4, controls centriole duplication at the heart of centrosome assembly, and elevated PLK4 levels promote centrosome amplification (CA), a founding event of tumorigenesis. Here, we investigate the transcriptional consequences of elevated PLK4 and find Unkempt (UNK), a gene encoding an RNA-binding protein with roles in mRNA translational regulation, to be one of only two upregulated mRNAs. UNK protein localizes around centrosomes and with CEP131-positive centriolar satellites, promoting CEP131 localization to and around centrosomes. UNK's RNA-binding activity is required for PLK4-induced centriole overduplication. Consistent with the loss in PLK4-induced centriole overduplication, UNK depletion disrupts PLK4 and centriole assembly protein localization. Finally, translation is enriched at centrosomes and centriolar satellites, with UNK and CEP131 promoting this localized translation. In summary, UNK and CEP131 promote PLK4 localization and local translation at centrosomes during centriole overduplication. <div widget-instance-contentmetadata_articlefulltext_article"=""> Subjects:

B086/P0666

Unbiased separation of insulin secretory granule subpopulations by direct current insulator-based dielectrophoresis

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The pancreatic β-cell contains several subpopulations of insulin secretory granule (ISG). These subpopulations include differences in maturity, age, and secretory capacity. Due to limitations in traditional separation methods, the extent of these subpopulations, any overlap between them, and how they are affected by insulinotropic drugs have not been determined. In this work, we adapted a recently developed separation technology, direct current insulator-based dielectrophoresis (DC-iDEP), to separate ISGs isolated from INS-1E cells, an immortalized rat insulinoma cell line model for β-cells, according to their electrokinetic mobility ratio (EKMr). We were able to separate ISG subpopulations from unstimulated cells to determine a baseline distribution and identify characteristic profiles for immature, young, and old ISGs. We then compared this distribution with the distributions of subpopulations from cells stimulated by GPR40 agonism or incretin signaling. We found significant changes in each subpopulation studied in response to stimulation, consistent with the increases in maturation, crystallization, and changes in size found in previous publications.

Organelles - Organelle Crosstalk and Cell Homeostasis

B160/P0740
A KDELR-mediated ER-retrieval system modulates mitochondrial functions via the unfolded protein response in fission yeast
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KDELR (Erd2 in yeasts) is a receptor protein that retrieves ER-resident proteins from the Golgi apparatus. However, the role of the KDELR-mediated ER-retrieval system in regulating cellular homeostasis remains elusive. Here we show that the absence of Erd2 triggers the unfolded protein response (UPR) and enhances mitochondrial respiration and reactive oxygen species (ROS) in an UPR-dependent manner in the fission yeast Schizosaccharomyces Pombe. Moreover, we perform transcriptomic analysis and find that the expression of genes related to mitochondrial respiration and the tricarboxylic acid cycle is upregulated in a UPR-dependent manner in cells lacking Erd2. The increased mitochondrial respiration and ROS production is required for cell survival in the absence of Erd2. Therefore, our findings reveal a novel role of the KDELR/Erd2-mediated ER-retrieval system in modulating mitochondrial functions and highlight its importance for cellular homeostasis in the fission yeast.

B162/P0742
Defining the Ciliary Proteome in Epithelial and Fibroblast Cells using a Proximity Labelling Approach
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Several postnatal tissues and almost every stage of embryonic development exhibit a diverse array of ciliopathy-associated dysfunctions, implying a unique role of cilia on various cell types, with outcomes dictated by the cilia proteome. Our hypothesis is that the cilia proteome allows cells to dynamically respond to environmental cues and varies between cell types resulting in the array of phenotypes observed in ciliopathy patients. Proteins enter and exit cilia through the transition zone (TZ), which serves as the gatekeeper for ciliary protein, while the ciliary Inversin compartment (IC) controls the transport of cargo proteins by forming functional networks. In present study, the proximity labelling approach has been used to dynamically map ciliary proteome. To evaluate the cilia proteome, epithelial and fibroblast cell lines from the Flp-In system have been exploited to target the promiscuous biotin ligase BirA*G3 to the TZ and IC by fusing it with B9d1 (TZ) or the first 203 amino acids of Nphp3 (IC). To validate the generation of stable cell lines, the expression of V5 epitopes and BirA included in the BirA*G3 fusion protein were confirmed using anti-V5 and anti-BirA antibodies through immunoblotting. Immunofluorescence staining revealed precise localization of B9d1-BirA *G3 and Nphp3-1-203-BirA-*G3 fusion protein at the base of cilia or along the length of the axoneme respectively, confirming the successful generation of both Flp-In-IMCD and Flp-In-3T3 cell lines expressing the BirA *G3-tagged protein. Functional characterization of the constructs was validated through incubation of BirA-expressing cell lines with biotin and detection of biotinylated proteins through western blot and immunofluorescence. A significant increase in biotinylated proteins was noticed in cell lines expressing B9d1-BirA *G3 and NPHP3-1-203-BirA*G3 fusion proteins compared to cell lines without BirA fusion protein. Additionally, biotinylated proteins were detected at the base of the cilia (B9d1-BirA-*G3-expressing cells) as they are transported into and out of the cilium or along the length of the axoneme (Nphp3-1-203-BirA *G3-

expressing cells) using streptavidin. The cell lines are currently being employed to pull down biotinylated proteins utilizing streptavidin affinity column for mass spectroscopy analysis. Henceforth, these new resources will allow us to define differences in ciliary proteomes of these two cell types and assess dynamic changes that occur in the cilia proteome in response to induction of signaling activity. Commentary mouse lines are being generated that will allow us to analyze ciliary proteomes across different cell types, developmental stages, and different diseases states.

B165/P0745

ERM proteins regulate the shape and number of Endoplasmic Reticulum-Plasma Membrane Junctions in neurons

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Endoplasmic Reticulum (ER) - Plasma Membrane (PM) Junctions (EPJs) are specialized contact sites between ER membrane and the inner leaflet of PM. These junctions are critical for lipid exchange and Ca2+ signaling. In excitable cells like neurons and muscle, EPJs further modulate membrane excitability by regulating Ca2+ homeostasis. The mechanisms controlling EPJ abundance and morphology remain poorly understood. Using in vivo fluorescence imaging and electron microscopy of C. elegans neurons, we showed that EPJs form discrete, patch-like structures distributed across the soma. Through a forward genetics screen, we identified two conserved ERM (Ezrin-Radixin-Moesin) proteins, FRM-4 and FRM-1, as key regulators of EPJ shape and abundance. Both proteins localize to EPJs and exhibit liquid-liquid phase separation properties (LLPS). in vitro, purified FRM-4 binds to FRM-1, and together bundle filamentous actin. However, their presence in LLPS condensates and actin-bundling activity are mutually exclusive. In vivo, F-actin cables surround—but do not penetrate—EPJs, where FRM proteins are enriched as phase-separated condensates. Loss of FRM-4, FRM-1, or disruption of F-actin led to increased mobility of EPJs that fused into fewer but enlarged junctions. Together, our findings demonstrate that FRM-4 and FRM-1 control EPJ morphology by organizing peri-junctional F-actin networks, thereby restricting EPJ mobility and fusion.

B167/P0747

Illuminating the Organelle Signature of SNCA Mutant iPSCs

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Although distinct in their clinical presentations, neurodegenerative diseases share numerous cellular hallmarks, including pathological protein aggregation, altered energy homeostasis, and cytoskeletal abnormalities. Parkinson’s disease (PD) is characterized by alpha-synuclein aggregation, synaptic dysfunction, and dopaminergic neuronal cell loss. A rare autosomal dominant mutation in the SNCA gene, p.Ala53Thr, increases the aggregation potential of alpha-synuclein monomers and causes early onset PD. The association of alpha-synuclein with numerous membrane-bound organelles within the cell, including mitochondria, endoplasmic reticulum (ER), Golgi, and the endolysosomal system, suggests that the dysregulation of organelle dynamics plays a critical role in Parkinson’s disease pathology. While the effect of mutant alpha-synuclein on individual organelles has been studied, multispectral imaging enables the simultaneous visualization of seven organelles in live cells, including the nucleus, ER, Golgi, lysosomes, mitochondria, lipid droplets, and

peroxisomes. When coupled with computational image analysis tools, these techniques provide quantitative information about organelle morphology, dynamics, and contacts between organelles, i.e., the overall organelle signature. We used this systems-level approach to identify changes in the Golgi morphology in KOLF2.1J human induced pluripotent stem cells (iPSCs) harboring the heterozygous p.Ala53Thr mutation when compared to wild-type or revertant iPSCs. The Golgi apparatus is uniquely sensitive to cellular stressors, and Golgi stress has been identified as an early marker of PD neurodegeneration, preceding alpha-synuclein aggregation. Furthermore, the SNCA mutant cells exhibited decreased interactions among mitochondria, peroxisomes, and lysosomes, suggesting perturbation of organelle membranes and setting the stage for altered metabolism and oxidative stress accumulation during aging. Functional interrogation of changes in the organelle signature throughout differentiation into dopaminergic neurons will aid in elucidating the cellular pathophysiology underlying neurodegeneration, opening new avenues for biomarker and therapeutic discovery.

B164/P0744

Microtubule-targeting agents as inducers of endoplasmic reticulum-mitochondria contacts

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Microtubule-targeting agents (MTAs) are one of the largest and longest-standing classes of cancer chemotherapeutics. Despite decades of usage in the clinic, the original purported mechanism of action (MOA) of these drugs has been repeatedly rebutted and continues to be debated in the field. As such, the MOA by which MTAs act remains to be fully clarified, leading to open questions concerning what other cellular phenotypes MTAs may be inducing to initiate downstream cell death. Recently, a drug screen by our lab suggested that a variety of MTAs are inducers of ER-mitochondrial contacts (ERMC), sites of close interface between the endoplasmic reticulum and mitochondria, allowing for dynamic signaling pathways and molecular exchanges. Here, our objective was to investigate this novel phenotype of MTAs & related stress pathway signaling as an initial study to explore and characterize this new phenotype. Concerning our methodology, we utilized a molecular fluorescent reporter to assess for induction of ERMC upon MTA-treatment. We further assessed ERMC-associated cellular stress pathways including the unfolded protein response & calcium homeostasis by western blot and flow cytometry. Finally, we performed Kaplan-Meier analyses to explore whether varying endogenous levels of an enzyme suggested to control microtubule-associated ERMC can have clinical implications on MTA efficacy in cancer patients. Significant ERMC induction by MTAs was observed via a validated fluorescent reporter in multiple cell lines when we utilized various MTAs; an accompanying dose-dependent response was additionally observed. The ERMC-linked cellular stress pathway known as the unfolded protein response was found to be induced as confirmed by significant elevation of unfolded protein levels assessed via flow and activation of the PERK pathway assessed via western blot. Similarly, significant increases in mitochondrial calcium as assessed by Rhod-2 AM dye flow cytometry was observed. Finally, Kaplan-Meier plots indicated that patients with predicted higher levels of MTA-induced ERMC responded better to MTA. In conclusion, this study identified a novel phenotype of MTAs as inducers of ERMC with strong indications of related stress pathway induction; this study additionally highlights the clinical relevance of exploring MTA-induced ERMC to improve cancer treatment response. This project acts as an initial step forward in better understanding the MOA of MTAs in the hopes of refining future ways of targeting cancers treated with MTAs.

B163/P0743
Mitochondria on the Move: Supporting RPE Phagocytosis Through Regulation of LD Dynamics
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The Retinal Pigment Epithelium (RPE) plays a vital role in maintaining photoreceptor function and visual health. Impaired RPE lipid metabolism and accumulation of lipid-rich deposits are common features of retinal degenerative diseases, including age-related macular degeneration (AMD). The RPE and photoreceptors are tightly metabolically coupled. To support photoreceptor glycolysis, the RPE conserves glucose and relies on oxidative phosphorylation of alternative fuels. It also performs daily phagocytosis of photoreceptor outer segment (POS) tips—removing photo-damaged components while gaining nutrient-rich substrates like lipids and amino acids. Therefore, we hypothesized that mitochondrial function and dynamics are critical for efficient POS phagocytosis. Using advanced light and electron microscopy, we found that POS phagocytosis triggers mitochondrial redistribution to the RPE apical region, driven by TRAK1-mediated microtubule transport. Blocking this motility slowed phagosome degradation without affecting binding, suggesting its role in phagosome processing. Since defective phagocytosis is liked to lipid accumulation, we investigated lipid handling further. We found that RPE cells transiently form lipid droplets (LDs) following POS or palmitate exposure. TRAK1-regulated mitochondrial motility is essential for both LD formation and turnover, suggesting mitochondria positioning influences RPE lipid processing. In turn, proper LD dynamics support sustained phagocytosis and lipid clearance. Together, our work identifies mitochondrial motility and LD dynamics as key regulators of RPE function and metabolism. This mechanism integrates spatially coordinated energy metabolism and lipid handling, offering new insight into AMD pathogenesis and potential therapeutic strategies targeting organelle interaction and positioning.

B158/P0738
Modulating Endoplasmic Reticulum-Mitochondria Contacts (ERMCS) to Identify Metabolic Vulnerabilities in Cancer
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In a nutrient scarce microenvironment, cancer cells must alter their normal metabolic program to meet the energetic requirements for cancer progression. Cellular metabolism is organized spatially with dedicated organelles, and at the nexus of cellular metabolism sits the mitochondrion. Mitochondria integrate multiple inputs and transduce metabolic signaling to support cancer cell growth. Additionally, the mitochondrial network must be dynamically regulated in cancer to provide metabolic plasticity necessary for adaptation to energetically unfavorable environments. Mitochondria can achieve this through physical contacts with the ER (ERMCS) to improve mitochondrial function through exchange of metabolites, such as phospholipids, and enzymatic cofactors like calcium. However, we lack mechanistic understanding of how ERMCS are regulated and integrated into cellular metabolism and tumor physiology. To investigate this, we employ a Split GFP based Contact Site sensor (SPLICS) to monitor ERMCS, and a tunable synthetic mechanism of ERMCS induction, Shield-1. First, we show that SPLICS faithfully reports ERMCS via fluorescent labelling of the ER and the mitochondria. We then identify various chemical perturbations that alter ERMCS dynamics by SPLICS, connecting ERMCS induction to lipid metabolism. Finally, we validate that the Shield-1 system is sufficient to induce a unique ER-mitochondria envelopment phenotype that we observe also with an alternative chemical

ERMCS inducer, fedratinib, allowing us to separate the metabolic effects of ERMCS induction from the off-target effects of canonical ERMCS inducers. Finally, we are validating murine models of both the SPLICS reporter and the Shield-1 system for ERMCS induction to identify how ERMCS are distributed in various tissues in normal and tumor physiology.

B157/P0737

Molecular Convergence of Cancer, Cell Bioenergetics, and Viral Infection Defined by Single-Organoid Proteomics

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Compared to conventional cell monolayers, three-dimensional (3D) organoids have emerged as powerful experimental models that more accurately recapitulate the architectural complexity of human tissues and hallmarks of tumorigenesis in patients. We leveraged organoids to dissect how tumors spatially and temporally reprogram inter- and intra-cellular signaling, further determining how these changes remodel the tumor microenvironment and establish vulnerabilities that can be exploited by common pathogens, including oncomodulatory viruses. To investigate cell remodeling events at the interface of cancer, metabolism, and virus infection, we used organoid models of the most frequent and lethal subtype of epithelial ovarian cancers, high-grade serous tubo-ovarian cancer. We applied a multidisciplinary approach that integrated quantitative single-organoid proteomics, targeted mass spectrometry, live metabolic flux assays, live super-resolution microscopy, and virology. Major ovarian cancer subtypes exhibited metabolically distinct proteome profiles, with the most aggressive subtype displaying enrichments in mitochondria-associated metabolism. Consistent with this, gene ontology comparison of 3D organoids relative to their 2D monolayer counterparts revealed enriched cellular respiration, electron transport chain activity, and lipid metabolism. Targeted proteomics uncovered abundance changes in proteins mediating organelle-organelle contacts (e.g., mitochondria with endoplasmic reticulum, ER). Follow-up analyses with super-resolution microscopy showed that the most aggressive subtype had more stable mitochondria-ER encapsulations, coincident with elevated calcium signaling and lipid droplet clustering. Live metabolic flux assays further demonstrated that this subtype had increased bioenergetic respiration through oxidative phosphorylation. As mitochondria-ER contacts are known to coordinate calcium homeostasis, lipid exchange, and metabolism, expression of a mitochondria-ER tether intensified lipid droplet clustering, suggesting a role for this altered inter-organelle communication in fueling the bioenergetic demands of ovarian tumors. These findings suggested that remodeled tumor-intrinsic metabolism not only fuels tumor growth but may also be exploited by microenvironmental viruses that depend on the same metabolic pathways. We interrogated this vulnerability upon infection with the oncomodulatory cytomegalovirus, which resulted in significant differences in viral susceptibility across cancer subtypes. Our findings explain how cancer cells exploit the metabolic capacity of remodeled organelles to sustain tumorigenesis, while reshaping infection outcomes within the tumor microenvironment.

B159/P0739

Novel crosstalk between TDP-43 oxidation and the GADD34-PP1 complex at RNA granule-mitochondria contact sites in ALS

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Inter-organelle contact sites are key hubs for organelle bidirectional crosstalk which are critical for cellular homeostasis and implicated in human disease etiology. However, whether mitochondria and RNA granules directly interact at contact sites, and the role of reactive oxygen species (ROS) generated from mitochondrial oxidative phosphorylation (OXPHOS) in mechanistically regulating this pathway is still not well understood. Here, using Super-Resolution live microscopy, we identify the dynamic formation of RNA granule-mitochondria contact sites in OXPHOS conditions. We find that ROS generated by mitochondrial OXPHOS is sufficient to recruit RNA granules containing TDP-43 to the cytoplasm and is dependent on oxidation of TDP-43 at cysteine residues. Mechanistically, RNA granule-mitochondria contact site tethering is mediated by TDP-43 binding to GADD34 on mitochondria, while contact untethering is driven by release of its binding from GADD34 and regulated by TDP-43's oxidation status. Functionally, this allows for the bidirectional crosstalk between TDP-43 RNA granules and a GADD34-PP1 complex on the mitochondria, whereby GADD34-PP1 regulates TDP-43 RNA granule dynamics, and conversely, TDP-43 oxidation status regulates PP1 granule phase separation dynamics. Furthermore, we demonstrate that this pathway is misregulated by disease-associated mutant TDP-43. Thus, RNA granule-mitochondria contacts represent an important site for the molecular crosstalk between TDP-43 oxidation and GADD34-PP1 and highlight a key role for inter-organelle contacts in cellular homeostasis.

B161/P0741

The role of tubulin code in organelle contacts during neuronal differentiation

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A functional nervous system depends on the correct neuronal morphology established during differentiation. Microtubule (MT) cytoskeleton is required for successfully completing all the morphological transitions of differentiation, providing structural support, positioning organelles, and enabling their transport. MT dynamics and properties are regulated by a variety of post-translational modifications (PTMs), which have been shown to interact with membrane-bound organelles in non-neuronal cells. However, how neurons rearrange organelles through modifying tubulin PTMs to meet their transition from stem cells remains to be fully delineated. We recently found that organelle contact increases during axon formation, coinciding with enriched tubulin PTMs. We hypothesize that neurons spatiotemporally coordinate the distribution of MT PTMs throughout neuronal differentiation to drive organelle reprogramming during differentiation. We derived cortical neurons (iNeurons) from human induced pluripotent stem cells and found that α tubulin K40 acetylation and polyglutamylation display distinct changes in abundance and subcellular localization during differentiation. We downregulated acetyltransferase, ATAT1, or polyglutamylase, TTLL1, then applied 3D multispectral imaging analysis to study how tubulin PTMs affect eight membrane-bound organelles simultaneously in day 7 iNeurons. We found that reducing MT acetylation alters multiple inter-organelle contacts in addition to diverse aspects of lysosomal biology, including morphology, function, autophagy, and dynamic regulation. In contrast, reduced tubulin polyglutamylation leads to Golgi fragmentation and disrupts Golgi-related organelle contacts. Moreover, reduction of tubulin acetylation or polyglutamylation differently affects iNeuron morphology. Together, our results suggest that changes in the amount and localization of MT PTMs are a mechanism of organelle remodeling throughout neuronal differentiation.

B166/P0746

The survival factor, renalase, is a multilocalizing protein (mitochondria, centrosome, midbody, nucleus) that is

also secreted by the kidney
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Background: Renalase (RNLS) is a pro-survival and anti-inflammatory plasma flavoprotein secreted by the kidney. In some cancers RNLS protein is upregulated and appears to drive select cancer development. The RNLS protein (342aa, 37 kD) structure appears bean-shaped with a FAD-binding domain and a putative amine oxidase domain. The N-terminus of RNLS has overlapping targeting motifs: a putative signal sequence (aa1-17), mitochondrial targeting sequence (MTS, aa1-22). Methods: For IF and immunoblots, two monoclonal antibodies to RNLS were used: m28 raised to aa220-240 (RP220) and m42 raised to the C-termini. N-terminal (aa1-22, 1-39) GFP fusion proteins were made in pEGFP-N3; plasmids were transfected into HEK293, MiaPaCa-2 or Panc1 cells. Results: The N-terminal 22aa were sufficient to direct GFP to the mitochondria where it colocalized with Tom20. Surprisingly, 1-22-GFP also localized to the nucleus but a strong ER signal was absent. The double arginines (R21, R22) appear to be important with either mutation (R21A, R22A) reducing mitochondria targeting and R21A/R22A (AA) largely cytosolic. Immunoblot of NP40 cell extract transfected with 1-39-GFP showed a doublet that could indicate MTS cleavage within the matrix; cells transfected with the AA mutation showed the GFP fusion protein remained in the cytosol. Using IF we found that endogenous RNLS localization depended on antibody and fixation. The m28 antibody recognized a strong mitochondria signal with PFA fixation. However, with methanol fixation, the mitochondrial signal diminished and m28 (but not m42) recognized RNLS within the nucleus at the histone locus body (HLB), colocalizing with NPAT. m28 also recognized RNLS at the midbody, colocalizing with MKLP and Rac-GAP1, and in midbody remnants collected from conditioned media. Conversely, the m42 antibody (but not m28) recognized RNLS at the centrosome; specifically, the proximal end of mother/daughter centrioles, colocalizing with Cep135, Cep250 (C-Nap1), pericentrin and ϒ-tubulin. Centrosomal fractions isolated by discontinuous sucrose gradient showed by immunoblot RNLS enrichment in Cep250-positive fractions. To summarize, RNLS localizes to mitochondria, HLB, midbody and centrosome: uniting these organelles is a cell cycle dependence. Using cell cycle synchronization, we determined that RNLS protein levels were lowest at G1 and peaked at S phase, a pattern that closely correlates with HLB formation and expansion. Conclusion: We found that RNLS is a multilocalizing protein with a N-terminal MTS. Low ER localization suggests RNLS secretion may be via an unconventional pathway. Since RNLS localization varied with antibody and fixation, our future studies will examine how RNLS conformation or its protein:protein interactions may differ by compartment.

Organelles - Peroxisomes

B114/P0694
Identifying and characterizing a missing peroxin-PEX8-in Arabidopsis thaliana
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Peroxisomes are dynamic organelles that perform widely conserved functions, like fatty acid β-oxidation, and many critical, specialized functions. In plants, peroxisomes contribute to carbon metabolism through fatty acid β-oxidation and photorespiration and to development and defense through the biosynthesis of phytohormones. Peroxisomes must continuously import newly synthesized proteins from the cytosol to perform these metabolic functions and are capable of importing fully-folded and even oligomeric proteins. The proteins mediating peroxisomal protein import are called peroxins, and most are conserved among fungi, plants, and vertebrates. An exception is Pex8, the only peroxin found fully in the lumen of the peroxisome. Pex8 is essential for peroxisomal protein import in yeast but has not been identified outside of fungi. Indeed, traditional sequence homology searches failed to predict a Pex8 ortholog in plants or vertebrates. We

identified this elusive peroxin in Arabidopsis thaliana using structural homology software. Like yeast Pex8, Arabidopsis PEX8 has a predicted Huntingtin, EF3, a-PP2A, TOR1 (HEAT) repeat structure and two peroxisomal targeting signals, PTS1 and PTS2. We found that an insertional mutation in our candidate PEX8 is lethal when homozygous, like other null pex mutations in Arabidopsis. Furthermore, knockdown of PEX8 using artificial miRNA results in defects in the import of both PTS1 and PTS2 cargo proteins. Visualizing fluorescent reporter fusions in live seedlings, we found that this protein localizes to foci associated with peroxisome membranes, with or without either PTS1 or PTS2, implicating that PEX8 may partition with the phase-separated peroxisomal import pore. Disrupting both peroxisomal targeting signals may mislocalize the protein to the cytosol. Together, these data suggest the Arabidopsis PEX8 is orthologous to yeast Pex8, and further characterization of PEX8 foci and interactions with other peroxins will refine our understanding of PEX8's role in peroxisomal protein import. This study also raises the intriguing question of whether other peroxins in plants and vertebrates have eluded discovery because of low primary sequence conservation. This research is supported by the NIH.

B116/P0696

Import mechanism of peroxisomal proteins with an N-terminal signal sequence

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Peroxisomes are conserved membrane-bounded organelles essential for human health, with crucial functions in lipid metabolism and redox homeostasis. Proteins are imported into peroxisomes from the cytosol by soluble receptors, which remarkably can transport even folded proteins and protein complexes across the peroxisomal membrane. Most peroxisomal proteins contain a C-terminal import signal that is recognized by the receptor PEX5, which then shuttles the cargo through a nuclear pore-like conduit formed by the tyrosine/glycine-rich YG domain of the peroxisomal membrane protein PEX13. After cargo release inside the organelle, PEX5 returns to the cytosol by being pulled out through a separate retrotranslocon. Some peroxisomal proteins instead contain an N-terminal signal that is recognized by the receptor PEX7, but how PEX7 transports its cargo into peroxisomes and then returns to the cytosol has been unknown. Here, we present a comprehensive model of the import cycle of PEX7. We show that PEX7 hijacks PEX5 to move cargo through the YG conduit into peroxisomes. After cargo release inside the lumen, PEX7 returns to the cytosol by moving back through the same conduit, a mechanism distinct from that used by PEX5. We further identify a chaperone called PEX39 that extracts PEX7 out of the YG conduit on the cytosolic side, and then reloads PEX7 with new cargo and a receptor to start another round of translocation. Our work unveils the unique pathway by which PEX7 imports proteins into peroxisomes, paving the way toward understanding how defects in this process cause the devastating human disorder rhizomelic chondrodysplasia punctata and Refsum disease.

B111/P0691

Investigating roles of the early-acting peroxin PEX16 in Arabidopsis

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Peroxisomes are conserved organelles involved in fatty acid beta-oxidation and reactive oxygen species metabolism; proteins called peroxins (PEX proteins) guide organelle formation and function. Mutations in peroxins often result in deleterious phenotypes, such as Zellweger spectrum disorder in humans and developmental defects in plants. Early-acting peroxins (PEX3, PEX16, and PEX19) work together to insert other

peroxisomal membrane proteins (PMPs) during peroxisomal biogenesis. However, our understanding of this process and the exact functions of these peroxins, in particular PEX16, remains limited. We used CRISPR-Cas9 to develop pex16 mutant alleles disrupting certain predicted domains to investigate the roles of these domains. We found that PEX16 is essential for life in Arabidopsis, as an early frameshift mutation confers embryo lethality. In viable mutants, we found multiple striking phenotypes. First, we found cytosolic/vacuolar localization of two different peroxisomal membrane reporters using confocal microscopy and lower levels of these reporters and some endogenous PMPs using immunoblotting, indicating decreased ability to insert PMPs. Second, we found that pex16 peroxisomes are large and mostly devoid of intralumenal vesicles. Lastly, we found that pex16 seedlings display slowed lipid droplet mobilization and beta-oxidation defects in growth assays without notable defects in lumenal protein import. Our findings reveal peroxisomal defects in pex16, and we plan to learn more about PMP insertion when PEX16 is dysfunctional by assessing the localization of various PMP-fluorescent protein reporters. Our goal is to expand our understanding of PEX16 roles in peroxisomal biogenesis, including its localization, effect on PMP insertion, and overall effect on peroxisome morphology.

B113/P0693
Mechanistic Studies of Peroxisomal Metabolite Transport
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Peroxisomes are essential eukaryotic organelles involved in fatty acid oxidation and bile acid synthesis that rely on ATP-binding cassette (ABC) transporters of the D subfamily to import CoA thioesters across their membranes. ABCD3 is the most abundant peroxisomal ABC transporter, facilitating import of diverse substrates including branched-chain fatty acids, very long-chain fatty acids, and bile acid intermediates. Here, we determined cryo-EM structures of full-length human ABCD3 in apo and phytanoyl-CoA-bound states at 3.33 Å and 3.13 Å resolution, respectively, and performed biochemical assays to characterize substrate-dependent ATPase activity. Our structures reveal that substrate binding induces significant conformational changes in ABCD3, bringing the nucleotide-binding domains into closer proximity. Biochemical analyses demonstrate that substrate binding specifically activates ATPase activity, and biophysical binding assays establish a direct interaction with acyl-CoA substrates. Structural comparison between apo and substrate-bound states provides molecular insights into the transport cycle. These findings illuminate the structural basis of substrate-induced activation in ABCD3 and establish a foundation for understanding peroxisomal metabolite transport mechanisms. The results have important implications for developing therapeutic strategies for ABCD3-related disorders and advancing our understanding of peroxisomal biogenesis diseases.

B112/P0692
PEX11 sculpts peroxisome architecture
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Peroxisomes are critical organelles housing numerous metabolic reactions including fatty acid β-oxidation and reactive oxidative species decomposition. Peroxins (PEX proteins) direct peroxisome biogenesis, and the membrane peroxin PEX11 is implicated in modulating peroxisome size and number in various species. We are examining PEX11 function in Arabidopsis because its large peroxisomes allow facile visualization of peroxisome

architecture using live-cell confocal microscopy, and its specialized peroxisomal metabolism enables robust whole-organism readouts of peroxisome function. We generated null mutations in various combinations of the five Arabidopsis PEX11 isoforms (PEX11A-E) using a CRISPR-based approach in a transgenic reporter line that allows visualization of peroxisome membranes and lumen. As expected of peroxin null mutants, the pex11 quintuple mutant displays seedling lethality. Interestingly, mutants lacking PEX11A and PEX11B have pronounced defects in generating peroxisomal intralumenal vesicles (ILVs), which are implicated in efficient fatty acid β-oxidation. Chemically or genetically disabling lipid mobilization in pex11a pex11b double mutants prevents peroxisome enlargement, revealing roles for PEX11A/B in ILV formation during lipid metabolism. A triple mutant lacking PEX11C, PEX11D, and PEX11E displays abnormally large peroxisomes, impaired mobilization of fatty acids stored in lipid droplets, and severe physiological deficiencies. In contrast to PEX11A/B function, aberrant peroxisome expansion in these triple mutants is apparent even in tissues that lack active fatty acid catabolism, suggesting that PEX11C/D/E governs peroxisome membrane architecture independently of lipid metabolism. We found that yeast Pex11 restores peroxisome size and ILV formation in Arabidopsis mutants lacking PEX11A and B, revealing evolutionary conservation of PEX11 functions. Beyond advancing our understanding of cell biology and organellar evolution, illuminating how PEX11 alters the cryptic peroxisomal processes of ILV formation and peroxisome division could enhance agricultural sciences and reveal the etiology of human pathologies, including peroxisome biogenesis disorders. This work was supported by the NIH and the Welch Foundation

B115/P0695

Resolving Peroxisome Heterogeneity in a Parasitic Model System Using High-Resolution Organelle Profiling

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Ancient eukaryotic parasites such as Trypanosoma brucei present a dual opportunity: they pose significant public health challenges while also serving as powerful model systems for uncovering novel and conserved aspects of eukaryotic cell biology. These organisms harbor specialized peroxisomes, termed glycosomes, which uniquely compartmentalize the glycolytic pathway. In addition to glycolysis, glycosomes also house diverse metabolic pathways, including purine salvage and pyrimidine biosynthesis. However, the degree to which these pathways are co-localized within the same organelles—or instead partitioned among distinct glycosome populations—remains unclear. Several studies suggest glycosomes are heterogeneous: density-based fractionation reveals subpopulations, and fluorescence microscopy frequently shows only partial co-localization of glycosomal enzymes. Yet, the extent and functional relevance of this heterogeneity remain unresolved, in part due to limitations in current imaging and separation techniques. Electron microscopy is low throughput, widefield fluorescence lacks resolution and multiplexing capabilities, and conventional flow cytometry is optimized for larger structures such as mammalian cells. To overcome these challenges, we are applying expansion microscopy (ExM) and fluorescence-activated organelle sorting (FAOS), a technology recently refined for extracellular vesicle analysis—to resolve and profile glycosome diversity at high resolution and throughput. These approaches enable us to systematically define glycosome subpopulations, investigate their biogenesis, and determine the biological consequences of disrupting specific glycosome classes. This work provides fundamental insight into organelle specialization and plasticity in eukaryotic pathogens and inform broader principles of peroxisome biology.

B118/P0698
The ER crossing guard: Djp1 facilitates peroxisomal membrane protein trafficking
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Peroxisomes can be formed de novo from the endoplasmic reticulum (ER). In this process, peroxisomal membrane proteins are inserted into the ER, sorted to subdomains and bud off into vesicles that mature into functional peroxisomes. While passing through the ER, peroxisomal proteins must escape being recognized as “foreign” and eliminated by ER quality control processes. However, the mechanisms that protect and sort peroxisomal membrane proteins in the ER prior to their exit remain unclear. We found that an ER-localized cochaperone, Djp1, facilitates these early steps of peroxisomal biogenesis in yeast. Deletion of DJP1 destabilized certain peroxisomal membrane proteins, including Pex13 and Pex15. Additional deletion of the ER quality control factor SPF1 rescued the observed dysregulation of Pex13 and Pex15, suggesting that Djp1 protects these proteins from ER quality control processes. The exposure to quality control systems could be due to their presence in the ER outside of specific subdomains. Using a split-GFP system, we visualized the transient ER localization of peroxisomal proteins. Pex13 and Pex15 showed a broad ER signal only in absence of Djp1. This supports a role for Djp1 in the sorting of these proteins to the correct ER subdomain by preventing their unspecific insertion into the ER. Induction of peroxisomal biogenesis, via growth in oleic acid-containing medium, revealed broad defects in both peroxisomal formation and function without Djp1. Altogether, our results suggest Djp1 functions to sort and protect peroxisomal proteins in the ER, thereby promoting efficient peroxisomal biogenesis.

B117/P0697
The Mechanism and Function of Organelle Hitchhiking
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To maintain proper cellular function, molecular motor-driven transport of organelles is essential for coordinating the distribution of proteins, lipids, and metabolites across large distances. A novel mode of transport was recently discovered by the Reck-Peterson lab in the filamentous fungus Aspergillus nidulans, where peroxisome “hitchhiking” on early endosomes (EEs) requires the protein PxdA. Aspergillus species are members of the Pezizomycotina subphylum, the only fungal taxon that contains PxdA and one that produces an exceptional diversity of secondary metabolites (SMs). SMs are at least partly synthesized in the peroxisome. However, why peroxisomes hitchhike on EEs and the molecular content they transport is unknown. Using genetics, live-cell fluorescence microscopy, and biochemical and biophysical techniques, my project investigates the role of hitchhiking peroxisomes in SM production and fungal development. Leveraging SM production in fungi may reveal new antifungal strategies, as the Pezizomycotina subphylum contains the most clinically significant human fungal pathogens.

Plant Cell Biology - Cell structure and organelles

B543/P1107
Protein folding efficiency influences ER export and vacuolar delivery of carboxypeptidase Y

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Carboxypeptidase Y (CPY) is a vacuolar protein transported via the endoplasmic reticulum (ER), Golgi, and prevacuolar compartment. In the ER, misfolded proteins are retained and eliminated by ER-associated degradation (ERAD). Although ERAD mechanisms have been extensively studied, how enhanced folding influences ER exit of secretory proteins remains unclear. Here, we examined the vacuolar trafficking efficiency of Arabidopsis CPY fused to green fluorescent proteins (GFPs) with different folding properties: normal GFP, superfolder GFP (sfGFP) with enhanced folding capacity, and aggregation-prone GFP[V30A]. As expected, CPY-GFP[V30A] failed to reach the vacuole and accumulated mainly as the precursor form. Notably, CPY-sfGFP was transported to the vacuole more efficiently than CPY-GFP. Similar results were observed with CPY95, a truncated version of CPY. Together, these findings suggest a novel mechanism in which the folding capacity of secretory proteins influences their vacuolar trafficking efficiency.

B542/P1106

Two DPLG motifs in chlorophyll a/b-binding protein 6 are critical for Photosystem I supercomplex assembly

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Chlorophyll a/b-binding (Cab) proteins, components of the Photosystem I (PSI) and Photosystem II (PSII) supercomplexes, are encoded by the nuclear genome, synthesized on cytosolic ribosomes, and imported into chloroplasts via the TOC/TIC (Translocon at the Outer/Inner envelope of Chloroplasts) complexes, with the N-terminal transit peptide playing a key role in this process. After import, the transit peptide is cleaved in the stroma, and mature Cab proteins are targeted to thylakoid membranes through an interaction between the DPLG motif in the L18 domain and the stromal chloroplast signal recognition particle 43 (cpSRP43). Among the various Cab isoforms, some – such as Cab6 – harbor not only a conserved DPLG motif in the L18 domain (the 2nd DPLG) located between the second and third transmembrane domains but also an additional DPLG motif near the N-terminal transit peptide (the 1st DPLG), whose function remains unclear. In this study, using an Arabidopsis protoplast system, we demonstrated that although neither motif is essential for import via the TOC/TIC translocons, both the 1st and 2nd DPLG motifs are required for proper incorporation of Cab6 into the PSI supercomplex.

B541/P1105

Unique intracellular dynamics of Arabidopsis myosin XI and its regulatory role in actin array remodeling

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Myosins are molecular motors that move along actin filaments. Plant-specific class XI myosins are major drivers of intracellular transport and play crucial roles in regulating diverse physiological functions. The subcellular localization of myosin XI and its association with organelle motility have been well-studied using a cargo-binding tail domain that lacks a motor domain. However, the localization and motility of full-length myosin XI are less well understood. To address this knowledge gap, we employed a transient expression system in tobacco leaves to visualize full-length myosin XI with high temporal resolution. Live-cell imaging revealed that several isoforms of full-length Arabidopsis myosin XI exhibited characteristic localization patterns that

differed from those of their corresponding tail domains. Of these myosin XI isoforms, we focused on myosin XI-2, a major driving force of cytoplasmic streaming. Full-length myosin XI-2 localized to dot-like structures that displayed unique dynamics characterized by accumulation and dissociation while moving along the actin filament network. Such behavior was not observed in cells expressing the myosin XI-2 tail domain, which lacks actin-binding ability, and was also absent when actin polymerization inhibitors were applied to cells expressing full-length myosin XI-2. Furthermore, cells expressing the ΔGTD mutant—deficient in cargo-binding—did not display such dynamics. These results suggest that accumulation and dissociation events were driven by both the interaction between the motor domain and actin filaments, as well as binding to the cargo via the tail domain. High temporal resolution imaging with simultaneous dual-color excitation revealed that accumulation events occurred at intersections of actin filaments and gradually shifted their location in conjunction with active actin filament translocation. Moreover, we observed active actin array dynamics in both full-length myosin XI-2-expressing and non-expressing cells, whereas these dynamics were suppressed in the cells expressing myosin XI-2-tail. Collectively, these results shed light on a previously uncharacterized role for myosin XI in coordinating intracellular transport and actin array remodeling through its motor and cargo-binding activities.

Plant Cell Biology - Development and morphogenesis

B544/P1108
Roles of the STM and ATH1 homeodomain transcription factors in establishing floral organ boundaries
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Plants shed leaves and floral organs through the programmed process of abscission. After Arabidopsis flowers are pollinated, detachment of their outer floral organs promotes successful fruit and seed development. Cell separation occurs within abscission zones that differentiate from cells in organ boundary regions. A pair of homeodomain transcription factors—STM and ATH1—regulate the development of boundary regions between the flower and its underlying pedicel (stem). Mutations in either STM or ATH1 alone disrupt stamen abscission, while mutations in both genes prevent sepal and petal abscission as well. Scanning electron microscopy has revealed that the sepal-pedicel organ boundary is less defined in stm and ath1 flowers and largely missing in stm ath1 flowers. As predicted, cells at the sepal bases of stm ath1 flowers are larger than those of wild type. Expression of a receptor-like kinase that activates floral organ abscission, as detected with a HAESA::GUS reporter, is absent from the sepal bases of stm ath1 flowers. Within the sepal abscission zones, lignifed cell layers provide mechanical support and enable precise organ detachment. Lignification of these cells (visualized with basic fuchsin dye and confocal microscopy) is disorganized in stm and ath1 sepals and does not occur or is displaced from the bases of stm ath1 sepals. These findings and others highlight key contributions of STM and ATH1 to the formation of proximal-distal organ boundaries and differentiation of both cell types found within sepal abscission zones.

Plant Cell Biology - Genomes, genomics and gene regulation

B545/P1109
A Novel CRISPR-Based Screening Approach to Identify DNA Methylation Readers and Downstream Pathways in Arabidopsis thaliana
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An equilibrium exists in the genome between heterochromatin and euchromatin mediated by chromatin modifications such as histone tail modifications and DNA methylation (DNAm). These epigenetic changes are deposited by writers, removed by erasers, and interpreted by readers to influence chromatin state and its accessibility. The Jacobsen lab produced a DNAm free mutant by eliminating all DNA methyltransferases and introducing mutants for the downstream pathways, including linker histone H1, the H3K9me2 reader ADCP1, DNAm readers MBD2, MBD5, and MBD6, and chromatin factor MOM1. Unpublished results from the Jacobsen lab indicate that in this mutant, silencing was not released at 348 loci, suggesting the presence of additional, unidentified DNAm readers and active downstream pathways in A. thaliana. To identify these new putative downstream pathways, we are developing a novel screening technique in A. thaliana. Initially, this technique involved growing A. thaliana in vitro, protoplasting, FISH, and FACS. However, protoplast autofluorescence—primarily from chloroplasts—impeded accurate GFP-based sorting in FACS. These results prompted a methodological shift toward the use of cells derived from suspension culture, which enables FISH and FACS without interference from autofluorescence. In future work, this screening approach will be integrated with a CRISPR library developed by Professor Shani's lab in the School of Plant Science and Food Security, Israel, to address high genetic redundancy in plants and facilitate the identification of DNAm readers and active downstream pathways. As a proof of concept, we will target ATMORC6 using the CRISPR library, which is expected to reduce methylation at SDC, alter protein expression detectable by FISH, and allow subsequent FACS-based sorting. Using this novel screening method in combination with the CRISPR library can contribute to a deeper understanding of DNAm readers and active downstream pathways in A. thaliana, along with significantly reducing the time required for discovery from years to months. Further research could investigate whether these findings are conserved in other eukaryotes, including humans.

Plant Cell Biology - Stress responses

B546/P1110
Deciphering the regulatory functions of plant B Cell Lymphoma 2-associated athanogene (BAG) family proteins in pathogen-induced programmed cell death
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Programmed cell death (PCD) is a fundamental biological process conserved across eukaryotes, where it plays a crucial role in development, stress responses, and immunity. In animals, PCD is orchestrated by well-characterized regulators, including pro- and anti-apoptotic Bcl-2 proteins, caspases, and Inhibitor of Apoptosis (IAP) proteins. While plants employ PCD similar to animals, they lack sequence conservation of canonical regulators, suggesting the presence of distinct components that govern this process in the plant kingdom. One emerging group of regulators is the Bcl-2-associated athanogene (BAG) family proteins, which are conserved across plants and animals and play a role in diverse cellular pathways, including cell death regulation. In the model plant Arabidopsis thaliana, seven BAG proteins (AtBAG1-7) have been identified. Previous work demonstrated that AtBAG6 and AtBAG7 aid in cell death regulation in response to fungal and viral pathogens, respectively. Yet, the underlying mechanism by which AtBAG6 and AtBAG7 execute PCD and the functions of the remaining BAG family members, particularly during bacterial pathogen infection, remain largely unknown. To investigate this, we assessed whether atbag1 to atbag7 mutants exhibit alterations in PCD using an ion leakage assay to measure time-dependent ion leakage as a proxy for cell death. In addition, we determined bacterial growth in these mutants to assess disease resistance. Interestingly, we discovered that atbag3 showed increased PCD and enhanced disease resistance, suggesting that AtBAG3 functions as a negative regulator of cell death via mechanisms that are still under investigation. Current work is focused on defining

how AtBAG3 contributes to PCD regulation, with a particular focus on its post-transcriptional modification. Overall, our findings posit AtBAG3 as a previously unreported negative regulator of pathogen-induced PCD and provide new insight into the evolutionarily conserved, yet mechanistically distinct regulation of cell death across kingdoms.

RNA Biology - Non-coding RNAs

B010/P0596
A RNA fragment of MALAT1 enhances Influenza A Virus replication
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The human genome exhibits extensive transcription of long non-coding RNAs (lncRNAs) with diverse biological functions. While lncRNAs were initially thought to lack protein-coding capacity, some are now known to encode functional micropeptides (miPEPs). Many lncRNAs exhibit functional versatility, serving as scaffolds, miRNA sponges, or sources of miRNAs and miPEPs. MALAT1 is an evolutionarily conserved, highly abundant intergenic lncRNA across species. It is a critical lncRNA with multiple biological activities. However, the extent to which it can encode miPEPs and the functions of any such encoded peptides remain unclear. Here, we report a significant upregulation of MALAT1 by influenza A Virus (IAV) infection. IAV-induced MALAT1 upregulation depended on the NF-κB/IL-6/STAT3 axis, but not by type I and III interferons signaling. Depletion of MALAT1 suppressed IAV replication, while expression of certain MALAT1 fragments enhanced IAV replication. Furthermore, we identified a biologically functional miPEP (miPEP-52) encoded by a RNA fragment of MALAT1. We found that miPEP-52 promoted IAV replication by strongly inhibiting IRF3 phosphorylation, impairing antiviral immunity. Interestingly, miPEP-52 greatly enhances the production of "weak viruses." These phenotypes are reversed by mutating the start codon of miPEP-52 or knocking out miPEP-52 from the MALAT1 fragment. Importantly, synthetic miPEP-52 showed similar effects in vitro and in vivo. Mechanistically, miPEP-52 bound MAVS via its DLAIS motif, inhibiting MAVS/TBK1/IRF3 complex formation required for IRF3 phosphorylation. Together, the results demonstrate that miPEP-52, a MALAT1-encoded miPEP, is involved in IAV pathogenesis. Our work provides new insights into the miPEP function in biological processes, and suggests a new solution to the production of viruses with weak ability of replication in vaccine development.

B012/P0598
Epithelial Adherens Junctions Recruit and Regulate a Novel Pro-tumorigenic LncRNA That Disrupts Cell Adhesion and Homeostasis
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Adherens junctions are cell-cell adhesion structures that are critical for epithelial integrity and regulation of cell behavior. Along these lines, we have previously shown that PLEKHA7, an epithelial adherens junction component, recruits the RNA interference (RNAi) machinery to suppress pro-tumorigenic transformation of well-differentiated epithelial cells. To further explore the interaction of PLEKHA7 with other potential RNA mediators we performed RNA cross-linked immunoprecipitation (RNA-CLIP) and subsequent RNA-seq analysis, which revealed association of PLEKHA7 with a large set of long non-coding RNAs (lncRNAs). LncRNAs are a class of >200 nt -long RNA transcripts that do not encode for protein and are largely unexplored. RNAseq analysis revealed a set of lncRNAs that are differentially expressed upon depletion of PLEKHA7. The top

upregulated lncRNA in absence of PLEKHA7, is a previously uncharacterized, alternatively spliced isoform of the oncogenic lncRNA MIR17HG, which we named MIR17HG_S. We have found that junctional localization of PLEKHA7 and the associated RNAi machinery is required for MIR17HG_S suppression. Examination of MIR17HG_S by fluorescent in-situ hybridization (FISH) using isoform custom made - specific probes confirmed its junctional localization. qRT-PCR analysis of RNAs extracted from tissues of colon cancer patients shows a strong upregulation of the short isoform MIR17HG_S in colon tumors, compared to normal matched tissues, and that this upregulation correlates with disruption of the junctional PLEKHA7-RNAi complex. Ectopic overexpression of MIR17HG_S in the well-differentiated colon epithelial Caco2 cells promotes pro- tumorigenic phenotypes in a dose-dependent manner, including increased anchorage-independent and cell migration. Moreover, immunofluorescence and confocal microscopy revealed a striking increase in stress fiber and focal adhesion formation in MIR17HG_S-overexpressed cells. These changes were accompanied by a marked disruption of the apical junctional complex in these cells, which was coupled by sequestration of junctional and cytoskeletal components in cytoplasmic condensates. Together, the data reveal a bidirectional mechanism, whereby the adherens junctions recruit and suppress a novel pro-tumorigenic lncRNA to maintain cellular homeostasis.

B015/P0601

Importance of Reference Gene Choice for Studying miRNA Expression in Infarcted Heart.

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Background: To understand the microRNA (miRNA) expression across cardiac diseases, an appropriate reference gene must be determined. We aimed to study the impact of reference gene on miRNA expression in different regions of the infarcted hearts. Methods: Myocardial Infarction (MI) was induced in 10 Yorkshire pigs. One week later, 5 pigs were randomly selected to be supported with mechanical circulatory assist device (MI+MCS group) and compared to those without support (n=5, MI group) to study the effect of acute (2 hours) left ventricle unloading on tissue miRNA profiles. Age matched 3 healthy Yorkshire pigs were included as sham group. Based on differential expression in the blood, we profiled for mir19b, mir20a, mir21, mir144, and mir146b. Using qPCR, we assessed three commonly used reference genes: SNORD48, 5S and U6. Results: CT values of SNORD48 were similar across the groups and the tissue regions (mean CT for border region: MI=30.4±1.3, MI+MCS=30.3±0.7, infarct region: MI=31.2±0.8, MI+MCS=31.2±0.7, Sham: 31.7±0.6), whereas 5S had more variation across regions (mean CT for border region: MI=8.9±0.5, MI+MCS= 9.2±0.9, infarct region: MI=10.5±3.17, MI+MCS=8.32±1.3, Sham: 8.3±1.4). CT value of U6 exhibited higher expression post-MI compared to sham tissue (mean CT for border region: MI=26±2.4, MI+MCS=26.65±1.1, infarct region: MI=26.39±0.4, MI+MCS=27.11±0.9, Sham: 29±0.5). Using SNORD 48 as a reference gene for data normalization, levels of mir20a, mir21, mir144, and mir146b were significantly lower at $p<0.05$ in infract compared to the border region in MI group. In addition, there was a significant difference in the levels of mir21 and mir146b between MI and MI+MCS groups in the border region. Using 5S as a reference gene, only mir144 was significant at $p<0.05$ for between-tissue comparison and only mir21 for between-group comparison. Whereas none of the miRNA reached $p<0.05$ across regions and groups when using U6 as a reference gene. Our data revealed that choice of reference gene affects the analysis and interpretation of miRNA profiling. Conclusion: SNORD48 expression was low compared to 5S and U6 yet stable across the groups and regions, making it a most suitable reference gene. Our study highlights the importance of assessing the stability of reference genes and identifying appropriate reference gene in various conditions to draw reliable conclusions.

B013/P0599
microRNA-1 Regulates Embryonic Mitosis
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Following fertilization, embryos of metazoans undergo rapid and cell divisions that demand precise regulation of chromosome segregation. Errors during this process can cause outcomes that compromise embryonic viability, such as aneuploidy, genomic instability, and developmental arrest. The goal of the study is to investigate microRNA-mediated post-transcriptional regulation during embryonic mitosis. miR-1 is an evolutionarily conserved miRNA known for roles in cardiac development, skeletal muscle differentiation, and angiogenesis. Its function during embryonic mitosis is limited. In the cleavage stage sea urchin embryo, miR-1 is enriched in the cell cortex, midzone, and at the chromosomes. To understand its function, we microinjected miR-1 mimic (overexpression) or inhibitor into sea urchin zygotes. miR-1 overexpression induced bent and multipolar mitotic spindle defects, as well as chromosomal segregation errors, such as lagging chromosomes, DNA bridges, compaction failure, and multinucleation. miR-1 inhibition resulted in monopolar or misaligned spindles, angular spindle bridges, and defects in DNA condensation. Together these results lead to our central hypothesis that miR-1 regulates mitotic spindle organization and chromosomal segregation. Based on miR-1 perturbation phenotypes, we identified downstream effectors of this regulation, and applied candidate and bioinformatics approaches to predict miR-1 targets. We validated CDC42, a Rho GTPase essential for spindle polarity, and NuMA, a spindle-organizing protein, as direct miR-1 targets. miR-1 overexpression led to decreased levels of CDC42 and NuMA proteins compared to control; miR-1 inhibition led to increased levels of CDC42 and NuMA proteins. Importantly, knockdown of CDC42 phenocopied many of the defects induced by miR-1 overexpression. However, the DNA bridging and spindle-bending defects are only observed in miR-1 overexpressed embryos, suggesting the involvement of additional miR-1 targets. To better understand the miR-1 regulatory network, we will use a novel, high throughput proteomics approach to identify potential miR-1 targets by comparing proteomes from control mimic, miR-1 mimic, FITC-injected control and miR-1 inhibited early cleavage stage embryos. Our findings identify miR-1 as a key regulator of spindle architecture and chromosome segregation during embryonic mitosis. This study shows how miR-1 controls post-transcriptional regulation of spindle dynamics, providing insight into the molecular mechanisms that ensure faithful chromosome segregation and spindle organization.

B011/P0597
Post-transcriptional regulation during embryonic mitosis
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Following fertilization, metazoan embryos undergo a series of rapid and coordinated mitoses. In the sea urchin cleavage embryo, we found miR-31 regulates local translation of cytoskeletal modulators, such as Fascin, Rab35, Gelsolin and β-actin, at the mitotic spindle during early development. Localization of miR-31 and its target transcripts to the mitotic spindle is evolutionarily conserved, as we observe this spindle localization in sea urchin embryos and mammalian cells. We found miR-31 inhibition and forced ectopic translation of miR-31 target Fascin at the cell periphery resulted in chromosomal segregation defects, developmental delay, and embryonic death. Interestingly, we recently identified another maternally abundant miRNA, miR-1, to follow a similar localization pattern as miR-31. We found that miR-1 overexpression resulted in developmental delay, mitotic spindle structural defects, and aberrant chromosomal segregation. miR-1 inhibition resulted in

monopolar or misaligned spindles, angular spindle bridges, and defects in DNA condensation. Using a candidate approach, we have observed a few transcripts that co-localize with miR-1 and validated Cdc42 and NuMA to be directly suppressed by miR-1. Although miR-31 and miR-1 perturbations both cause chromosomal defects, miR-1 perturbation also leads to microtubule structural defects, not observed in miR-31 perturbation. These results indicate that miRNA-mediated post-transcriptional regulation is likely a general and evolutionarily conserved regulatory mechanism for ensuring proper embryonic mitosis. To comprehensively understand the function of miR-1, we will use proteomics to identify systems-level proteins that alter upon miR-1 perturbation (knockdown and overexpression) at the cleavage and blastulae stages of development. Overall, this work contributes to fundamental understanding of dynamic post-transcriptional regulation, RNA localization, and cell division during early development. This work is funded by NSF MCB 2103453 to JLS.

B014/P0600
RNA-seq analysis of HSATII knockdown in cancer cells reveals downregulation of genes implicated in oncogenic pathways
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Human satellite II (HSATII) is a tandemly repeated DNA sequence that is normally silenced in healthy cells but aberrantly transcribed in many cancers. The resulting HSATII RNA accumulates in the nuclei of cells and contributes to genomic instability, making it a potential therapeutic target. This study investigates the effects of HSATII knockdown using GapmeRs, antisense oligonucleotides designed to target and degrade HSATII RNA, with a focus on RNA-seq analysis, downstream changes in the transcriptome, and pathway enrichment analysis. RNA-sequencing data from U2OS cells transfected with a 16-base pair HSATII GapmeR, a MALAT1 GapmeR, or no GapmeR control were analyzed to assess the efficiency of HSATII knockdown and genome-wide changes. A computational pipeline was developed in RStudio incorporating Kallisto for pseudoalignment, tximport for mapping transcripts to genes, DESeq2 for differential expression, and g:Profiler for pathway enrichment analysis. Computational analysis confirmed that GapmeR treatment effectively reduced HSATII RNA, with knockdown observed across nearly all HSATII sequences. Differential expression analysis revealed broad changes in gene expression, including downregulation of oncogenic signaling pathways such as EGFR and MAPK signaling. These findings suggest that HSATII may influence cancer cell survival and proliferation through its impact on oncogenic signaling networks, highlighting its potential as a therapeutic target.

RNA Biology - RNA in health and disease

B020/P0604
Dysregulated subcellular localization and alternative translation in rare human diseases
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Mitochondrial endosymbiosis was a pivotal event in eukaryotic evolution, requiring core proteins to adapt to function both within the mitochondria and in the host cell. Alternative start codon selection of a single mRNA generates alternative N-terminal protein isoforms with shared core sequences but distinct N-termini. These alternative N-terminal isoforms of a gene may localize to distinct subcellar locations to enable the same function in different organelles. Here, we systematically profile the localization of alternative protein isoforms generated by alternate start codon selection during translation. Using protein language models, we identify

498 of pairs of differentially-localized protein isoforms, many of which affect mitochondrial targeting and are essential for mitochondrial function. By tracing the evolution of alternative start codons, we find that the emergence of dual-localized mitochondrial isoforms coincides with mitochondrial acquisition in early eukaryotes. We uncover multiple different mechanisms by which alternative translation controls N-terminal localization, including signal sequence masking, a novel strategy that conceals targeting signals to prevent organelle import. Finally, we leverage the identification of these alternative N-terminal isoforms to reshape our interpretation of disease alleles, understand clinical manifestations, and potentially treat rare human diseases. Pathogenic nonsense and frameshift mutations are typically assumed to completely eliminate a gene's function. We demonstrate that alternative start codon selection can bypass these genetic disruptions, selectively eliminating specific isoforms of a gene while preserving others—a phenomenon we term Isoform-Specific Alleles (ISAs). We found that ISAs in the gene TRNT1 eliminate a specific alternative N-terminal isoform of TRNT1, explaining the distinct clinical symptoms among patients with TRNT1-associated diseases. We further identify thousands of ISAs in rare human diseases and demonstrate that these alleles result in isoform-specific effects on the protein. Together, our findings illuminate the evolutionary and pathological relevance of alternative translation initiation, offering new insights into organelle biology and the molecular basis of rare disease.

B022/P0606

Estrogen Disrupts an Epithelial Adherens Junction - Associated RNAi Machinery to Promote ECM Remodeling and Fibrosis in Women

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Pro-inflammatory diseases, such as Systemic Sclerosis (SSc) and Crohn's disease (CD), are often characterized by extensive remodeling of the extracellular matrix (ECM). The result of this remodeling is tissue fibrosis, a term that refers to excess stromal tissue and ECM buildup. There are no treatment options for fibrosis and current therapies fail to provide relief for this condition. Additionally, SSc and CD have higher incidence rates in women; however, the underlying mechanism behind this discrepancy remains unclear. At the cellular level, these diseases are characterized by compromised epithelial integrity, which occurs due to disruption of adherens junctions. Our lab has shown that loss of PLEKHA7, an epithelial adherens junction component, results in the disruption of junctional integrity, mislocalization of an adherens junction – associated RNAi machinery, and upregulation of ECM markers. Interestingly, it has been shown that estrogens can also disrupt adherens junction integrity, through a non-canonical pathway that involves EGFR and Src activation. Immunofluorescent staining of PLEKHA7 and of the key components of the RNAi machinery in colon epithelial (Caco2) cells, including the microprocessor, DICER, and AGO2, revealed mislocalization of DICER following estrogen (17 β-Estradiol) treatment. Furthermore, estrogen treatment results in increased AGO2 phosphorylation at Y393, which has been shown to negatively affect AGO2 silencing activity and to disrupt the association between DICER and AGO2. Our data show that estrogen also results in downregulation of miRNAs that are regulated by the junctional RNAi machinery. Our recent in vitro and in vivo work has shown that these miRNAs target enzymes that mediate ECM remodeling and pro-inflammatory regulators, downstream of disruption of the junctional RNAi machinery. We are currently examining whether estrogen indeed promotes upregulation of ECM remodeling markers and fibrotic phenotypes, through blocking of the DICER-AGO2 interaction and miRNA function. We are also investigating whether estrogen performs these activities through EGFR or Src activation. Taken together, the work aims to identify a previously unknown regulation of miRNAs at adherens junctions by estrogen that could explain the increased incidence of fibrotic diseases in women.

B019/P0603

Exon-skipping morpholinos targeting the SUMO1 and SUMO2 paralogs enhance the efficacy of chemotherapy in a non-small cell lung cancer

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Lung cancer remains a leading cause of cancer-related mortality, highlighting the urgent need for novel therapeutic strategies. SUMOylation, a post-translational modification regulating DNA repair, replication, and cell cycle progression, is frequently elevated in cancers and represents a promising therapeutic target. We previously identified SUMO2 as the most abundant transcript in human cells and characterized alternatively spliced SUMO transcripts and their encoded non-conjugatable isoforms, SUMO1α, SUMO2α, and SUMO3α, that may act as endogenous inhibitors of global SUMOylation. We hypothesized that upregulating these isoforms would attenuate SUMOylation and enhance chemotherapeutic efficacy in non-small cell lung cancer (NSCLC). To test this, we designed exon-skipping morpholinos (ESMs) targeting SUMO1 and SUMO2 transcripts and evaluated them in A549 and HCC827 cell lines. Treatment with our ESMs increased the respective SUMOα mRNAs by over two orders of magnitude, resulting in altered cell cycle progression, reduced proliferation, and pronounced S-phase arrest. When combined with cisplatin or etoposide, our ESMs increased cell death by 50% and 60%, respectively. These findings demonstrate that SUMOα upregulation sensitizes NSCLC cells to chemotherapy, supporting our ESMs as a candidate class of chemosensitizers. Validation in HEL-299 cells suggests broad applicability, and forthcoming in vivo studies will assess their therapeutic potential for NSCLC.

B017/P0602

Interaction of Estrogen Receptor 1 (ER1) mRNA with microRNA 18a and 19a in Canine Mammary Tumor Cells.

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Breast cancer is among the most prevalent malignancies in humans. Canine models are valuable for comparative studies of breast tumors due to their spontaneous tumor development and shared molecular and hormonal characteristics with human breast cancer. Loss of estrogen receptor 1 (ER1) in mammary tumors is associated with increased aggressiveness and poor prognosis in both species. Recent evidence suggests that small non-coding RNAs, microRNAs (miRNAs), may regulate the expression of ER1 in breast cancer. We have previously shown that miR-18a and miR-19a are upregulated in canine mammary tumors (CMTs). Based on these findings, we propose that miR-18a and/or miR-19a can modulate ER1 expression in breast cancer, affecting its neoplastic potential. Bioinformatic analyses predict that these miRNAs may bind to the ESR1 3’ untranslated region (UTR), although experimental validation in CMTs is currently limited. To validate our predictions, we measured ER1 mRNA, miR-18a, and miR-19a levels using quantitative real-time PCR (qRT-PCR) in various cell lines from our lab, including CMT12, CMT27, CMT28, and CMT47, and normal epithelial cells (validated by sequencing). We then performed co-transfections with ER1 3' UTR reporter constructs containing Gaussia luciferase, and secreted embryonic alkaline phosphatase, along with vectors expressing either miR-18a or miR-19a tagged with green or red fluorescent proteins as reporter genes, and/or anti-miR oligonucleotides. Luciferase activity was measured to assess direct miRNA–target interactions. Gaussia luciferase activity decreases by at least 30% upon miRNA transfection. In contrast, anti-miR co-transfection restores activity to nearly control levels, confirming the miRNA's knockdown effect on the target gene. Luciferase reporter assays have confirmed that miR-18a and miR-19a directly target the ER1 gene, underscoring differences in ER1

expression between breast cancer cells and normal cells. Further levels of mRNA and protein of ER1 will be measured using quantitative real-time PCR (qRT-PCR) and Western blot techniques after transfection with either miRNA vectors or anti-miRNA oligonucleotides. This investigation will also examine how the suppression of ER1, driven by miRNAs, affects critical processes, including cell proliferation, migration, and invasion. In summary, these insights enhance our knowledge of miRNA-mediated control of ER1 and its impact on tumor development, paving the way for new therapeutic approaches.

B023/P0607

Leveraging Drug Off-Target Effects for Therapeutic Innovation

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Small molecules developed to target proteins and DNA can also bind RNA, but the extent and functional consequences of these interactions remain largely unexplored. We systematically profiled RNA interactions of clinically approved chemotherapeutics and uncovered widespread RNA interactions in a substantial fraction of tested small molecules. Cisplatin, a chemotherapeutic for solid tumors, emerged as the most prominent RNA binder. Cisplatin and its analogues (hereafter referred to as platins) are the most commonly used chemotherapeutic agents worldwide. To map cisplatin–RNA interactions in cancer cells, we developed a chemogenomic assay called Plat-RNAseq. It is a click-chemistry-mediated transcriptome-wide assay that allows the identification of platin-interacting RNAs in an open-ended manner. Our analysis revealed that platin-binding was enriched at guanine-rich regions capable of forming RNA G-quadruplexes (rG4s) and preferentially accumulated at the 5′ ends of nascent transcripts. The platin-interacting RNAs tend to have elevated levels of rG4s and a tendency to form RNA-DNA hybrid formation. Mechanistically, cisplatin stabilized pre-formed rG4s while preventing their de novo assembly. Drug combination studies reveal that the recruitment of cisplatin to rG4s partially contributed to its cytotoxicity, revealing a noncanonical mechanism of action for this widely used chemotherapeutic agent. Collectively, these findings redefine platinum pharmacology by establishing RNA interactions not as passive off-targets, but as a biologically relevant and previously underappreciated mechanism of action. We will discuss the therapeutic relevance of our discovery and also how this information can be leveraged to enhance drug efficacy and inform next-generation therapeutic strategies.

B021/P0605

Spatiotemporal mapping reveals principles of multiphase nucleolar organization and bottlenecks in cancer ribosome biogenesis

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Biomolecular condensates are key features of intracellular compartmentalization. The nucleolus, a multiphase liquid-like structure, orchestrates ribosomal RNA (rRNA) transcription and processing to assemble small (SSU) and large (LSU) ribosomal subunits. Elevated rRNA biogenesis and enlarged nucleoli are commonly observed in cancer, yet how rates of rRNA processing steps are coupled to the layered nucleolar organization—and how their dysregulation contributes to tumor progression—remains unclear. We developed spatiotemporal mapping combining imaging and sequencing, revealing that rRNA processing is spatially segregated, with sequential maturation driving outward movement through nucleolar phases. Applying these tools in oncogene-

activated cells, this mapping uncovered bottlenecks in ribosome biogenesis that emerge from accelerated rRNA production. These bottlenecks highlight the trade-offs cancer cells face to sustain high ribosome output and point to nucleolar vulnerabilities that could be exploited to selectively disrupt tumor growth.

RNA Biology - RNA processing and modification

B016/P0585
Investigating the Quality Control Mechanisms that Cope with Defective Ribosomal RNA
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Protein synthesis is one of the most energy-intensive processes in the cell. Disruptions in protein production can compromise cell viability and lead to cellular stress and complex diseases, such as cancer and neurodegeneration. To maintain continuous protein synthesis, cells must monitor the integrity of key translation components: mRNA, tRNAs, and ribosomes. While quality control mechanisms for defective mRNAs and tRNAs are relatively well understood, less is known about how cells manage faulty ribosomes. The eukaryotic ribosome is a complex molecular machine consisting of 4 ribosomal RNAs (rRNA) and 80 proteins, and all these components are susceptible to mutations, oxidative stress, and UV radiation. Notably, the rRNA carries out the core enzymatic functions of the ribosome during translation. Despite the importance of rRNA to ribosomal function and human health, the quality control mechanisms that cope with the presence of defective rRNA remain poorly defined. To address this knowledge gap, we developed a system to investigate the quality control pathways that cope with ribosomes harboring defective ribosomal RNA in human cells. We engineered a reporter ribosomal RNA carrying tags that allow for fluorescent detection and purification. To model defective rRNA, we engineered a mutation in the conserved rRNA decoding center that disrupts protein synthesis and is actively degraded by an unknown quality control pathway(s). To identify novel factors involved in the degradation of defect, we performed a genome-wide CRISPRi screen. Follow up biochemical characterization of top hits confirmed their crucial role in faulty ribosome detection and degradation. This work advances our understanding of quality control mechanisms that eliminate defective ribosomal RNA in human cells. These insights into ribosomal quality control are essential for understanding cellular proteostasis and how accumulation of faulty ribosomes can drive human disease.

B018/P0586
Loss of indiscriminate GLDR-2 tailing enhances body length and reduces fertility and embryonic viability in C. elegans
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During the oocyte-to-embryo transition RNA tailing is dynamic. Yet, we don’t fully understand how tailing patterns are established in gametes and early embryos, how they coordinate RNA biology, or why they are required for embryonic viability. gldr-2 is one of two C. elegans orthologues of the vertebrate noncanonical poly(A) polymerase Gld2, critical for mouse oocyte maturation. In vitro, CeGLDR-2 displays indiscriminate transferase activity, while in vivo, it is responsible for A-tailing miRNA precursors. Its effect on other RNA biotypes and its physiological relevance, however, remain elusive. Its paralogue, GLD-2 has a key regulatory function in C. elegans meiosis and oogenesis, which our work, using a Nanopore-based direct cDNA sequencing method monitoring RNA tail length and composition (Nano3P-seq), has recently expanded on. Here, we use traditional genetics and Nano3P-seq to determine the molecular targets, and the cellular and

physiological function of GLDR-2-mediated tailing. Imaging of GFP tagged GLDR-2 reveals a discrete but robust expression in both hermaphrodite and male germlines. Yet, gldr-2 null hermaphrodites remain fertile while male mutants are severely subfertile. Interestingly, in a pup-1/2 mutant background, loss of gldr-2 in hermaphrodites results in smaller brood and an exacerbated decrease in embryonic viability of F1 offsprings, suggesting an epistatic relationship between gldr-2 and the pup genes. Nano3P-seq data shows a loss of A-tails in the absence of GLDR-2. We also observed a loss of U- and mixed tails, further supporting some redundancy with PUP-1/2. We identified 433 genes with a decrease in average transcript tail length and a decrease in the proportions of A-, U- or mixed-tailed reads in gldr-2 null mutants. Gene Ontology analysis shows that these putative GLDR-2 targets are enriched in processes important for early embryonic viability and cell proliferation, including energy metabolism and cell division, and correlates well with the observed GLDR-2 expression and mutant phenotypes. Future work will determine the effects of A-, U- and mixed-tail loss on stability and translation, confirm physical interactions between targets and GLDR-2 and validate their function. Without an RNA recognition motif, GLDR-2, like GLD-2, must rely on RNA-binding proteins to interact selectively with targets. Our data also shows an enrichment for RNA Pol III and ETR-1 associated transcripts amongst GLDR-2 targets, providing insight into the mechanisms behind GLDR-2 recruitment. This work expands the list of RNA tailing enzymes and the mechanisms responsible for complex gene expression patterns during the oocyte-to-embryo transition and provides novel candidates for therapies targeting RNA mechanisms to impact embryonic viability.

B002/P0588

Moloney leukemia virus 10 (MOV10) is phosphorylated at S970 by c-Jun N-terminal kinase (JNK) to modulate its stress granule localization

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MOV10, an ATP-dependent RNA helicase, has emerged as an important factor in RNA-induced mRNA silencing. We and others have shown that MOV10 associates with Argonaute (AGO) proteins within the RNA-induced silencing complex (RISC), where it facilitates target recognition and mRNA repression. MOV10 also localizes to membraneless organelles found in mammalian cells like P-bodies (PBs) and Stress granules (SGs). PBs are constitutively present in the cells and formed by coalescing RNA, RNA decay and translation suppression factors. Although PBs are enriched in RNA decay enzymes, they are not required for mRNA decay and may function as storage for poorly translated mRNAs. Stress granules on the other hand are caused by induction of stress such as translation inhibition, oxidative or osmotic stress and viral infection. Many proteins including MOV10, shuttle between the two granules upon induction of stress and modulate mRNA stability, translation and turnover. Understanding what determines the recruitment of MOV10 to these granules is crucial to understanding how it alters MOV10 function. We previously reported that MOV10 is phosphorylated at S970 and the phospho-mimic mutant of MOV10 (S970D) downregulates its target mRNAs in an AGO2-dependent manner. Here, we identified JNK as the kinase that phosphorylates MOV10 at S970, using an inhibitor screen. We show that MOV10 phosphorylation increases upon induction of stress by sodium arsenite and, this may contribute to the localization of MOV10 to the SGs. We also show that under normal conditions, MOV10 is dispersed from PBs upon expressing constitutively active JNK, which also leads to downregulation of MOV10 targets, indicating that PB localization is not required for degradation of MOV10 targets. It has been previously reported that JNK inhibition results in failure to form SGs due to reduced expression of scaffold protein, G3BP1 and that JNK also phosphorylates mRNA decapping protein DCP1a to affect its PB localization. Our data suggests that MOV10 is another stress responsive protein that is affected by JNK stimulation to modulate SG assembly. Importantly, MOV10 is a well-known anti-viral protein and JNK-mediated phosphorylation can play a

crucial role in its anti-viral activity. Together these data show that JNK phosphorylates MOV10 to control its localization in RNA granules in the cytosol.

B003/P0589
Rational sequence design for enhanced editing specificity of RNA sensors
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The objective of this study is to enhance the editing specificity of CellREADR through the incorporation of stem-loop (SL) structures and nuclear retention sequences (NRS). CellREADR is a programmable RNA sensor that couples detection of cell-defining RNAs to effector protein translation, enabling precise control of gene expression in specific cell types, with broad relevance to biomedical research and therapeutic applications. Its design relies on an ADAR-editable STOP codon within sesRNA to prevent payload translation upon target RNA recognition. However, this atypical STOP codon is prone to ribosomal read-through and re-initiation, compromising sensing specificity. It was hypothesized that introducing SL elements into the mRNA sequence would enforce translation termination, while NRSs would spatially separate RNA from the translational machinery to further reduce leaky translation. Methods: For in vitro validation, CellREADR constructs driven by a CAG promoter (CAG–mCherry–sesRNA–mNeonGreen) were tested in HEK293T and Neuro-2A cells through transient transfection, fluorescence imaging, and flow cytometry. For in vivo testing, CellREADR sensors and reporters were packaged inAAV (PHP.eB serotype) and delivered into the mouse brain via retro-orbital injection into 3–4-week-old wild-type C57BL/6 mice. Brains were collected and analyzed 3–4 weeks post-injection by HCR in situ and immunostaining. Results: In HEK293 assays, sesRNAs containing SLs with a variety of GC content and thermal stability significantly reduced unintended mNeonGreen translation ($p < 0.01$). This effect was position-dependent, with SL-75 (82% GC, −40 kcal/mol ΔG, located 75 bp downstream of the editable UAG) producing the strongest suppression. For in vivo testing, AAV delivery of this sensor, sesRNAvGAT(SL-75), increased targeting specificity of cortical GABAergic interneurons from ~50% to >80%. Additionally, NRSs reduced non-specific payload expression in HEK cells. Ongoing studies aim to evaluate ADAR editing efficiency in cell culture, test NRS sequences in vivo, and assess combined NRS + SL strategies in vivo. In summary, SLs and NRSs cis-elements substantially improves CellREADR specificity, establishing CellREADR as a next-generation toolkit for programmable decoding and engineering with broad biomedical applications.

B001/P0587
RNA-editing in a large cell for rapid environmental response
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All cells must reorganize biochemistry to respond to rapid environmental fluctuations. This is especially important for large cells where local composition is challenging to regulate by de-novo transcription. RNA editing, or modifications to RNA that result in base pairing changes, represents a fundamental yet underexplored layer of reorganization. RNA base substitutions can alter encoded amino acid sequences and modify RNA structure. Importantly, RNA editing, which has the capacity to diversify transcriptomes and proteomes, acts as a rapid-response regulatory mechanism in lieu of slower transcriptional or evolutionary changes. The multinucleate slime mold, Physarum polycephalum is remarkable for its ability to show rapid and coordinated environmental response. Despite being one giant, single cell, this organism can solve mazes, compute shortest-path problems, and optimize transport networks through global and local cytoplasmic

reorganization. Importantly, Physarum utilizes extensive RNA editing across mitochondrial encoded RNAs. However, less is known about the mechanism or distribution of RNA editing events more generally across other compartments. Using computational approaches and spatially sensitive sequencing data, we show that (i) RNA editing occurs across nuclear encoded RNAs, (ii) RNA editing is spatially patterned within the cell, and (iii) that RNA editing can drive both changes in RNA structure as well as protein recoding. Specifically, we find that mRNAs in regions of the cell involved in foraging have a different RNA editing profile than mRNAs contained within regions of the cell involved in transport. These data support a role for RNA editing in creating distinct cytoplasmic territories that direct local function and chemistry, enabling cell coordination over centimeter-scale distances.

RNA Biology - RNA structure and function

B005/P0591
Role of MicroRNA in Endostatin-mediated Effects on Endothelial Cells
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Background: Pulmonary arterial hypertension (PAH) is a disease that impacts vascular function both within the pulmonary circulation and the right ventricle (RV). Endostatin (ES), a circulating peptide derived from the extracellular matrix protein collagen, COL18A1, is a potent inhibitor of angiogenesis with dramatic effects on endothelial (EC) gene expression and function. COL18A1/ES is significantly increased in both the PAH lung and diseased RV and circulating ES is associated with clinical functional impairment, and mortality. Despite the established angiostatic effects of ES, the molecular mechanisms by which COL18A1/ES contribute to EC dysfunction in disease are unknown. Method: Using primary human pulmonary microvascular EC (HMVEC-Ls), we aimed to define the contribution of ES-miR-dependent gene expression on the angiostatic functions of ES in vitro. HMVEC-Ls were challenged with either control miR or miR-155 for up to 24 hours and were analyzed using bulk RNAseq, miR array, qPCR, and Western blotting. Differential Gene expression (absolute fold change > 1.2 and FDR <0.05) were performed on filtered (low counts), normalized (TMM-normalized) counts using edgeR. Pre-ranked gene set enrichment analysis were performed on up- and down-regulated genes, using Gene Ontology and KEGG databases. Transcription factors and pathway activity were further assessed using decoupleR. Results: ES treatment significantly increased the expression of multiple microRNA, inducing miR-155. MiR-155 contributes to abnormal EC function and can modify gene expression in EC. A total of 6390 genes (3242 upregulated and 3148 downregulated genes) were differentially expressed in human EC in response to miR-155 mimetic in vitro. Functional enrichment identified multiple immune and inflammatory processes among the top miR-155-modified pathways and implicated miR-155 in the altered activity of multiple transcription factors dysregulated in disease, including HIF1, NF-KB, and SMAD3. Conclusion: ES suppresses pivotal genes in EC homeostasis and PAH pathogenesis, including multiple genes within the TGF-beta signaling pathway, the most notable of which is bone morphogenic receptor 2 (BMPR2). MiR-155 mimetic suppresses BMPR2 and is implicated in altered transcriptional regulation of inflammatory pathways in EC. This implicates miR-155 as an effector of ES-mediated EC dysfunction. We predict that ES-miR-mediated signaling networks may contribute to the pathogenesis of PAH.

B004/P0590
The post-transcriptional regulation mechanism and functional importance of a key maternal mRNA, glp-1

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Translational control of maternal mRNAs is a major form of gene regulation during germline development and embryogenesis. C. elegans Notch homolog glp-1 maternal gene is necessary for germ cell proliferation, and embryonic fate determination. The RNA binding proteins POS-1 and GLD-1 directly regulate the translation of GLP-1 protein by binding to the specific elements within the glp-1 3' UTR. When POS-1 or GLD-1 binding is disrupted by mutation of their respective elements, the expression pattern of a glp-1 3' UTR reporter transgene changes in both the germline and in embryos. The mechanism by which POS-1 and GLD-1 mediate translation repression is not well understood. Previous work showed that loss of pos-1 increases the average polyA tail length of endogenous glp-1 transcripts in embryos. In this study, we show that mutation of either the GLD-1 or POS-1 binding motifs in transgenic reporters does not change polyA site selection. This result rules out alternative polyA site selection as a mechanism of regulation. We also show that wild-type glp-1 transgenic reporter embryos have a shorter average polyA tail length compared to transgenic reporters with mutated GLD-1 or POS-1 binding motifs. We have studied the effect of cytoplasmic polymerases, deadenylases and translation initiation factors on our transgenic reporters. Our RNAi experiments show that two cytoplasmic polyA polymerases, GLD-2 and GLD-4, have strikingly different effects on the expression of reporter transgenes harboring GLD-1 or POS-1 binding motif mutations. We also observed strong derepression of all reporters upon reduction of the translation initiation factor ife-3. The results reveal that POS-1 and GLD-1 exert their repressive effects in different ways through cytoplasmic polyA polymerase activity, while IFE-3 mediated translation repression is independent of both POS-1 and GLD-1. Lastly, we have examined the biological significance of glp-1 3'UTR to the worm reproduction by using CRISPR/Cas9 mutagenesis to generate glp-1 3'UTR mutations in the endogenous locus. Characterization of a 71 base pair mutation that deletes the GLD-1 and POS-1 binding sites in the glp-1 3'UTR reveals a 2-fold reduction in the number of embryos produced and a 4-fold reduction in the hatch rate. Imaging results show that the mutant embryos appear to have patterning defects. Together, our results show that the glp-1 3'UTR contributes to reproductive health.

RNA Biology - RNA transport and localization

B006/P0592
Competition Between Diffusive Search and Translation Kinetics Controls mRNA Targeting to Mitochondria
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Most mitochondrial proteins are encoded by DNA in the nucleus, which is transcribed into mRNA and then translated into proteins in the cytosol that are imported into mitochondria. Experiments show that mRNAs for many mitochondrial proteins are associated with the mitochondrial surface. This association is mediated by the mitochondrial targeting signal (MTS) region of these proteins, which allows the mRNAs to bind to mitochondria. We aim to understand how translation kinetics and mitochondrial network geometry together regulate the degree of mRNA association with the mitochondrial surface. We simulate mRNA association with many three-dimensional experimentally-measured mitochondrial structures and with mRNA parameters corresponding to many different genes to investigate how these factors lead to diversity in mRNA association with mitochondria. While we recapitulate the experimental result that mitochondrial networks that occupy a higher fraction of the cell volume tend to have higher mitochondrial mRNA association, we also show that mitochondrial networks that occupy a similar fraction of the cell volume can have substantial variation in mRNA association. We find that mitochondria that are more evenly distributed throughout the cell volume have higher mRNA association via shorter diffusive search times for the mitochondrial surface. We reinforce earlier results to describe differences in mitochondrial mRNA association between genes, with longer mRNAs,

higher initiation rates, and lower elongation rates leading to higher mitochondrial mRNA association. We find that the duration that an mRNA has at least one binding-competent MTS, enabling mitochondrial attachment, largely explains differences in mitochondrial association between genes. Overall, we find that a small ratio of diffusive search time (controlled by mitochondrial structure) to MTS exposure duration (determined by translation kinetics) leads to a high level of mRNA association with mitochondria. Our work thus explains how competition between search and translation kinetics regulates mRNA association with mitochondria.

B007/P0593

LENG8 interacts with TREX2 subunit PCID2 and promotes accurate mRNA processing

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Inside the nucleus, eukaryotic RNA transcripts become co-transcriptionally assembled into messenger ribonucleoprotein complexes, processed and then exported to the cytoplasm through the nuclear pore complex (NPC). The TRanscription and EXport 2 (TREX2) complex plays a central role in bridging the transcription and export machinery. It is conserved across eukaryotes. Mammalian TREX2 consists of GANP, PCID2, ENY2, DSS1, and either CETN2 or CETN3. Although TREX2 subunits clearly function in tandem to facilitate RNA export, mutations of individual subunits cause distinct phenotypes and the subunits show subtle differences in their expression, localization and dynamics, suggesting that they may also have distinct cellular roles. To understand the roles of individual TREX2 components, we used Immuno Precipitation (IP) of each subunit coupled to Mass Spectrometry (MS) and AlphaFold (AF) analysis to identify their separate and overlapping interactomes. Among PCID2-bound proteins, we found two paralogs of human GANP - Leukocyte Receptor Cluster Member 8 (LENG8) and SAC3 Domain Containing 1 (SAC3D1). GANP, LENG8, and SAC3D1 each possess a SAC3-homology domain and exhibit extensive conservation with the ySAC3 domain. AF analysis of predicted multimers suggests that all three proteins interact with PCID2 in a mutually exclusive fashion. Our data further show that GANP and LENG8 alternatively control PCID2 intracellular localization. Notably, transcriptome analysis after LENG8 depletion shows that it plays a role in splice site selection. Consistent with this idea, IP-MS analysis of LENG8 binding partners indicates extensive interactions with the mRNA processing machinery.Together, our findings indicate that PCID2 acts in mRNA processing and alternative splice site selection in conjunction with LENG8 and separately in mRNA export in conjunction with GANP. We are currently investigating how these distinct roles of PCID2 may be coordinated and regulated with respect to each other. Notably, our findings support the more general idea that TREX2 subunits can assemble into functionally distinct subcomplexes, where interacting partners define their specificity and alternative functions. We are further analyzing additional interactions partners for each of the TREX2 subunits.

B009/P0595

Posttranscriptional control of cytoskeletal regulators during neuronal remodeling

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A neuron’s function relies on its form. In some neurons, establishment of the cell’s morphology continues after embryonic development through neuronal remodeling. Neuronal remodeling is a developmentally controlled process that refines connectivity through pruning of exuberant neurites followed by regrowth of new neurites

to establish a mature, refined structure. A central question that still remains is: how is the cytoskeleton rapidly reorganized to support neurite regrowth at local sites during neuronal remodeling? We hypothesize that posttranscriptional regulation of mRNAs encoding cytoskeletal regulating proteins supports neurite regrowth in remodeling neurons. Posttranscriptional regulation, such as RNA trafficking and local translation, allows for local regulation at sites distal to the cell body. In Drosophila, the central nervous system mushroom body γ neurons and peripheral sensory class IV dendritic arborization neurons undergo stereotyped remodeling of their axons and dendritic arbor, respectively, and are amenable models to elucidate mechanisms underlying neuron remodeling. This project focuses on posttranscriptional regulation of transcripts encoding the actin binding proteins profilin and ciboulot in these remodeling neurons. We utilize genetic knockdowns of these transcripts to determine the protein's contribution to remodeling and live imaging of fluorescently labeled profilin RNA to characterize profilin RNA localization and motility. This study provides insights into the role of posttranscriptional regulation in neuronal morphogenesis.

B008/P0594
The Drosophila melanogaster TENT5 homolog, isep, is required for spermatogenesis
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TENT5s are non-canonical poly(A) polymerases (PAPs) that add non-templated adenines to mRNAs post-transcriptionally. The TENT2 family of non-canonical PAPs are important for reactivation of translationally repressed mRNAs in oocytes and in dendrites of neurons and are required for long term memory. Human TENT5s have been implicated in multiple diseases and cancers suggesting a tumor suppressive role. TENT5D has been linked to human male infertility and TENT5C has also been shown to be essential during spermatogenesis in mice. However, the mechanisms by which TENT5s polyadenylate mRNAs remain elusive and they may have other biochemical roles besides poly(A) polymerase activity. Drosophila melanogaster has one TENT5 ortholog, isep. We have generated a loss of function allele of isep and have demonstrated that homozygous males are sterile with defects arising during spermatid individualization. Individualization is the last stage of spermatogenesis where a syncytium of 64 spermatids must be resolved to encase each spermatid in its own plasma membrane and strip away unneeded organelles and cytoplasm. Restricted localization of caspase-3 and synchronous actin cone progress along the axoneme is disturbed in isep loss of function. In-situ hybridization shows that isep is expressed in two phases: first during the meiotic divisions of spermatocytes, and again post-mitotically in elongating spermatids, where transcripts exhibit a characteristic "comet" localization pattern. Structural defects are also evident in the axonemes of individualizing spermatids, yet our data indicate that isep's PAP activity is dispensable for spermatogenesis. Together, these findings suggest that isep performs an essential function in male germ cell differentiation that does not depend on its PAP activity. Ongoing work aims to elucidate the molecular mechanism of isep action, providing insights into how TENT5 orthologs operate across species.

Scholarship of Diversity - Evidence-based approaches to improve recruitment and retention in the biomedical research workforce

B548/P1112
Peer Mentoring Impacts Science Retention of Latina and Black Female Urban Students Enrolled in First-Year Biology Courses

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Latina and Black female students are more likely than their White and Asian counterparts to leave science majors after their first year of college. Supportive peer mentorship has been shown to have a positive effect on retention and to improve the academic performance of STEM students. Limited data has explored the impact and experience of Latina and Black female students engaged in peer mentoring during the first year science courses. The objective of this research was to determine whether a one-year peer mentoring experience could impact student outcomes for participants enrolled in first-year biology courses. The mentoring experience took place between 2020 and 2022, spanning two academic years, mostly during the COVID-19 pandemic lockdown. Students attending an urban commuter 4-year college were recruited through flyers on their course website. The recruitment encouraged students to join by mentioning specific activities they could find relevant including: Black female and Latinas peer mentors, a network of Black female and Latinas alumni scientists and presentations about career and professional opportunities. A total of 44 students participated in the program. Each student was assigned to a small group of four students with a peer mentor, which were upper level, junior and senior, Latinas and Black female students majoring in the sciences. Students met at least once a month during the semester, and joined whole cohort meetings designed to build connections among first year students and in which scientists, also Latinas and/or Black females, were invited to share their personal journey and professional experience. Students completed surveys at the end of each meeting. We also collected the Peer Group Interaction scale, to understand their experience in comparison to other first-year students. 1-year science retention was 79% for participants compared to 43% for all non-White female students, after 2 years 58% of participants were still enrolled in a science major when compared to only 30% of other females of color. Participants reported meetings focused on academic topics (46%) versus personal (24%) and professional (30%) topics. Over 90% of participants found the meetings productive and looked forward to the following meeting. Responses to the scale showed that mentored students, that included the peer-mentored group, had higher scores for positive peer interactions than not mentored students. They developed more close personal relationships ($p=0.09$), and felt that peers had a positive influence in their personal ($p=0.07$) and intellectual ($p=0.019$) growth. Our findings are limited because this is a pilot study, but they add to the current knowledge that supports the relevance of peer-mentoring in the retention of diverse student populations.

Scholarship of Diversity - Individual and institutional barriers to workforce diversity

B547/P1111
Longitudinal Insights from the Faculty Job Market Collaboration
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Securing a tenure-track faculty position remains one of the most opaque and consequential transitions in the biomedical sciences. Since our initial eLife paper (Fernandes et al., 2020), the Faculty Job Market Collaboration (FJMC) has built the largest longitudinal dataset on biomedical faculty hiring, integrating over 4,500 applicant survey responses (collected in years 2018–2024), job posting data, and qualitative interviews. Across these data sources, we examine how applicant metrics, institutional context, and committee practices shape outcomes on the academic job market. Recent publications from our team (Frontiers in Research Metrics and

Analytics, 2024; Studies in Graduate and Postdoctoral Education, 2024 & 2025) demonstrate that traditional scholarly benchmarks—such as publications, citations, and career transition funding—are associated with reaching the interview stage but are poor predictors of final job offers. Instead, hiring success reflects the interaction of multiple factors, including institutional prestige, number of postdoctoral positions, field norms, and applicant demographics. Statistical modeling and machine learning approaches reveal structured, multivariate patterns in faculty employment that defy reduction to a single profile of success. Our most recent qualitative pilot (2024–25) follows biomedical applicants longitudinally through the full hiring cycle, capturing decision-making, perceptions of fit, and responses to shifting political and policy climates. Participants describe navigating inconsistent communication, unclear evaluation criteria, and a strong reliance on peer networks and informal mentoring. These narratives contextualize our quantitative findings, highlighting both the structural and experiential dimensions of gatekeeping in academic hiring.

Science Education - Graduate and medical school

B568/P1130
MBL Physiology: Advanced Quantitative Cell Biology Research Training, from Discovery to Community Impact
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The Advanced Research Training Courses (ARTCs) at the Marine Biological Laboratory (MBL) subscribe to a fundamental principle of conducting fearless science. The strategy of combining experimental creativity with scientific talent has resulted in the course serving as a catalyst for scientific networks that span career stages, occupational sectors, national and international institutions and organizations that focus on the biological and physical sciences. The course is uniquely poised to accomplish this goal as it has long been a meeting ground for students with backgrounds in both the biological and physical sciences as well as an incubator for research discovery and networking that creates career launching points through innovative research collaborations. Here we show the results for published research discoveries that originated in the course, as measured by article citation metrics and Altmetric scores. In addition, we show how the course, powered by collaborations and publications, results in the expanded network of professional development.

Science Education - K-12

B550/P1822
Conveying Science With Art in Everyday Settings
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Information can be communicated through a wide range of mediums, limited only by our creativity, and science communication is no exception. Traditional forms typically involve sharing facts orally or in writing, but these methods often reach a self-selecting audience already inclined to engage with science. There are alternative approaches to science engagement that prioritize connection over content, such as highlighting the stories of individual scientists or sharing science-inspired art. By embracing these other creative methods to relate with others, we can reach a broader and more diverse audience. Moreover, the growing “anti-science” sentiment is often not a rejection of the scientific method itself, but from distrust in academic institutions, corporations, and researchers. One effective way to rebuild public trust is through casual and regular interactions that humanize scientists and make them more relatable. This talk will center on the speaker’s

personal experiences and community involvement, with the goal of inspiring listeners to connect more deeply with their own communities.

B549/P1113
Exploring South America's Nature Secrets: Hands-on Ethnobotany for Underserved Children in the Historic Barrio Huemul (Chile)
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Objective The objective of this initiative was to foster early scientific curiosity, communication skills, and cultural identity among children living in foster care by integrating Chilean ethnobotanical knowledge with basic cell biology through a structured, hands-on outreach program in the University Bernardo O Higgins. Methods We designed and implemented a multi-stage educational initiative for children aged 4–8 residing at the “Guadalupe Acoge Foundation” in Chile. The program included: (1) interactive workshops introducing botanical sciences and traditional medicinal plants through storytelling, illustrations, and simple experiments; (2) laboratory sessions at the Integrative Center for biology and applied chemistry, where children prepared non-toxic plant extracts, added them to pathogen-free cell cultures, and observed cell viability using fluorescence microscopy accompanied by students of Medical Technology; (3) visit to the university’s Innovation laboratory (Living Lab), where 3D printers are located, observing their operation in the company of Mechatronics Engineering students; and (4) creation of age-appropriate scientific posters documenting their findings, with the posters made available for viewing by university authorities, students, scientists, and community members. Results Children demonstrated improved observational and descriptive skills, accurately identifying plant species, describing experimental procedures, and communicating results visually. The children learned to follow laboratory safety rules and to wait their turn to use equipment, which represents a valuable behavioral gain transferable to their regular school environments. From the perspective of cell biology outcomes, the plant extracts prepared by the children protected cell cultures from hydrogen peroxide–induced cell death, as visualized through the counting of apoptotic nuclei using DAPI staining. The children showed great enthusiasm during the laboratory sessions, as it was their first time visiting a university. They were particularly fascinated by observing university students studying and engaging in recreational activities on campus. According to their own expressions, this experience inspired many of them to envision themselves attending university in the future. Conclusion This initiative effectively combined traditional ethnobotanical knowledge and basic cell biology to promote STEM engagement among underserved children. The results demonstrate that culturally grounded, hands-on science programs can enhance scientific literacy, communication skills, and a sense of belonging, while strengthening university–community partnerships for sustainable educational impact. Supported by the COMPASS Outreach Grant (ASCB).

Science Education - Primarily Undergraduate

B564/P1126
A 'research in the classroom' bioinformatics study to analyze wasp virulence proteins of Leptopilina heterotoma.
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Parasitic wasps of Drosophila melanogaster present a unique host-parasite model to analyze the mechanisms underlying innate immunity in Drosophila melanogaster's response to the wasps, and the wasp's ability to suppress such responses. The wasp, Leptopilina heterotoma(Lh) is highly successful in blocking cellular and humoral host responses. The success is driven by the immunosuppressive activities of Mixed Strategy Extracellular vesicles (MSEVs), specialized microvesicle-like organelles that transfer protein cargo (a complex cocktail of venom proteins containing diverse virulence factors) from the parasite to the host to manipulate immune responses during infection. In a uniquely designed 'research in the classroom' bioinformatics course, we have characterized the family of Lh metallopeptidases (MEPs), specifically neprilysin-like M13 MEPs as venom proteins. Students in this upper division, writing intensive course developed specific hypotheses, devised bioinformatics approaches to design in silico experiments, analyze, interpret, and presented their findings in a term paper modeled after a peer reviewed journal article. Here we present select analyses of the Lh MEPs from the summer 2025 course. Analysis of sequence and domain architecture showed conservation of peptidase domains across proteins, along with secretion signals consistent with venom function. Interestingly, despite global structural conservation, our analysis revealed key differences within the binding sites of the Lh MEP family members. While some of wasp proteins retained the classic catalytic motifs and the canonical HExxH zinc binding motif, others lacked the motif, suggesting possible pseudoprotease function. In catalytically competent homologs, active site motifs and zinc coordination were intact, whereas in predicted pseudoenzymes, substitution in conserved motifs disrupted the catalytic environment. Surface electrostatic profiles further revealed that wasp enzymes often displayed increased charged and polar residues in the substrate binding pocket compared to the smaller hydrophobic residues of human neprilysin, implying altered substrate preference. These findings indicate that Lh venom contains both enzymatically active neprilysin-like proteases involved in degrading host signaling peptides as well as structurally conserved but catalytically inactive pseudoenzymes that may have evolved to adopt novel and non-proteolytic roles. These findings also highlight the functional diversity of venom MEP in wasps and their contributions to host immune modulation.

B559/P1121

Biodesign curricula engage diverse learners in biomaking and biomaterial investigation outside the classroom.

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This study examines the implementation of biodesign and biomaking curricula in different out-of-school contexts and assesses their impact on students' biology learning and STEM identity. The programs were developed and run by scientists at BioBus, an independent nonprofit organization that works with K–12 and college students in NYC and beyond to discover, explore, and pursue science, with a focus on students historically excluded from the scientific community due to factors such as race, gender, economic status, and physical access. All BioBus programs aim to create spaces where youth voices and lived experiences are central to science learning. We analyzed four out-of-school programs facilitated by BioBus scientists, including: (1) weekly after-school programs for middle and high school students, (2) a multi-institutional STEM mentoring program for middle school students, and (3) a youth program at a community-based nonprofit. Across these settings, students engaged in project-based biodesign investigations that integrated scientific, creative, and community knowledge. A central case study features the Environmental Leaders of the Brotherhood Sister Sol (BroSis) in West Harlem, where 28 youth and two near-peer mentors developed and prototyped COMPOSTourri, an infused biopolymer that aimed to mitigate compost odor and repel pests. Grounded in students' expertise managing a local composting system, this project demonstrated how place-based biomaking can cultivate agency, collaboration, and applied biological understanding. Importantly this case study shows how microscopy and cell biology can be incorporated in different contexts where no formal lab is available. Participants met weekly from February to May 2025, exploring soil science, material properties, and

design thinking while preparing their projects for presentation at the BioDesign Challenge and BioBus Harlem Science Showcase. We conclude that engagement in project-based biodesign projects positively influenced participants' scientific learning, confidence, and STEM identity, while fostering core BioSkills they can carry forward in their STEM journeys.

B567/P1129
Collaborating with Tribal Colleges to develop targeted workshops
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The Trails to Research Program aims to increase research capacity at Tribal Colleges and to help students transfer from Tribal Colleges to 4-year universities. We have built strong relationships with all seven Tribal Colleges (TCs) in Montana and with TCs beyond Montana. In phase one of our program, we developed intensive one-week long courses based on zebrafish development, which we have recently opened to TC students beyond Montana. In phase two, we are helping TC faculty develop their own research-based courses according to their interests. This is ongoing at Aaniiih Nakoda College, and we are beginning to work with faculty at other Tribal Colleges. Phase three of our program is to develop and teach workshops that are more targeted and technique-focused, according to the wishes of the collaborating TC. We developed immunofluorescence workshops with Salish Kootenai College and with Blackfeet College. We stained the zebrafish embryos generated in the week-long course (phase 1) with antibodies to visualize the state of their nervous systems. Thus, the courses connected directly and we covered the importance of antibodies in research and medical diagnostics. With Chief Dull Knife College, we developed an RT-PCR workshop. We screen honey bees from the hives on the Chief Dull Knife College campus for picornaviruses, which bees frequently carry (and are thought to be involved in colony collapse syndrome). Thorough discussions ensure that students understand how RT-PCR works and students learn about a variety of viruses. We are beginning collaboration with other Tribal Colleges on additional projects. This work is funded by the NSF and USDA.

B565/P1127
Differential Effects of Alcohol on Breast Cancer Cell Lines with Distinct Hormone Receptor Statuses
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Alcohol consumption is a known risk factor for breast cancer, yet its cellular effects vary depending on tumor subtype. This study investigates the impact of ethanol exposure on three breast cancer cell lines - MCF7 (ER+/PR+/HER2−), MDA-MB-231 (ER-/PR-/HER2-), and SkBR3 (ER-/PR-/HER2+). These lines represent distinct molecular subtypes with differing hormone receptor statuses, which may influence their response to alcohol. Cells were treated with physiologically relevant concentrations of ethanol, and assays were conducted to assess cell proliferation, migration, as well as changes in key signaling pathways. MCF7 cells exhibited increased proliferation but not migration, suggesting a mitogenic effect of alcohol potentially mediated through estrogen receptor signaling. SkBR3 cells demonstrated significant increases in proliferation in response to ethanol exposure which was accompanied by altered HER2 signaling. In contrast, MDA-MB-231 cells showed only modest increases in cellular proliferation in response to ethanol, but significant changes in ROS production and migratory behavior. Ethanol treatment also altered the cellular morphology of the MDA-MB-231 cells, especially at higher concentrations. These findings underscore the subtype-specific effects of alcohol on breast cancer biology and suggest that alcohol may exacerbate tumor progression through distinct

molecular mechanisms depending on receptor status. Understanding these differential responses is critical for developing targeted prevention strategies and informing public health recommendations.

B553/P1115

Examining the influence of Vision & Change and a mentoring network on teaching philosophies and strategies

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The 2011 report Vision and Change: A Call to Action (V&C) resulted from a national effort to rethink biology curriculum. V&C outlines core concepts and core competencies for biology undergraduates and promotes evidence-based pedagogy, undergraduate research, and inclusive practices. However, it is unclear how much biology educators know about V&C and what motivates educators' development of their teaching philosophy and practices. We leveraged the Promoting Active Learning and Mentoring (PALM) Network, a group that introduced evidence-based instructional practices (EBIPs) to instructors through mentoring, journal clubs, and a community of practice, to investigate how much V&C has influenced educator knowledge and motivation. Through focus groups, 16 mentors and 22 fellows were asked about their motivations to join PALM, familiarity with V&C, how they learned about V&C, and how PALM and/or V&C shaped the development of their teaching philosophies and strategies. We found that the teaching philosophies and practices of these educators align strongly with V&C principles. V&C provided expectancy (established value), while PALM contributed to greater instructor self-efficacy in EBIPs, overall resulting in reformed teaching philosophies and practices. This model highlights the importance of mentorship and community to successfully drive biology education reform.

B560/P1122

Fly-CURE and Connecting Curriculum: Multi-Institutional Course-Based Undergraduate Research Experiences in Genetics and Beyond

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The Fly-CURE is a course-based undergraduate research experience (CURE) centered on the genetic mapping of cell growth mutants identified in an EMS screen in Drosophila melanogaster. There are currently over 30 faculty implementing the Fly-CURE in genetics classrooms across a wide variety of institution types, including community colleges, MSIs, public and private universities. As of 2025, over 3,000 undergraduate students have participated in the project, resulting in 17 peer-reviewed publications, with over 800 student co-authors. Assessment data demonstrates that students who participate in the Fly-CURE report gains in their sense of science identity, self-efficacy in research methods, and intent to pursue future research experience, regardless of their institution type, or demographics. The Fly-CURE curriculum is in the process of expanding via an NSF-funded Research Coordinated Network (RCN) to develop courses in bioinformatics, behavioral genetics, molecular biology, and developmental biology. Each RCN module is designed such that it can be implemented as a stand-alone CURE, mixed and matched to create unique interdisciplinary CUREs, or scaffolded with the genetics Fly-CURE. We are currently recruiting faculty to participate in our RCN, including the molecular biology CURE. Faculty participants will be provided stipends for curriculum training and implementation, a

social network of faculty, a community of like-minded scientists, continued scholarship opportunities, and support for tenure and promotion. Supported by NSF Awards DUE #2021146 and RCN-UBE #2316218.

B562/P1124

How do undergraduates learn to communicate science? A systematic review of the literature

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Effective science communication has been identified as an essential skill not only for professional scientists already in the field, but also for undergraduate and graduate students. While some studies have been done to identify priorities for science communication education, there is not a broad synthesis of literature about science communication education available for scientists and students, making it challenging to identify existing programs that have been successful, establish evidence-based best practices, and uncover areas that need additional research. To address this lack of a comprehensive overview, we have designed a systematic literature review to document the scope and focus of existing science communication education research. Since the overall goal of this project is to generate a broad view of current science communication education literature, we chose search methodology that would allow us to highlight trends in this emerging field and identify existing knowledge gaps. We assembled a broad list of search terms and searched four electronic databases and key journals in science communication and science education. Articles are included in this review if they were published in peer-reviewed journals, described science communication education, and were written in English. We also chose to explore the subset of articles describing undergraduate science communication education and categorize them according to the type of communication, evidence provided for effectiveness of instruction, and collaboration between authors. Of over 2,400 science communication education articles identified, 142 describe undergraduate science communication. Over 80 percent of these articles describe audience or scientific communication, while only 8.5 percent of articles described interpersonal science communication education. While approximately half of the instructional articles provided evidence of effectiveness, very few provided any validity or trustworthiness evidence for their evaluation methods. Most of the undergraduate science communication education research was conducted by science educators, with only one sustained collaboration that included communication scholars. These preliminary findings suggest that there is a strong need for more interpersonal science communication education and for stronger collaboration between scientists and communication scholars to inform evidence-based instructional practices in science communication education.

B561/P1123

Identity-Specific Scientist Spotlight Boosts Exam Performance and STEM Identity in Introductory Cell Biology

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The Scientist Spotlight assignment is a tool has been shown to improve students' science identity and success in a biology class (Schinske et al., 2017 a,b). Likewise, increased representation of historically excluded minority students has been associated with improved achievement equity in STEM courses (Bowman et al., 2023), Conversely, stereotype threat theory suggests that salience of minoritized group status can harm students' achievement and persistence in STEM fields (Totonochi et al., 2021). Objective: The aim of this study is to combine positive representations of student identities with the Scientist Spotlight activity to generate a

stereotype boost (Shih et al., 2011) and improve exam performance and STEM identity in an introductory cell biology class. Methods: Over three semesters, students were assigned the Scientist Spotlight activity and were randomly divided into three groups, control, shared identity, and self-imagining, when choosing the scientist they wanted to learn about. The control group was instructed to choose a scientist, while students in the other groups were asked to find a scientist that shared a component of their identity. Both groups were instructed to find an image of their scientist, however, half of these students were also instructed to generate an image that reimagined themselves as the scientist they chose (self-imagining). Students were instructed to bring the image with them to all course exams during the semester. Results: Students who brought the image achieved significantly higher exam scores than students who did not. Furthermore, exam scores of students in the self-imagining group that brought their images with them performed significantly better than their control and shared identity counterparts as determined by a one-way ANOVA followed by Bonferroni post hoc test. Finally, students in the self-imagining group had a significant increase in STEM identity as measured by the previously validated single-item measure also improved (McDonald et al., 2019). Assessment of retention into following-semester biology courses is ongoing, as is identification of differences among demographics. Conclusion: Providing students with opportunities to learn about scientists with shared identity and keeping those scientists salient during exams can provide a stereotype boost that correlates with improved exam performance and STEM identity.

B563/P1125

Initiating Science Advocacy Training at a Primarily Undergraduate Institution

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Given the current landscape where skepticism about the value of science has increased and federal scientific funding is being reduced, there is a great need for scientists to become more engaged advocates. Despite success in teaching our students about the process and value of science, this information is not reaching our lawmakers or the general voting public. This is particularly true of funding for studies focused on specific populations critical for understanding the effects of gender, race, and other social determinants on health and disease, studies aimed at developing preventive vaccines and other public health measures, and training programs aimed at broadening access to STEM education. As faculty at a research-active, predominately undergraduate institution (PUI), we wanted to increase our own advocacy for science and train STEM and non-STEM students in the skills of science advocacy. We hypothesized that students were not currently active in this space because they lacked knowledge of the tools and resources for doing science advocacy and predicted that, with training, students would be more open to engaging in effective science and other advocacy work. We thus combined information from the advocacy arms of multiple professional scientific societies to develop a flexible two-session workshop while also sharing this information in different formats with Butler faculty/staff. The workshop sessions provided information about the sources of federal science funding, an overview of current cuts and delays, and advocacy tools including the “two-minute speech” from the ASCB Advocacy Toolkit and tips for contacting and interacting with legislators from materials provided by the Council on Undergraduate Research (CUR). We did this in a non-partisan manner with the aim of sharing information and promoting open dialogue among people of all political affiliations. Informal feedback suggested students appreciated the information, and we received verbal affirmations along with invitations to present to other student groups. More formal survey assessments of participating students indicated that 93% found the workshops helpful or very helpful and, while a few stated prior interest in advocacy, 64% of respondents indicated increased inclination to engage in the future, ~3/4 thought we should run the workshop again, and 100% indicated it is important or very important for college students to engage in advocacy. Written comments supported our hypotheses that students did not previously think they had the ability or tools to do

advocacy but afterwards felt they do. Future work is aimed at increasing exposure to advocacy training for students at Butler and other institutions and expanding the program to include advocacy for the arts and humanities, as well as STEM.

B556/P1118
Learning Biological Physics via Modeling and Simulation: a Course for Science and Bioengineering Undergraduates
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Undergraduate physical-science curricula are heavily mathematicized, despite research showing that learning should begin with qualitative phenomena of broad current interest. Meanwhile, undergraduate life-science curricula are often rooted in descriptive approaches, despite the fact that much current research involves quantitative modeling. I'll describe an alternative, an intermediate-level course now embodied in a new edition of the textbook Physical Models of Living Systems. The course is the foundation of Penn's biophysics undergraduate major, as well as a response to rapidly growing interest among students in a broad range of science and engineering majors. Students acquire several research skills that are often not addressed in traditional undergraduate courses. The combination of experimental data, modeling, and physical reasoning used in this course represents a new mode of "how to learn" for many of our students. These basic skills are presented in the context of case studies from cell biology, including: •Mechanobiological morphogenesis in chick embryo; •Virus dynamics in single patients, and in populations; •Bacterial genetics and evolution of drug resistance; •Statistical inference, with applications to superresolution microscopy and cryo-EM; •Catch bonding in immune cell receptors; •Synthetic cell circuits. Outcomes include student reports of improved ability to gain research positions as undergraduates, and greater effectiveness in such positions, as well as students enrolling in more challenging later courses than they would otherwise have chosen. [NSF CMMI–1548571, PHY--2210452.]

B566/P1128
Small Publications, Big Impact: microPublications as a Path to Scholarship for PUI Faculty and Their Undergraduates
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The Cell Biology Education Consortium: Path to Publication (CBEC:P2P) is a collaborative research network designed to help faculty at primarily undergraduate institutions (PUIs) publish their work in peer-reviewed journals. Recognizing the challenge of balancing research productivity with heavy teaching loads, CBEC has developed modular resources (“Cell Blocks”) that integrate cell culture–based research into the classroom. Although course-based undergraduate research experiences (CUREs) produce valuable data, generating sufficiently robust and reproducible datasets for full-length manuscripts remains a common obstacle. To address this gap, CBEC:P2P leverages the collective strength of its network through a triage-style system that supports faculty and students in preparing single-figure manuscripts for submission to microPublication Biology. The structured workflow, hosted on QUBES, guides participants through each stage of the process:

submission of a draft to network leaders, iterative feedback and resubmission, and final submission to the journal. Publication costs are covered by CBEC funding. To date, nine manuscripts have been submitted and reviewed within the CBEC network, with seven officially submitted to microPublication Biology (four accepted and three under review). This process has established a clear and supportive pathway to publication, enabling faculty and students to transform classroom-based research into peer-reviewed contributions to the field. Importantly, because microPublication Biology is PubMed searchable and recognized by both the NIH and NSF, publications in this journal can also strengthen faculty advancement and professional development.

B551/P1823
The (sci)art of microscopy
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Historically, many great scientists were also great artists and vice-versa. However, with the advent of modern science came a dichotomy between art and science. Yet biology leaves a lot of room for art: drawing and illustrating is an integral part of the learning process, and that was an important (if long unconscious) factor in my decision to become a biologist. Cell biologists know all too well that microscopy images and videos do not need to be pretty to answer biological questions, and trying to make them beautiful can often be fatal to your experiment. Yet as a microscopist, I now have the chance to work constantly at the intersection of science art and to bridge them again. And generating beautiful images can often open unsuspected avenues in your research, and help reach out to a bigger audience and communicate about science.

B552/P1114
The Council on Undergraduate Research (CUR) Biology Division: A Dynamic Community Promoting Best Practices in Undergraduate Biology Education
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The life sciences have long been a fertile testing ground for developing best practices in undergraduate research. The Biology Division of the Council on Undergraduate Research (CUR) has worked to serve as both an agent of change for the development and implementation of such best practices and a clearinghouse for the dissemination of new and exciting trends in undergraduate research to the life sciences community. In this presentation, we describe the current work of the Biology Division to meet these goals. The Division's projects include active award programs to assist students in being able to purchase supplies for research projects and travel to professional meetings to disseminate their research, a professional development program for the integration of course-based undergraduate research experiences (CUREs) into the undergraduate classroom, a recognition program for outstanding research mentors, and an outreach initiative for maximizing the spread of Division work to as wide of an audience as possible. For each initiative, Biology Councilors are tasked with using appropriate assessment tools to maximize the benefits that they bring to the undergraduate life science education community. We encourage our colleagues in the life sciences to consider becoming a Division Representative and joining a dynamic and creative group dedicated to bringing undergraduate research opportunities to all Biology students.

B557/P1119

Undergraduate research journals as a pedagogical tool for student writing and publication: The Hampden-Sydney Journal of the Sciences

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Undergraduate student research plays a vital role in the undergraduate setting, serving as both a proving ground for young academics and mentorship opporutnities for professors. Research generated from such partnerships, while often unique and valuable to the student and professor, often lacks the scope and duration necessary to submit to traditional scientific journals. To provide more opportunity for undergraduate publication as well as expose students to the scientific publication process, college-run science journals can prove to be an excellent platform for young academics. Students submitting research to such journals gain invaluable experience in proper scientific writing as well as a publication venue for their scientific achievements to be displayed. Meanwhile, student editors gain rare experience in the construction, editing, and management of such a journal. To emphasize the unique community of students found at each distinct institution, college research journals can also highlight cross-departmental initiatives and collaborations, yielding novel articles which will entice readers of all disciplines. Here, we discuss our experiences in the publication of The Hampden-Sydney Journal of the Sciences, a journal showcasing student research across all STEM disciplines at Hampden-Sydney College. In publication since 2012, the 2026 edition of the Journal also happens to coincide with the 250th anniversary of the College, making this a rare opportunity to further expand collaborative efforts with Hampden-Sydney's Social Science and Humanities Departments to generate novel content. Such strategies employed in the creation of the Journal will be discussed, including recent products and results, to aid and inspire other institutions to attempt similar projects.

B554/P1116

Undergraduates' sense of belonging in science is enhanced by a curriculum on preprint peer review

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Undergraduates are taught that manuscript peer review is integral to science. Yet, they also learn that undergraduates are not invited to participate in the peer review process, creating an implicit sense of exclusion from the scientific community. Few training opportunities exist in manuscript peer review and none are designed for undergraduates (McDowell et al., eLife, 2019). We envision flipping this paradigm: teaching students how to peer review manuscripts could be used as a tool to enhance undergraduates' sense of belonging in science (McDowell et al., MBoC, 2021). Intentional engagement in the peer review process would unmask part of STEM's hidden curriculum, the untaught knowledge required for success in STEM which disproportionately hinders minoritized students. With NSF support, we created and evaluated a curriculum in which biology undergraduates write and publish their own peer reviews of preprints. By authentically reviewing works-in-progress created by practicing scientists, undergraduates meaningfully contribute to the scientific literature and community. As a result, we hypothesize that our preprint peer review curriculum enhances students' science identity and sense of belonging (McDowell et al., Learned Publishing, 2022). To test this hypothesis, we used validated pre-post surveys and thematic analysis to measure changes in identity formation as a result of the curriculum. At previous CellBio meetings, we presented results that the curriculum increases students' scientific literacy (now published as Otto et al., JMBE, 2023). Here, we report that students'

sense of identity as a scientist also increases significantly as a result of the curriculum ($p < 0.0001$ by RM ANOVA), with a particular impact on enhancing identity prominence and self-efficacy. Gains in science identity formation are most significant in students with minoritized racial and ethnic identities (e.g. BIPOC, Hispanic students). We demonstrate that these gains are transferable to different instructors, persist across diverse educational settings (a liberal arts college, a land grant university), and can be adapted to introductory, intermediate, and advanced courses (100- through 400-level). The curriculum will be freely available through an open educational resource (Fankhauser, in prep). By enhancing students' identity as scientists, this peer review curriculum has the potential to broaden participation in STEM and prepare students for success in the scientific workforce.

B555/P1117

Using Science Practices to Enact Vision and Change in the Introductory Biology Laboratory

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The three-dimensional (3D) learning model for science teaching and learning, which was introduced by the Framework for Science Education and Next-Generation Science Standards (NGSS), includes guidance towards teaching science practices in biology. The science practices embody both content knowledge and skills, describing activities that scientist participate in their work. As such, science practices are generally aligned with the core competencies described in the Vision and Change and could be used to scaffold learning activities toward those core competencies. We used the 3D-Learning Assessment Protocol (3D-LAP), which defines criteria required for each science practice, to evaluate the extent to which introductory biology laboratory course series have the potential to engage students in science practices. Using the 3D-LAP, we found that 24/25 of the lab sessions we looked at had the potential to engage students in at least one science practice, with a total of 152 instances over the three courses. Across all three courses we found that there was a strong emphasis on Analyzing and Interpreting Data (80% of sessions), Constructing Explanations and Engaging in Argumentation Using Data (64% of sessions), and Engaging in Argument from Evidence (64% of sessions), with others at lower frequencies. However, three practices–Planning Investigations, Communicating Information, and Defining Problems/Designing Solutions–were not identified in any of the biology laboratory sessions. We also found that potential for students to engage in science practices would increase by 89% if activities that failed to meet just one 3D-LAP criterion ('near misses') were modified to meet them all. Moreover, if these 'near misses' were available to students, they would have the opportunity to engage in two otherwise absent science practices. Our analysis provides insight into how instructors can align introductory-level, course-based laboratory work with the criteria in the 3D-LAP. Through practices-focused curricula, students can gain technical facility and participate in science practices that build towards the course level and curriculum level outcomes described in Vision and Change's core competencies and refined in the BioSkills guide.

B558/P1120

Work Design in Undergraduate Research: Links Between Task Characteristics, Social Characteristics, and Student Development

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Background: Undergraduate Research Experiences (UREs) are a powerful way for students to learn, but we know less about how the day-to-day setup of the work supports positive outcomes. Using Job Characteristics Theory (JCT), we asked how various features of work design of URE affect positive outcomes, such as the development of science self-efficacy and science identity, understanding of scientific process, and the development of scientific and other skills. Methods: We held cognitive interviews with 18 undergraduates, including bilingual students, to check that items adapted from the Work Design Questionnaire were clear and relevant and to hear how students made sense of them. Those insights shaped a field survey completed by 130 URE participants. We measured task characteristics (e.g. autonomy, task significance, and task variety), along with social characteristics (feedback and support) and previously characterized URE outcomes, such as development of science identity, science self-efficacy, research skills, and understanding science. Results: Across methods, more social support and feedback, higher autonomy, greater task significance, and more task variety were linked to stronger engagement and self-reported skill growth. Students described both bright spots and pain points: opportunities to build analytical and problem-solving skills contrasted with repetitive work and projects whose purpose or broader context felt unclear. The survey mirrored these themes; social support and mentor's feedback correlated with a wide range of positive outcomes. Conclusions: Viewing UREs through JCT clarifies how everyday task design shapes motivation and performance. Small, targeted changes—explaining the "why" behind projects, increasing variety and appropriate autonomy, and giving timely, informative feedback—can boost learning and satisfaction and create more inspiring research settings. We offer concrete, practice-oriented recommendations to help mentors and program leaders improve URE quality at scale. We are also looking to partner with programs that are interested in assessment and formative feedback for their undergraduate research experiences. This study was supported by the National Science Foundation under IUSE Award #2142404. Any findings, conclusions, or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of NSF.

Science Education - Tools and Technology for Science Education

B570/P1132
Democratizing science through low-cost microscopy: lessons from rural schools
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Access to meaningful science education remains an urgent challenge in rural and low-resource communities worldwide, including Peru, where schools often lack laboratories, materials, and teacher training for hands-on learning. The objective of this project was to democratize access to science by integrating Foldscope, a low-cost paper microscope, into classrooms through teacher professional development. In 2024, in-person workshops were delivered to 101 teachers serving rural public schools in high-poverty regions of Peru. Each 3.5-hour session included Foldscope assembly, sample preparation, and strategies for curricular integration aligned with national standards. Teachers received microscopes, slides, and activity guides, and ongoing support was provided through regional networks that encouraged sharing of experiences and classroom evidence. Pre- and post-training surveys measured prior exposure to microscopes, frequency of classroom use, confidence levels, and perceived pedagogical value. Results showed that one-third of teachers had never used a microscope, and nearly four out of five reported rarely or never using them in class. After training, over 90 percent of participants reported confidence in using Foldscope, and 78 percent indicated improved teaching strategies. Confidence scores rose significantly ($M = 3.64$ pre vs. 3.97 post, $p < 0.001$). Teachers rated the workshops highly, with 87 percent assigning scores of 9 or 10 out of 10. Within weeks, classrooms documented students examining insect wings, plant tissues, fibers, and rainwater, often for the first time. Approximately 2,900 students directly engaged in microscopy activities that fostered curiosity, observation, and critical

thinking. This study shows that frugal technologies, when combined with targeted training and support, can help close long-standing gaps in science education. The Foldscope intervention provided an affordable tool and positioned teachers as agents of change by equipping them to deliver experiential science without laboratories or specialized infrastructure. While implemented in Peru, the model offers a replicable framework adaptable to rural and resource-limited settings globally. By enabling authentic and locally relevant scientific inquiry, low-cost microscopy advances educational equity and nurtures future scientists.

B571/P1133

From Pixels to Partnerships: Navigate Your BioImaging Journey with BINA

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Visualizing biological structures and processes at the cellular, tissue, or organismal level is crucial to advancing knowledge and making discoveries in the health and life sciences. Many institutions lack dedicated bioimaging platforms; however, even when the equipment is available, support mechanisms for the dedicated staff who enable advanced imaging research are insufficient. BioImaging North America (BINA), established in 2018, addresses this gap by fostering a vibrant, inclusive community that connects research scientists and other professionals within Canada, the USA, Mexico, and beyond. With over 1800 members, BINA offers a range of professional development initiatives, including educational webinars, community-led working groups, and funding for microscopy and image analysis courses. The organization also provides space for discussions across various imaging specialties. These efforts ensure opportunities for engagement at all career stages and technical interests and across key interest groups, including industry, publishers, and government. BINA continues to expand its community and grow partnerships with global and continental organizations, connecting, empowering, and giving a unified voice to the global bioimaging community. BINA membership is free and open to all. We invite cell biologists and imaging professionals to explore how BINA can support their work and connect them with the broader imaging ecosystem. Please visit us at: https://www.bioimagingnorthamerica.org/ to learn more!

B569/P1131

microPublication Biology: a novel scientific publication platform

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microPublication Biology publishes brief scholarly reports of research findings based on data presented in a single multi-panel figure. The journal is Open Access, manuscripts are peer-reviewed, articles are PubMed-indexed, and for subject areas associated with databases (e.g., ZFIN), the content is curator-vetted. The goals of the journal include (1) increasing the entry of peer-reviewed findings into the public domain, meeting authors' obligation for taxpayer-funded research; (2) providing a peer-reviewed publication platform for research conducted by undergraduates; and (3) enabling researchers who conducted the study and wrote the scholarly article to receive credit. Manuscripts undergo a rigorous peer review for the work being scientifically sound, the conclusions supported by the data, and being well-written. This is facilitated by the manuscripts

being brief and thus reducing reviewer burden. The journal publishes novel findings, new methods, and reagents. Unlike many other journals, negative results, replications that are successful (including being scooped), and replications that are unsuccessful, are also published. Studies can be observational or interventional/mechanistic. A few examples of situations to micropublish include (a) findings that are of value to the community now, but the project it is part of won't be published for a year or more; (b) concrete findings, but the project was discontinued or tangential to current work; (c) solid findings, but the student or postdoc has left the lab and it is not clear if work on the project will be reinitiated; and (d) findings from a well-designed, circumscribed undergraduate project. microPublication Biology has published more than 1400 articles thus far, including contributions from authors who are Nobel laureates, National Academy of Sciences members, and Howard Hughes Investigators.

B572/P1824
Molecular Animation: Past, Present, and Future
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In recent years, there has been a rapid growth in the use of animation as a means to communicate complex biological processes to a wide range of audiences. My group and others have seen that 3D animation software can be used to synthesize spatial and dynamic information from a variety of experimental modalities, and that this process can provide important scientific insights and provide a contextualized view of how molecular and cellular systems operate. These visualizations have served not only to make molecular concepts more accessible to students and the public, but have also proven to be extremely useful for researchers seeking to build and refine their hypotheses. In this talk, I will describe projects that have used animation to communicate and explore different topics in cell biology and discuss new tools my group is developing to enable molecular biologists to create, share, and annotate animated three-dimensional models of molecules.

Signal Transduction - Intracellular signaling pathways and second messengers

B455/P0439
A PPP1Cß-PPP1R14a FLARE Biosensor Demonstrates PKC-Driven Phenotypic Changes in Prostate Myofibroblasts
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Benign prostatic hyperplasia (BPH) and lower urinary tract symptoms (LUTS) are associated with abnormal contraction of prostate stromal cells, a process regulated in part by protein phosphatase 1 (PP1c) and its regulatory subunits (PPP1Rs). PPP1R14a inhibits PP1c activity when phosphorylated by protein kinase C (PKC), yet the dynamics of this regulation in living cells remain poorly defined. To address this, we developed a FLARE biosensor co-expressing PPP1Cβ–YFP and PPP1R14a–CFP in WPMY-1 and expressed this new biosensor prostate myofibroblasts, enabling real-time monitoring of protein–protein interactions by FRET microscopy. As an initial positive control, we used the phorbol ester PMA, a well-established PKC activator that phosphorylates PPP1R14a and enhances its binding to PP1c. Consistent with expectation, PMA stimulation induced a robust increase in FRET, indicating dynamic association between PPP1Cβ and PPP1R14a. Unexpectedly, overexpression of these proteins caused rapid contraction and, in some cases, cell death following PMA treatment. These findings suggest that altering the stoichiometry of PP1c and PPP1R14a sensitizes stromal

cells to acute cytoskeletal stress. Ongoing experiments are testing whether these responses arise from PPP1Cβ catalytic activity, PPP1R14a regulation, or both, and whether dihydrotestosterone or estradiol similarly influence PP1c–PPP1R interactions. We demonstrate successful construction and validation of a PPP1Cβ–PPP1R14a FLARE biosensor in prostate myofibroblasts. Importantly, our data reveal that expression levels of PPP1Cβ and/or PPP1R14a profoundly alter cellular responses to PKC activation, providing a new platform for dissecting phosphatase regulation in hormone- and kinase-responsive signaling contexts.

B457/P0441

Characterization of RNF111 as a new positive regulator of Wnt/beta-catenin signaling

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The canonical Wnt signaling pathway plays a critical role in developmental processes and is frequently dysregulated in human diseases, including cancer. However, there are no FDA-approved inhibitors of this pathway due to incomplete knowledge of its regulatory mechanisms and a lack of druggable targets. A crucial step in Wnt pathway activation involves inhibition of the beta-catenin destruction complex, which, in the absence of Wnt ligand, promotes ubiquitin-mediated degradation of beta-catenin and prevents Wnt target gene transcription. Besides beta-catenin, many key Wnt components are regulated by ubiquitylation at each step in the pathway. Thus, identifying new ubiquitin ligases in Wnt signaling will provide important insight for better understanding the pathway's regulatory mechanisms and developing drugs for treatment of Wnt-driven diseases. We conducted CRISPR/Cas9 screens using a curated Mouse Ubiquitinating enzymes-Deubiquitinases-Phosphatases CRISPR Library, monitoring changes in beta-catenin levels by FACS, followed by deep sequencing. From this screen, we identified RING finger protein 111 (RNF111)/Arkadia, a SUMO-directed E3 ligase essential for embryonic development and TGF-beta signaling, as a new positive regulator of Wnt signaling. Our follow-up studies demonstrate that RNF111 depletion inhibits Wnt signaling in human cell culture and causes heart malformations in zebrafish. Previously published results have demonstrated that RNF111 interacts with Axin, a component of the destruction complex that mediates turnover of beta-catenin, the primary effector in the Wnt pathway. Through co-immunoprecipitation studies, we demonstrated that, in addition to Axin, RNF111 also interacts with the kinase GSK3 and with beta-catenin. Further, we show that RNF111 remains localized primarily in the nucleus upon Wnt stimulation and interacts with the Wnt nuclear components TCF4 and TLE3. Together, these data indicate that RNF111 is a conserved positive regulator in the Wnt pathway, and we hypothesize that it stimulates Wnt signaling through ubiquitylation of nuclear pathway components. Determining RNF111's mechanism of action is integral for better understanding the role of ubiquitylation in regulating the Wnt signaling pathway and for identifying a previously undescribed link in the complex cross-talk between the Wnt and TGF-beta pathways.

B456/P0440

Characterization of the STK38 kinase in wnt signaling and cardiac development

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Precise coordination of Wnt signaling dynamics is essential for heart development and its dysregulation underlies many congenital heart defects (CHDs)—the most prevalent class of birth anomalies. We have

identified the serine/threonine kinase STK38 as a novel and evolutionarily conserved regulator of Wnt signaling, with pivotal functions in cardiogenesis across drosophila, zebrafish, mice, and mammalian systems. Loss-of-function studies in zebrafish reveal that STK38 is essential for proper cardiac morphogenesis, positioning it as a critical node in Wnt-dependent developmental processes. In human cells, STK38 depletion leads to a marked reduction in Wnt/β-catenin reporter activity and downstream target gene expression, acting downstream of the β-catenin destruction complex. These findings point to a functional role for STK38 at the transcriptional level, beyond the cytoplasmic signal cascade. Mechanistically, co-immunoprecipitation and mass spectrometry uncover a direct interaction between STK38 and the transcriptional co-repressor TLE3, suggesting that STK38 may modulate nuclear Wnt signaling output by influencing repressor–activator dynamics within the Wnt transcriptional complex. Ongoing work leverages induced pluripotent stem cells (iPSCs) to map STK38 expression and function across stages of cardiac differentiation. Through temporally controlled depletion, we aim to define critical windows of STK38 activity and its role in directing Wnt-driven transitions from progenitors to differentiated cardiomyocytes. Together, our findings illuminate a previously unrecognized nuclear function of STK38 in shaping Wnt signaling outcomes during heart development. This work provides a new lens through which to understand cell fate dynamics in cardiogenesis and opens the door to novel therapeutic strategies for CHDs.

B445/P0429

Chronic exposure to IL-13 alters calcium and ciliary dynamics in human nasal epithelial cells through activation of IL-13Ra2

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Persistent Th2 inflammation is a major contributor to the pathophysiology of chronic airway diseases like asthma and chronic rhinosinusitis (CRS). IL-13, a Th2 cytokine, induces airway hyperresponsiveness, goblet cell hyperplasia, and increased MUC5AC mucus secretion in airway epithelial cells. Because these effects have been attributed to the canonical heterodimeric IL-13 receptor formed by the IL-13Rα1 and IL-4Rα subunits, much of the therapeutic development effort has focused on blocking the IL-13Rα1/IL-4Rα receptor using monoclonal antibodies, such as dupilumab targeting IL-4Rα. Here we further investigated the mechanistic effects of chronic exposure to IL-13 on nasal epithelium cell composition, intracellular calcium dynamics, and cilia function. We initially hypothesized that Th2 airway hyperresponsiveness might be driven by increased calcium signaling. Primary human nasal epithelial cells were treated with IL-13 for 14 days during differentiation at the air-liquid interface. IL-13-induced modifications in epithelium cell composition, gene expression, and intracellular calcium dynamics were assessed by immunohistochemistry, quantitative PCR, and live cell fluorescence imaging, respectively. Cilia beat frequency was measured by high-speed imaging. As expected, exposure to IL-13 resulted in goblet cell hyperplasia associated with increased MUC5AC gene expression and a reduction in the ciliated cell population. Surprisingly, cytosolic calcium responses after treatment with various stimuli were consistent with a decrease in ER calcium stores. In addition, the cilia lost their sensitivity to stimulation by calcium elevation. Blockade of IL-13Rα1/IL-4Rα failed to neutralize these effects. However, specific inhibition of ERK restored healthy cytosolic calcium responses and cilia sensitivity to calcium in the presence of IL-13. The non-canonical IL-13 receptor IL-13Rα2, once considered a mere decoy receptor, has been linked to ERK. Gene expression of IL-13Rα2 was increased with chronic IL-13 stimulation, supporting its involvement in IL-13 signal transduction. Our results suggest that, in the upper airways, chronic IL-13 signaling involves IL-13Rα2, rather than solely IL-13Rα1/IL-4Rα. IL-13Rα2 activation involves downstream activation of ERK. Thus, reducing the detrimental effects of IL-13 in diseases like CRS may require blocking IL-13Rα2 signaling in addition to IL-13Rα1/IL-4Rα.

B441/P0425
CSF1R-CAR T cells induce CSF1R signalling and promote cancer cell growth
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Chimeric antigen receptor (CAR) T cells have revolutionised cancer immunotherapy and show tremendous success in treating relapsed/refractory blood cancers, however the interaction and effects of CAR T cells on their targets remains unclear. Most research has focused on optimising CARs for effective T cell activation, largely ignoring any impact or response the targeted tumours may have. Here, we use LC-MS/MS based phophotyrosine (pY) proteomics to evaluate the signalling capacity of a second generation CSF1-targeted CSF1R-CAR and a CD19 scFv-targeted CD19-CAR in Jurkat T cells. We find that coculture of CSF1R-CAR T cells with CSF1R-expressing THP1 cells induces CSF1R-like pY signalling in THP1 cells, whereas coculture of CD19-CAR T cells with Raji cells show no response in Raji cells. Using small molecule inhibitors of CSF1R, Lck, and actin polymerisation, we show that the kinase activity of CSF1R, and not Lck activity or actin polymerisation in T cells, is necessary for CSF1R-CAR induced CSF1R phosphorylation. Further, primary human CSF1R-CAR T cells induce growth of THP1 cells at low effector-to-target (E:T) but prevent THP1 growth at high E:T ratios. Together, our data demonstrate an unintended consequence of CARs, CAR-induced signalling and growth in cancer cells, which is important to evaluate when designing new CARs.

B442/P0426
Decoding Amino Acid Sensing: Cryo-EM Structures of Sensors Interacting with the Human GATOR2 Complex
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Mammalian cells regulate growth by integrating environmental cues through the mTORC1 signaling pathway. The human GATOR2 complex plays a central role in regulating mTORC1 activity. Under conditions of amino acid deprivation, GATOR2 is inhibited through interactions with the cytosolic leucine sensor Sestrin2 and the arginine sensor CASTOR1. When amino acids are abundant, this inhibition is relieved, enabling GATOR2 to antagonize the repressor GATOR1 and activate mTORC1 signaling. Despite its key regulatory role, the precise mechanisms of GATOR2 inhibition by amino acid sensors have remained unclear. We determined cryo-electron microscopy (cryo-EM) structures of GATOR2 in three inhibitory states: bound to CASTOR1, bound to Sestrin2, and simultaneously bound to both sensors. Additionally, hydrogen-deuterium exchange mass spectrometry (HDX-MS) highlights dynamic structural motions in apo-GATOR2 and its complexes with amino acid sensors, as well as the conformational effects of amino acid supplementation. These findings provide key insights into the molecular interactions between GATOR2 and amino acid sensors, offering a comprehensive perspective on how nutrient-sensing machinery regulates the mTORC1 pathway. By elucidating the structural basis of GATOR2 inhibition and its release, this study advances our understanding of mTORC1 signaling and its role in cellular growth regulation.

B461/P0445
Epac inhibitor ESI-09 increases TRP channel Ca2+ influx induced by bitter receptor agonist denatonium

benzoate in airway epithelial cells

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Inflammatory respiratory diseases like asthma and chronic rhinosinusitis (CRS) are common diseases worldwide, resulting in significant public health burdens. CRS itself affects 11-16% of adults globally – in the United States this results in >$10 billion in direct healthcare costs per year and ~20% of antibiotics prescribed to U.S. adults annually. CRS continues to be a public health burden and exacerbates the growing antibiotic resistance crisis, emphasizing a need for alternative therapeutics. One alternative therapeutic strategy is to leverage the airway's innate immune system. Airway epithelial cells (AECs) are the airway's first line of defense and initiators of innate immune responses. Calcium (Ca2+) and cAMP are master regulators of innate immune responses in AECs downstream of GPCRs, and Ca2+ is a key factor in several innate immune responses. Better understanding how to manipulate AEC Ca2+ signaling may prove useful in reducing Ca2+-dependent inflammatory responses or enhancing Ca2+-driven innate immune responses. One downstream effector of cAMP, Epac (Exchange protein directly activated by cAMP) can regulate Ca2+ signaling in other cell types. The role of Epac in AEC Ca2+ signaling has not been investigated. We found that the Epac1 isoform was the predominantly expressed isoform in AECs and nasal tissue, and Epac1 expression was not affected by CRS disease status. We initially tested the effects of pan-Epac inhibitor ESI-09 on Ca2+ signaling in two AEC lines – 16HBEs and CFBEs. We found that ESI-09 pre-treatment significantly reduced Ca2+ responses to a variety of AEC GPCR agonists, including bitter taste receptor T2R14 and purinergic receptor P2Y2R agonists. This is likely due to a substantial decrease in ER Ca2+ stores. However, the Ca2+ response to denatonium benzoate, a bitter taste receptor T2R4 agonist, was significantly increased after ESI-09 pre-treatment. Our data suggests this increased response is due to enhanced Ca2+ influx through TRP channels, independent of the classic Orai/STIM store operated calcium entry model AECs typically utilize. Thus, Epac-targeting drugs may be able to differentially modify Ca2+ signaling through multiple mechanisms that we continue to elucidate.

B451/P0435

ERK signaling dynamics and alveolar epithelial cell fate

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The extracellular signal-regulated kinase (ERK) pathway regulates several cellular functions including proliferation, survival, and differentiation. Given its ubiquity, the specific cellular outcome downstream of ERK signaling depends greatly on the dynamics of ERK activity. The alveolar epithelium of the lung is important for gas exchange and consists of two types of alveolar epithelial cells — type 1 or AT1, and type 2 or AT2 — that are both derived from a common bipotent progenitor (BP) cell. Activation of ERK has been shown to promote differentiation of BP cells into AT2 cells and to prevent differentiation into AT1 cells. Paradoxically, however, ERK signaling is still required for survival of the latter. These findings suggest that the differences between differentiation into AT1 and AT2 cells may lie in the dynamics rather than the magnitude of ERK signaling. Here, we use a fluorescent ERK kinase translocation reporter (ERK-KTR) to track the dynamics of ERK signaling in real time as both mouse embryonic stem cell (mESC)-derived and primary BP cells differentiate into AT1 or AT2 cells. KTR localizes to the cytoplasm when ERK is active and to the nucleus when ERK is inactive. We isolated primary BP cells from ERK-KTR-expressing transgenic mouse embryos at embryonic day 16.5 and treated them with growth factors to induce differentiation. We performed time-lapse imaging to track the dynamics of KTR localization in BP cells with and without growth factors, and calculated the KTR cytoplasmic-to-nuclear (C/N) ratio as a measure of ERK activity, from which we quantified its amplitude, frequency, and duration. We used immunofluorescence to confirm expression of MUC1 (AT2 marker) and PDPN (AT1 marker) at the end of each

experiment in order to correlate ERK dynamics to final differentiated fate. We found that the baseline ERK activity in BP cells is pulsatile, but once BP cells are stimulated with ligands that induce differentiation, ERK activity loses its pulsatility and becomes static. We are currently implementing optoSOS, an optogenetic tool, to mimic the temporal patterns of ERK signaling in BP cells and to test whether these dynamics are sufficient to control alveolar epithelial cell fate. We anticipate that the principles uncovered by our work will be broadly applicable to other tissues and organoid models.

B460/P0444

Identifying the subcellular compartment(s) that serves as the major signaling platform for PDGFR homodimers and heterodimers

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Craniofacial development is a complex morphogenetic process, disruptions in which result in prevalent human birth differences. Signaling through the platelet-derived growth factor receptor (PDGFR) family of receptor tyrosine kinases (RTKs) plays critical roles in this process in humans and mice. Pdgfra mutant mouse models display midline clefting, subepidermal blebbing and hemorrhaging, due to aberrant cranial neural crest cell (NCC) migration and defective proliferation of the NCC-derived craniofacial mesenchyme at mid-gestation. Conditional ablation of Pdgfrb in the mouse NCC lineage results in increased nasal septum width, delayed palatal shelf development and subepidermal blebbing, primarily due to decreased proliferation of the craniofacial mesenchyme past mid-gestation. PDGFRα and PDGFRβ can homodimerize or heterodimerize in response to PDGF ligand binding, unleashing an intracellular signaling cascade. We recently adapted a bimolecular fluorescence complementation (BiFC) approach that enabled us to visualize and purify individual PDGFR dimers in vitro, revealing differences in the timing and extent of dimer activation, signal molecule binding, internalization, trafficking and downstream signaling. Moreover, our results supported an emerging theme in the RTK field that these receptors signal at sites other than the plasma membrane and require internalization to maximally activate downstream signaling. Here, we will identify the subcellular compartment(s) that serves as the major signaling platform for the various PDGFR dimers using biologically-relevant primary mouse embryonic palatal mesenchyme (MEPM) cells derived from embryos in which the stop codon of the endogenous Pdgfra and/or Pdgfrb locus was replaced with a BiFC fragment(s). These studies will provide significant insight into the mechanisms underlying the spatial and temporal regulation of RTK signaling during mammalian craniofacial development.

B452/P0436

Interleukin-10 Secretion in Lipopolysaccharide-treated RT4-D6P2T Schwann Cells

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Schwann cells have been found to play a key role in inflammation and repair following nerve injury. During nerve injury, the myelin sheath becomes damaged, stimulating Schwann cells to secrete cytokines and initiate an inflammatory response. They begin the process of repair by stimulating macrophages to make pro-inflammatory cytokines, such as Tumor Necrosis Factor-Alpha (TNF-a), and anti-inflammatory cytokines, such as Interleukin-10 (IL-10), which promotes healing and injury. Although it is clear that Schwann cells play a role in nerve repair, the exact mechanisms are unknown. In vitro, lipopolysaccharide (LPS) can induce inflammation and stimulate the production of TNF-α and IL-10. Reports on LPS-stimulated microglial cells treated with

forskolin at various time points revealed an increase in IL-10 mRNA levels. A similar study conducted on LPS-treated RT4-Schwann cell lines that were stimulated with forskolin for 3 hours resulted in the secretion of TNF-α. To further explore the effects of forskolin in LPS-stimulated Schwann cells, the role of IL-10 secretion was examined. It was hypothesized that IL-10 secretion will be increased in LPS-treated Schwann cells and higher in LPS-treated Schwann cells supplemented with forskolin. Cells from the RT4-D6P2T Schwann cell line were treated with 0.1,1, or 10 µg/mL of LPS, or 1, 2, or 3 µM forskolin (F), or combinations of LPS and F treatments for 3 and 24 hours in growth media (N2). Cell culture supernatants were collected and assayed for IL-10 secretion using ELISA. Secretion of IL-10 was augmented by forskolin in LPS-treated cells when compared to LPS or forskolin treatment only. For instance, IL-10 secretion was increased at 1 µg/mL of LPS with all treatments of forskolin at 24 hours (404 + 231.3%, 586 + 226.2%, 474 + 299.1%). Surprisingly, cells treated with 0.1 µg/mL of LPS for 3 hours secreted more IL-10 when stimulated with forskolin at concentrations of 1 µM (155.85+16.85%) and 3 µM (140.1+37.23%) in contrast to 24 hours (126.8+24%, 135+35.8%). In summary, it appears that LPS and forskolin may act in unison to upregulate IL-10 secretion. Moreover, these findings suggest that, during nerve injury, the ability of the cAMP pathway to counteract inflammation by secreting IL-10 in LPS-stimulated Schwann cells is dependent on concentration and time. A better understanding of the balance between pro- and anti-inflammatory cytokines during nerve injury may potentially unlock the mechanisms that mediate these pathways.

B462/P0446

Investigating the role of FTT-2, a conserved 14-3-3 protein, in smooth muscle contraction in C. elegans

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Proper regulation of cell contractility is essential for regulating various biological systems, including the respiratory, digestive, and cardiovascular systems. In the hermaphroditic nematode C. elegans, the reproductive system includes a contractile tube of myoepithelial cells called the spermatheca. This structure stores sperm and serves as the site for oocyte fertilization. Controlled contraction of the spermatheca facilitates the embryo's movement into the uterus. Cell contractility in the spermatheca relies on actin and myosin. It is also, in part, controlled by Ca2+ signaling through the phospholipase PLC-1, which facilitates the release of Ca2+ from the endoplasmic reticulum. Previous research has demonstrated the involvement of the heterotrimeric G-protein alpha subunit, GSA-1/Gαs, and protein kinase KIN-1/PKA-C in ovulation and Ca2+ release. However, the upstream regulators of these pathways remain unidentified. The spermatheca is not under neuronal control. So, how do the spermathecal cells know when to contract? To identify novel regulators of spermathecal contractility, we performed a candidate RNAi screen to look for genes that caused an increase in the number of occupied spermathecae. One of the candidate genes, ftt-2, is one of the two putative 14-3-3 encoding genes in C. elegans. The 14-3-3 proteins have been implicated in a growing number of cellular biology processes; however, their involvement in regulating cellular contraction remains largely unknown. Therefore, studying the FTT-2 gene may reveal a new function of the 14-3-3 protein.

B453/P0437

MS4A8B regulates Ca2+ signaling in small cell lung cancer.

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Small cell lung cancer (SCLC) is a neuroendocrine cancer that comprises approximately 15% of lung cancer diagnoses. It is a high-grade cancer considered one of the most aggressive types due to high relapse rates and metastatic potential. SCLC can be divided into different genetic lineages characterized by key transcription factors. ASCL1 is the primary transcription factor for SCLC subtype A that is the most prevalent. Nonetheless, the SCLC heterogeneity and short life-expectancy highlight the need to resolve molecular mechanisms specific to SCLC subtypes that may be used to propose better biomarkers and preclinical therapies. We surveyed differential gene expression analysis from SCLC cells of patient donors and immortalized cell lines to find the MS4A8B gene is among the most highly correlated genes to ASCL1. MS4A8B is a member of the MS4A family that are putative chemoreceptors and hypothesized to regulate Ca2+ transport. A clear understanding on the molecular mechanisms underpinning these functions has not been elucidated, especially in the context of SCLC. We used siRNA knockdown methods to test MS4A8B's role in Ca2+ signaling and found Ca2+ responses to agonists such as carbachol were diminished in siMS4A8B cells compared to non-targeting siRNA. We further ectopically over-expressed MS4A8B that demonstrated a potentiation of store-operated Ca2+ entry. Co-immunoprecipitation experiments revealed protein-protein interactions with Orai 1. We propose that MS4A8B is a central regulator of Ca2+ homeostasis that offers a target to kill SCLC cells.

B454/P0438

Negative feedback regulation by the scaffold Sms1 restricts MAPK signaling at the plasma membrane

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The three-tiered MAPK signaling cascade is one of the best characterized and most conserved in evolution. MAPK cascades convert a plethora of stimuli into context-dependent outputs, for which key factors are MAPK scaffolds that enhance signal transduction and confine signaling to specific cellular compartments. For example, the prototypical budding yeast scaffold Ste5 and the metazoan scaffold KSR anchor the MAPK module at the plasma membrane. In fission yeast, pheromone-induced MAPK signaling, orthologous to human and budding yeast, promotes sexual differentiation and orchestrates partner selection and cell-cell fusion during sexual reproduction. The MAP kinases localize to polarity sites at the plasma membrane. However, no scaffold has been identified. Using the MAP3K cofactor Ste4 as a bait for immunoprecipitation-mass spectrometry, we identified the poorly characterized protein Sms1, essential for mating, as one of its strongest interactors. Sms1 is a disordered, unstable protein that becomes stabilized at the plasma membrane. During mating, Sms1 localizes together with Ste4 and active Cdc42 at dynamic polarity patches that explore the cell periphery in search of a partner. Upon partner recognition, the Sms1 patch is stabilized at the point of contact between the two cells. This recruitment relies on interaction of Sms1 hemi-arrestin domain with both phospholipids and the pheromone-activated Gα. Sms1 further binds active MAP2K and MAPK, thus spatially restricting MAPK activity to a defined domain at the plasma membrane. We further demonstrate that phosphorylation of Sms1 by the MAPK leads to its release from the plasma membrane and terminates signaling. A phospho-mutant version of Sms1 shows a strong interaction with the membrane, even in non-mating condition, stabilizes polarity patches at low pheromone concentration and induces mating behaviours in the zygote. Thus, Sms1 is the MAPK scaffold for the pheromone-MAPK cascade in fission yeast. Similar to the structurally unrelated Ste5 and KSR, it binds all MAP kinases, recruits them to the plasma membrane and is under negative feedback by the associated MAPK, which ensures timely MAPK activity at the site of fusion between mating partners. These functional commonalities indicate that MAPK scaffolds are coopted from diverse molecular structures through convergent evolution.

B450/P0434

Novel burst-like cargo transport through tunneling nanotubes

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Cell-to-cell communications is essential for organisms to respond to stimuli and maintain homeostasis as an integrated system. Tunneling nanotubes (TNTs), recently identified in vivo as intercellular communication pathways, form bridges that connect nearby or distant cells and mediate the transfer of cargo ranging from small ions to large organelles such as mitochondria. Although an open-ended TNT structure is generally assumed to be required for material exchange, the mechanisms governing membrane opening remain poorly understood. A major challenge in studying TNT-mediated transport has been the reliance on rare, spontaneous transfer events. Here, we introduce an optogenetically engineered motor protein (OptoMotor) to actively drive cargo transport through TNTs. Unexpectedly, OptoMotors did not move continuously from donor to recipient cells. Instead, they accumulated at TNT tips for tens of minutes before abruptly crossing over in a burst. We further uncovered the molecular mechanism underlying this transfer process. These findings challenge the prevailing view that TNTs mediate cargo transport primarily through open-ended conduits, and instead point to an alternative, predominant mechanism of intercellular cargo transfer.

B446/P0430

Protons as a second messenger: pH-dependent allostery in signaling proteins

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Transient changes in intracellular pH (ranging from 6.8 to 7.6) drive various cellular behaviors. Furthermore, pHi dynamics are dysregulated in cancer, contributing to tumorigenic cell behaviors. However, the specific pH-sensitive proteins (pH sensors) responsible for these cell behaviors remain largely unknown. To identify candidate pH sensitive proteins, we developed an in silico computational pipeline that uses structural data to predict residues with pKa shifts that may function as pH sensing nodes in proteins. We applied this approach to SH2 domain-containing signaling proteins, a class of proteins that are dysregulated in cancer. Our method showed that modular SH2 proteins have predicted pH sensitive networks clustering at the interface between regulatory SH2 domains and catalytic domains. Using biophysical, biochemical, and cell biological approaches, we validated the role of pH in tuning the wild-type signaling activity of two oncogenes, c-Src and Src homology region 2 domain-containing phosphatase-2 (SHP2). At low pHi, these wild-type proteins have high activity with decreasing activity as pHi increases. Additionally, we validated the predictive power of our computational pipeline by mutating the identified residues to abrogate pH dependent activity. Importantly, when we analyzed databases of somatic mutations in cancer, we found that recurrent cancer-associated mutations in these signaling proteins cluster at these pH sensitive residues, highlighting their importance in regulating these crucial signaling proteins. Together, our computational, biophysical, and cellular analyses reveal a conserved role for modular SH2 proteins as mediators of pH-dependent signaling. Moreover, our data suggest that cancer mutations cluster in these conserved networks to abrogate pH-sensitive regulation of activity and contribute to the hyper-activation of cell signaling in cancer.

B448/P0432

Rap1B Maintains VEGFR2 Signaling by Regulating Receptor Trafficking in Endothelial Cells

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Two Rap1 isoforms, Rap1A and Rap1B, share identical effector domains that regulate endothelial adhesion yet have distinct physiological functions. The mechanisms underlying these isoform-specific effects remain unclear. Rap1B uniquely supports full activation of vascular endothelial growth factor receptor 2 (VEGFR2), a central mediator of angiogenesis, vascular permeability, and nitric-oxide production. Because the C-terminal hypervariable regions of Rap1 isoforms determine lipid interactions, and Rap1B uniquely binds cholesterol—a key lipid-raft component—we hypothesized that Rap1B controls VEGFR2 organization within plasma-membrane microdomains that govern receptor signaling, endocytosis, and turnover. VEGFR2 internalization was assessed by antibody uptake in serum-starved HUVECs treated with or without dynasore (100 µM) and stimulated with VEGF (20 ng/mL). Cells were stained for VEGFR2, pY1175-VEGFR2, and endosomal markers (EEA1, CD63, Rab11A) and analyzed by confocal microscopy; colocalization was quantified using Pearson correlation analysis. VEGFR2–VE-cadherin interactions were examined by co-immunoprecipitation, and surface and total receptor levels were quantified by biotinylation with densitometric analysis and by flow cytometry. Rap1B-deficient endothelial cells (siRap1B) showed increased VEGFR2 colocalization with EEA1 under basal conditions (0.723 ± 0.073 vs. 0.613 ± 0.077; n = 10; p = 0.021) and with CD63-positive late endosomes after VEGF stimulation (0.898 ± 0.021 vs. 0.808 ± 0.026; n = 5; p = 0.027), consistent with enhanced endosomal retention and trafficking toward degradation, while Rab11A colocalization was unchanged. Phospho-VEGFR2 intensity decreased by ~40% ($p < 0.0001$), indicating attenuated receptor activation. Surface biotinylation revealed decreased surface (12 928 ± 1 954 vs. 9 176 ± 4 182; n = 3; p = 0.23) and total VEGFR2 (0.75 ± 0.20 vs. 0.33 ± 0.22; n = 3; p = 0.07) levels. Although variability limited statistical power, results were consistent across experiments and supported by flow-cytometry data showing modest but significant reductions in surface (~7%; p = 0.0006) and total (~3%; $p < 0.0001$) VEGFR2 fluorescence. VEGFR2 - VE-cadherin association was unaffected, indicating that Rap1B does not influence VEGFR2 activity by modulating its interactions with VE-cadherin. These findings Rap1B maintains VEGFR2 signaling by preventing premature endocytosis and limiting trafficking toward degradative compartments, thereby preserving surface receptor availability. Loss of Rap1B reduces VEGFR2 abundance and phosphorylation, leading to attenuated VEGF signaling thereby sustaining receptor signaling and endothelial responsiveness to VEGF.

B449/P0433

Rapid in vitro Characterization of PKA Signal Termination Reveals Accelerated Signal Termination in Acrodysostosis Mutations

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cAMP-dependent protein kinase (PKA) functions as a master metabolic regulator and central component of the cAMP-signaling pathway. Although the regulatory and catalytic subunits of PKA have been studied extensively over many decades, fundamental questions remain about how PKA signaling is regulated in cells. In particular, there is a major knowledge gap in the phosphodiesterase (PDE) mediated termination of cAMP-PKA signaling and the critical role that ATP plays in the termination step has largely been overlooked. Impaired cAMP-signaling is correlated with a variety of disease phenotypes including Acrodysostosis, which is a rare syndrome associated with severe skeletal dysplasia. Elucidating how cAMP-signaling is terminated and the role that both

ATP and PDE play is crucial for understanding the biochemistry behind these disorders. It has previously been thought that PDEs terminate cAMP-signaling by passively hydrolyzing cAMP in proximity. However, recent studies have shown that PDEs and PKA may physically interact in vitro, suggesting a more active role. To characterize the role of ATP and PDE in stabilizing the fully inhibited, high-affinity PKA holoenzyme, we have utilized analytical gel-filtration, co-immunoprecipitation and a modified fluorescence polarization-based assay to monitor the formation of the holoenzyme over time. A spectrophotometric kinase activity assay was utilized in tandem to correlate PKA activity with holoenzyme fidelity. Our results show a synergistic effect between ATP and PDE in the stabilization of the inhibited holoenzyme and consequently the termination of PKA signaling. Furthermore, expression of point mutations which impair cAMP binding within specific cyclic nucleotide binding domains of PKA have been used as surrogates for Acrodysostosis disease mutations. Characterization of these mutations has revealed significantly accelerated PDE-driven signal-termination kinetics, indicating that mutations which reduce cyclic nucleotide binding domain dynamics may stabilize a direct interaction between PKA and PDE and provide a mechanistic explanation for the impaired PKA signaling observed in Acrodysostosis and related disorders.

B447/P0431

Revealing the Mucin-Induced Signaling Cascade in Batrachochytrium dendrobatidis Encystation

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Batrachochytrium dendrobatidis (Bd) is a chytrid fungus that infects amphibians and has caused devastating population declines worldwide. Bd has two life stages: unicellular zoospores that swim through water with a cilium and migrate across surfaces using actin, and non-motile sporangia that grow and produce new zoospores. Bd infection relies on encystation, the developmental transition from the dispersal zoospore stage to the growth sporangium stage. Encystation can be induced by host factors such as amphibian mucus or mucins within five minutes. This transition involves dramatic cellular changes, including actin cytoskeleton remodeling, cilium retraction, and cell wall synthesis. Understanding how these events are coordinated is critical to understanding Bd pathogenesis. In other systems, rapid developmental transitions are controlled by signaling cascades with hierarchical organization. For example, egg activation requires calcium flux coordinated with kinases and other messengers, and oocyte maturation involves cyclic nucleotide signaling. We previously showed that, like for egg activation, calcium flux is both necessary and sufficient for Bd encystation and that it proceeds without new protein translation. Given the rapid timescale of encystation and the fact that calcium fluxes typically function within broader networks, we hypothesize that Bd encystation is regulated by a signaling cascade involving kinases and/or cyclic nucleotides. To test this, we used small-molecule inhibitors or analogs to probe kinases and cyclic nucleotides, measuring cell wall formation as a readout for encystation. Kinase inhibition significantly blocked cell wall assembly even with mucin present, demonstrating that kinase activity is required for encystation, while encystation was not robustly altered when using analogs of cyclic nucleotides. We are currently using an epistasis approach with kinase inhibitors and a calcium ionophore, ionomycin, to determine the hierarchy of the pathway. These results suggest that a signaling cascade coordinates calcium signaling, kinase activity, and the cellular events of this critical developmental transition in Bd.

B444/P0428

Selective TSC2 dephosphorylation on the lysosomal surface ensures complete mTORC1 activation that occurs

only upon simultaneously sensing amino-acids and growth factors

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The evolutionarily conserved serine-threonine kinase mammalian target of rapamycin (mTOR) serves as the catalytic core of the structurally and functionally distinct multiprotein complexes, mTOR complex 1 (mTORC1) and complex 2 (mTORC2). The mTORC1 signaling pathway, composed of amino acid (AA)-sensing (Ragulator(LAMTOR)-Rag) and growth factor (GF)-sensing (PI3K-AKT-TSC1/2-Rheb) axes, plays a pivotal role in the regulation of intracellular anabolism (e.g., protein synthesis and metabolism) and catabolism (e.g., autophagy and lysosomal biogenesis). mTORC1 deregulation is associated with various metabolic diseases, including cancer and diabetes. As a master regulator of nutrient signaling, mTORC1 integrates a variety of nutrient signals, but signal integration and crosstalk in the mTORC1 pathway remains incompletely understood, because of the lack of quantitative data such as the intracellular concentrations of mTORC1 signaling module proteins and their reaction rates. Therefore, in this study, we aimed to understand the complex mTORC1 signaling cascade by constructing an integrated mathematical model of temporal mTORC1 regulation using two AA-sensing (Ragulator-Rag) and GF-sensing (PI3K-AKT-TSC1/2-Rheb) axes. Using the mathematical simulation and experimental quantitative data including immunofluorescent staining and mass spectrometry-based proteomic data (iMPAQT and Lyso-BioID), we revealed robust AKT phosphorylation (P-T308 and P-S473) after insulin stimulation, regardless of the intracellular AA levels. In contrast, AKT-mediated inhibitory TSC2 phosphorylation (P-T1462) substantially diminished during AA deprivation compared to AA treatment. Mechanistically, we found lysosomal PP2A-mediated TSC2 dephosphorylation upon AA deprivation, which ensures full mTORC1 activation only upon the concurrently sensing of intracellular nutrients Insulin and amino acids (Life Science Alliance 2025). Furthermore, we also found that deregulation of PP2A-TSC2 can cause Tuberous Sclerosis Complex, an intractable disease, and various kinds of carcinomas (Life Science Alliance 2025).

B459/P0443

TFF3 inhibits beta2 agonist-induced bronchodilation in Translational Models of Human Airways.

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Introduction: Hallmarks of obstructive airway diseases asthma include increased airway hyperresponsiveness, mucus hypersecretion and detrimental remodeling. Recent studies have linked Trefoil Factor 3 (TFF3), a peptide produced by the airway epithelium, as a contributor to airway remodeling in chronic obstructive pulmonary disease (COPD). In the gastrointestinal tract, TFF3 is known to support epithelial repair and integrity, and is associated with mucous secretion via epithelial goblet cells and submucosal cells. We posit that TFF3 exposure of human airway smooth muscle cells (HASM) drives airway occlusion through a combination of disruption of intracellular signaling pathways controlling HASM relaxation and increased mucus production. The combination of these factors will thereby serve to impair the efficacy of bronchodilator therapies and contribute to airway occlusion. Methods: To test this hypothesis, we investigated the effects of TFF3 on isoform specific G protein signaling, cAMP production, relaxation with β2 adrenergic receptor (β2AR) agonist in HASM, and dilation of small airways utilizing human precision cut lung slices (hPCLS). Spatial transcriptomics and immunostaining of fatal asthma-derived tissues was performed to assess TFF3 expression and localization. Results: In airways with overproduction of Mucin-5AC (MUC-5AC), TFF3 expression was elevated compared to airways with low MUC-5AC, suggesting mucus occluded airways are associated with

increased levels of TFF3. Exposure of HASM to TFF3 attenuates β2AR induced relaxation and cAMP production. Utilizing BRET (bioluminescence resonance energy transfer) sensors and following TFF3 exposure, inhibitory Gαi1 protein signaling increased in response to carbachol, a physiologically relevant bronchoconstrictor in asthma. In hPCLS, we show that TFF3 impairs formoterol-induced relaxation in airways pre-constricted with carbachol. Conclusions: Collectively, these findings demonstrate that TFF3 negatively impacts airway function and bronchodilator responsiveness by altering the cAMP signaling pathway and may contribute to the airway occlusion found in airways from asthma subjects. In obstructive airway diseases, TFF3 can serve as a therapeutic target to enhance bronchodilator efficacy and airway occlusion.

B443/P0427

TMEM65 Functions as the Mitochondrial Na+/Ca2+ Exchanger

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Mitochondrial Ca2+ homeostasis depends on the coordinated balance between uptake by the mitochondrial Ca2+ uniporter complex and efflux through two distinct pathways: the Na+/Ca2+ exchanger (mito-NCX) and the H+/Ca2+ exchanger. These tightly regulated processes are essential for controlling mitochondrial metabolism, shaping intracellular Ca2+ signaling dynamics, and safeguarding cell survival under both physiological and stress conditions. Disruption of either uptake or efflux can profoundly impact bioenergetics, redox balance, and susceptibility to cell death. Here, by integrating complementary biochemical, structural, and functional assays, we identify TMEM65 as the protein responsible for mito-NCX activity, overturning the prevailing model that attributed this function to NCLX. We show that TMEM65 assembles into a stable homodimer, with essential ion-coordinating residues positioned at the dimer interface, and harbors a specific binding site for the classical mito-NCX inhibitor CGP-37157. In parallel, our data reveal that NCLX, rather than mediating Na+-driven Ca2+ extrusion, instead functions as a mitochondrial H+/Ca2+ exchanger. These findings redefine the division of labor between mitochondrial Ca2+ efflux systems, providing a revised mechanistic framework for mitochondrial Ca2+ handling. This new understanding opens avenues for selectively targeting TMEM65 or NCLX in disorders where dysregulated mitochondrial Ca2+ transport drives pathology, including neurodegeneration, ischemia-reperfusion injury, and cardiovascular disease.

B458/P0442

Visualising cellular mechanisms of psychedelic drugs through fluorescence microscopy of serotonin receptors

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Psychedelic drugs show promise for treating affective disorders such as depression and anxiety. These effects are thought to occur primarily through the serotonin 2A receptor (5-HT2A). Additionally, it is hypothesised that the properties of psychedelics arise from internalised serotonin receptors as opposed to signalling from the cell surface. However, the precise pathways by which psychedelics signal through 5-HT2A and how they differ from the endogenous ligand serotonin remain unclear. Here we employed an ALFA-tagged 5-HT2A construct with its corresponding α-ALFA nanobody to specifically visualise newly internalised 5-HT2A. We unexpectedly found that 5-HT2A endocytosis occurs constitutively, with similar rates of endocytosis in untreated cells and in those treated with serotonin or the psychedelics LSD and psilocin. Stimulation with psychedelics promoted less coupling of canonical effectors (β-arrestin2 and the mini-G protein mGsq) to cell surface 5-HT2A than did serotonin. The mGsq probe was also less enriched on 5-HT2A-containing endosomes upon psychedelic

treatment, and intracellular β-arrestin2 recruitment was not observed under any condition. Most endosomes colocalised with endolysosomal markers (EEA1, Rab7) regardless of treatment, with less colocalisation to recycling markers (trans-Golgi network or Rab11 vesicles). Our findings suggest that psychedelics do not engage with the same 5-HT2A signalling effectors as serotonin. We predict they instead induce signalling through alternative pathways at either the cell surface or endosomes. Since 5-HT2A trafficking patterns were similar across treatments at the time points examined, differences in subcellular location and sorting alone are unlikely to underpin the signalling outcomes of psychedelics. Nevertheless, the ALFA-tag approach provides a specific tool for tracking receptor internalisation in live cells. When combined with organelle markers or biosensors, we anticipate that this strategy will be valuable for investigating the trafficking and signalling of other therapeutically relevant receptors.

Signal Transduction - Receptor ligand interactions

B436/P0420
Chemosensory Role for MS4A8B in Motile Cilia and Neuroendocrine Small Cell Lung Cancer for the Detection of Steroid Hormones and Ca2+ Signaling
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The MS4A family of genes encode for putative chemoreceptors. Within this family of 18 homologs, most are highly expressed in a variety of immune cells, but one homolog, MS4A8B, is unique in its expression within the airway epithelium. MS4A8B is highly expressed in ciliated cells, specifically enriched in motile cilia, and in pulmonary neuroendocrine cells (PNECs). Both of these cell types are chemosensory cells that sense the environment through inhaled air and may possess novel chemosensory signaling pathways. We tested both cell types for the detection of steroid hormones on the genetic basis of MS4A8B. PNECs are a rare cell type making up less than 1% of the lung airway epithelium which has prevented the generation of human PNEC cell lines that can be used for mechanistic studies. To overcome this limitation, we tested neuroendocrine small cell lung cancer (SCLC) cells that are derived from PNECs and thought to share genetic similarities. We tested 10 steroid hormone compounds by Ca2+ imaging and found that glucocorticoids and 17β-estradiol generated cytosolic Ca2+ responses. Steroid-induced responses were attenuated by siRNA targeting MS4A8B. These responses required extracellular Ca^{2+} and were blocked pharmacologically by La3+. Primary human nasal epithelial cells were differentiated at air-liquid interface to produce multi-ciliated cells whose ciliary beating could be monitored by high-speed video analysis. Hydrocortisone treatment increased the proportion of actively beating cilia, linking this signaling mechanism to airway clearance. Our findings suggest MS4A8B contributes to steroid signaling, however, whether it directly binds steroids remains to be determined in future studies. Glucocorticoids are a first-line treatment for respiratory disease yet long-term use is associated with deleterious side effects. Our results define alternative or off-target signaling pathways that will be critical for the development of safer therapies. Additionally, SCLC is a neuroendocrine cancer that has been clinically shown to secrete steroid hormones into the tumor microenvironment. Future studies will continue to investigate how steroid hormones drive cancer cell signaling.

B434/P0418
Differential Endocytosis of Type I BMP Receptors Mirrors Their Kinase Functions in the Signaling Complex

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Traditional models of Bone Morphogenetic Protein (BMP) signaling propose that ligand-receptor binding is the primary driver of signal transduction; however, a growing body of work challenges this dogma, finding that high affinity ligand-receptor pairs are not sufficient for signaling in many contexts. Instead, signaling is mediated through distinct subfunctions of the receptors in the signaling complex, including differential receptor trafficking and kinase activity, though how these individual receptor activities are regulated remains unknown. We previously demonstrated that a heteromeric receptor complex with Acvr1l and Bmpr1a is required for BMP signaling in the zebrafish gastrula. Interestingly, only Acvr1l phosphorylates Smad5, while Bmpr1a kinase activity is dispensable, suggesting each receptor contributes a different function to signaling. Through live-imaging, we found that Acvr1l is trafficked intracellularly via endocytosis, while Bmpr1a is predominantly localized to the membrane. As differential kinase requirements correlate to sub-cellular localization, we hypothesized that receptor endocytosis was required for BMP signaling in these cells. Type II Acvr2b receptors activate Acvr1l and we found that they colocalize in endosomes. Ectopic expression of Acvr1l and Acvr2b increased signaling and endocytosis. Surprisingly, ectopic Bmpr1a and Acvr2b also increased endocytosis and BMP signaling through phosphorylation of Smad5, despite the kinase activity of Bmpr1a not being required normally. This ectopic signaling is abrogated when Acvr1l is knocked down, confirming that Acvr1l is the required kinase, even when Bmpr1a is ectopically expressed. Additionally, Acvr1l loss reduced trafficking of Bmpr1a, indicating that endocytosis is mediated by Acvr1l. To define the molecular basis of differential receptor activity, we performed structure prediction with AlphaFold 3 to identify domains with different structures and protein-protein interactions between Acvr1l and Bmpr1a. Expression of domain swapped chimeric receptors will define the residues that confer or inhibit receptor kinase activity and endocytosis. These findings reveal critical mechanisms regulating BMP signaling and will enable precise control of signaling in the pursuit of therapeutics and tissue-engineering approaches that require BMP signaling for differentiation.

B437/P0421

Investigation of the potential ligand-binding partners of the G protein-coupled receptor FSHR-1 in the intestine of C. elegans

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Proper nervous system function depends on proteins to regulate intra- and inter-cellular signaling, such as gut-brain signaling, allowing organisms to respond to diverse physiological conditions, including oxidative stress. G protein-coupled receptors (GPCRs) coordinate responses within and between a variety of tissues, including the gut and nervous system; however, the mechanisms by which many of these GPCRs orchestrate inter-tissue communication remain unknown. We recently showed that FSHR-1, the sole Caenorhabditis elegans ortholog of a family of vertebrate glycoprotein (GP) receptor GPCRs involved in a diverse array of physiological functions, is necessary and sufficient in the intestine to regulate neuromuscular signaling, where its effects require genetic interactions with its known ligands, GPLA-1/GPA2 and GPLB-1/GPB5. Intestinal FSHR-1 is implicated in controlling oxidative stress responses in C. elegans; yet, the mechanisms of this regulation and its potential links to FSHR-1's regulation of neuronal signaling are unknown. Here, we show intestinal FSHR-1 is necessary and sufficient to promote motility in response to chronic, low-level oxidative stress; however, unlike in standard growth conditions, our results suggest FSHR-1 may act separately from at least one of its GP ligands during chronic oxidative stress. Additionally, we found FSHR-1 has opposing effects on neuromuscular function in chronic versus acute oxidative stress, suggesting different regulatory pathways. We thus hypothesized FSHR-

1 interacts with distinct sets of ligands to control neuromuscular function in different oxidative stress environments. To identify additional candidate FSHR-1 ligands, we expressed and isolated the extracellular domain (ECD) of FSHR-1 from yeast via affinity chromatography and immunoprecipitation. Ligand fishing assays will be performed by exposing purified FSHR-1ECD proteins to worm lysates grown under standard, acute, or chronic oxidative stress conditions. Pulldown samples will then be analyzed by mass spectrometry to identify novel ligands, followed by further behavioral tests of genetic mutants to determine players involved in the different FSHR-1-regulated pathways. Because oxidative stress and disruptions in mammalian GP receptor signaling are implicated in neurological and neurodegenerative diseases, our findings may provide unique insight into human neurobiology.

B440/P0424

Role of proteoglycans in the modulation of semaphorin-plexin signaling

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Semaphorins are secreted or membrane-associated proteins that guide axons during nervous system development. They are recognized by cell-surface plexin receptors, and semaphorin–plexin interactions trigger intracellular signaling cascades that remodel the cytoskeleton and direct axonal navigation toward targets. In addition to binding plexins, semaphorins also interact with components of the extracellular matrix, particularly proteoglycans; however, the molecular roles and mechanisms of these interactions remain poorly understood. We found that Drosophila semaphorins bind specifically to glycosaminoglycan (GAG) chains of proteoglycans. Our data indicate distinct functions of GAG chains for secreted versus transmembrane semaphorins. In secreted semaphorins, the highly basic C-terminal tail interacts with GAG chains that anchor the semaphorins to cell membranes, preventing their diffusion away from production sites. This local retention likely facilitates efficient presentation to plexin receptors on navigating axons. In contrast, transmembrane semaphorins appear to engage GAG chains both in cis and trans, promoting their clustering on the cell surface. Moreover, we show that cell-surface GAG chains enhance plexin-mediated cell collapse, possibly by orienting plexin receptors into signaling-competent conformations. Together, these findings reveal a previously underappreciated role of proteoglycans in shaping semaphorin–plexin signaling by spatially organizing semaphorin distribution and modulating plexin receptor activation.

B439/P0423

Shaping tissues using peptide signaling

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Tissue patterning requires coordinated cell migration in animals. However, organisms with cell walls, such as plants, must orchestrate tissue patterning via cell expansion. How cell expansion within tissues occurs, such that a fully developed tissue emerges with the correct shape and size, is far from understood. The moss Physcomitrium patens provides an excellent model system to pursue this question, as most of its tissues are a single cell layer thick, thereby reducing the complexity of the problem. Sulfated peptides, potentially acting through Leucine Rich Repeat Receptor Like Kinases (LRR-RLKs), the largest family of cell surface receptors in plants, modulate organ growth in seed plants. Sulfated peptides, such as the family of peptides known as PSY, are produced by cleavage of secreted proteins that are sulfated by the Golgi-resident enzyme Tyrosyl Protein

Sulfotransferase (TPST). Lack of TPST function inhibits leafy shoot expansion and leads to accumulation of the PSY receptors PSYR1 and PSYR2 on the plasma membrane. A null mutant lacking the PSY receptors (Δpsyr1/Δpsry2) does not have significant defects in leafy shoot expansion. However, the triple null mutant of Δtpst/Δpsyr1/Δpsyr2, rescues the leafy shoot expansion defect of Δtpst, suggesting that PSYR1 and PSYR2 normally inhibit expansion, and upon binding to PSY, the repression is released, allowing normal organ formation. To identify regulators of organ formation, we performed a suppressor screen of the Δtpst mutant. Strikingly, two of four suppressors were null mutants in a receptor, RPK2, which binds to a completely different family of signaling peptides. We show that loss of RPK2 results in lower levels of PSYRs on the plasma membrane in both wild type and Δtpst, suggesting that RPK2 regulates the amount of PSYR on the membrane. Future studies aim to investigate how RPK2 affects PSYR signaling.

B438/P0422

Transmembrane protein 184A modulates VEGFR2 signaling and internalization in response to heparin and VEGF delivery to cells in culture

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Angiogenesis in wound healing and repair requires the balance of proliferative and migratory signals that ensure proper vessel formation in acute injury. Our research group has identified a novel heparin receptor, Transmembrane protein 184A (TMEM184A), which we aim to characterize in vascular wound healing paradigms. TMEM184A elicits signaling in response to soluble heparin in both vascular endothelial cells (ECs) and smooth muscle cells that dampens proliferation through ERK dephosphorylation. Our recent study in bovine aortic ECs showed that TMEM interacts with the heparan sulfate proteoglycan (HSPG) Syndecan-4 (Sdc4) and colocalizes with Rab GTPases in response to VEGF growth factor. TMEM was also required to maintain vascular endothelial cadherin (VE-cadherin) levels, suggesting that TMEM184A functions to regulate adhesion dynamics in proliferative cells. Vascular endothelial growth factor receptor-2 (VEGFR2) signal cascades induced through soluble VEGF ligand binding and autophosphorylation of Tyrosine residues drives the proliferative and migratory signals that form new blood vessels. Both Y951 and Y1175 are important phosphorylation sites that promote cell proliferation and migration, and several factors including the trafficking regulation of VEGFR2 and dephosphorylation schemes modulate these signals in the presence of growth factors and inflammatory mediators. The objective of this study is to understand how TMEM184A trafficking and or signaling may modulate VEGFR2 activity in response to VEGF and heparin ligands. Here, we investigated whether TMEM184A interacts with Syndecan-2 (Sdc2), an HSPG that forms trimeric complexes with VEGFR2 and VEGF ligand, and whether TMEM184A expression and function elicits regulation of pVEGFR2 responses to VEGF or heparin delivered to cells in culture. Using immunofluorescence and western blotting techniques, we observed that TMEM indeed interacts with Sdc2, and that both Y951 and Y1175 events are consistently dysregulated in TMEM184A knockdown cells compared to standard control cells in response to both soluble heparin and VEGF. Collectively, our results suggest that TMEM184A modulates VEGFR2 signaling in proliferative ECs potentially through endogenous Sdc2 interactions. Funding source: NIH grant HL54269 to LJLK.

B435/P0419

Using Single Particle Tracking to Quantitatively Study the Phosphorylation of Epidermal Growth Factor Receptor (EGFR)

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The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that regulates various cellular processes, including differentiation, apoptosis, and proliferation. EGFR exists in a monomer-dimer equilibrium, that shifts upon ligand binding. When stimulated by one of its seven ligands, the receptor dimerizes and autophosphorylates some of its 10 different tyrosine residues on the C-terminal tail, which then serve as docking sites for adaptor proteins, allowing them to activate downstream signaling pathways. However, it is still unclear which tyrosine residues are phosphorylated upon activation of EGFR by its different ligands, or if there is an order or pattern in which they are phosphorylated. While it is known that adaptor proteins are recruited to EGFR in response to stimulation, the specific phosphorylated tyrosine residues to which they bind, and the kinetics of that binding remain unknown. We aim to elucidate the residue(s) to which different adaptor proteins are recruited in response to stimulation. We have generated several adaptor protein fusion constructs to HaloTag7, a self-labeling fluorescent tag. Using single particle tracking (SPT) and genetic code expansion (GCE) with bioorthogonal labeling, we can track adaptor protein recruitment to specific tyrosine residues on EGFR in response to stimulation with different ligands. In brief, we can add fluorescent tags with exquisite site specificity to our receptor near different phosphotyrosine residues and study their phosphorylation and the recruitment of adaptor proteins with single-molecule Förster resonance energy transfer (smFRET) in living cells. The preliminary findings of these studies have shown that we are able to monitor adaptor protein recruitment to activated EGFR and specific tyrosine residue phosphorylation of the receptor in response to stimulation by different ligands. Together, these studies will help to elucidate the specific binding sites of adaptor proteins, as well as patterns of phosphorylation in response to stimulation by different EGFR ligands.

Signal Transduction - Spatial organization and temporal dynamics

B468/P0452
Localized Action of the Cell Wall Integrity Pathway in Saccharomyces cerevisiae
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Fungal polar growth is achieved through a balance between local cell wall degradation and synthesis to enable directional expansion while preventing lysis (Davì et al., 2018). This balance is tuned by the Cell Wall Integrity (CWI) pathway (Levin, 2011), which is highly conserved across fungi (Dichtl et al., 2016). The CWI kinase Pkc1 promotes cell wall synthesis through a downstream MAPK cascade (Levin, 2011). Pkc1 localizes to sites of cell wall remodeling (Andrews and Stark, 2000), except in the context of cell-cell fusion, when cells allow local removal of the intervening cell walls between fusion partners. During mating of Saccharomyces cerevisiae, Pkc1 was mostly absent from the contact site between partners (Curtis and Lew, 2025). Mutants that failed to expel Pkc1 from contact sites showed mating defects, suggesting that Pkc1 at that site might impair mating (Curtis and Lew, 2025). We found that artificial targeting of active Pkc1 to the contact site reduced mating by impairing local cell wall thinning, assessed in live cells using membrane probes. Imaging with other probes further indicated that localized Pkc1 reduced the polarization of secretion. These findings suggest that Pkc1 acts in a spatially restricted manner to regulate local remodeling of the cell wall. References: Andrews, P. D., & Stark, M. J. R. (2000). Dynamic, Rho1p-dependent localization of Pkc1p to sites of polarized growth. https://doi.org/10.1242/jcs.113.15.2685 Curtis, E. R., & Lew, D. J. (2025). Regulation of the cell wall integrity pathway at the contact site between mating partners in yeast. https://doi.org/10.1101/2025.08.12.669915 Davì, V., et al. (2018). Mechanosensation Dynamically Coordinates Polar Growth and Cell Wall Assembly to Promote Cell Survival. https://doi.org/10.1016/j.devcel.2018.03.022 Dichtl, K., et al. (2016). Cell wall integrity

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B464/P0448

Memory of growth proteins Rax1/2 promote old-end dominance through initiation of Ras1-GTPase activity

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During cell division, growth stops to maintain cell integrity and prevent errors during the division process. The daughter cells must then reinitiate growth at the correct location to maintain proper shape and polarity. While the regulation of cell polarity is well studied, the mechanisms for how the cell determines the site of growth and reinitiates polarized growth at that site after each round of cell division is not fully known. In fission yeast, growth begins first at the old end of the cell inherited from the previous generation through activation of the small GTPase Cdc42. Only later does the cell transition to bipolar growth when new-end take-off (NETO) occurs. The two ends compete for activation of Cdc42, with the old end maintaining its advantage and growing at a faster rate. The old end displays this dominance only when it has grown in the previous generation, indicating the existence of a memory of growth. To function as a memory of growth the signal needs to localize to the cell ends in a growth dependent manner, persist at that end during division and activate growth at the onset of the next cell cycle. We find that the transmembrane proteins Rax1/2 fulfill these requirements and regulate the memory of growth. Rax1/2 localize to cell ends in a growth dependent manner and persist there through cell division. When we disrupt Rax1/2 through deletion of rax1, cells undergo NETO prematurely and Cdc42 activation at the cell ends during cell separation is delayed. We posit that this delay is just long enough to decrease old-end dominance thus enabling early NETO and precocious bipolar growth. In budding yeast, Rax1/2 act upstream of a pathway that promotes Cdc42 activity through a Ras-GTPase. A similar pathway exists in fission yeast, where Ras1 promotes Cdc42 activity through its GEF Scd1. We observe a loss of Ras1 activity at the ends of rax1Δ cells before separation, as well as decreased localization of its GEF Efc25. These findings suggest that the memory of growth Rax1/2 establish old end dominance by promoting Ras1 activation at the ends of dividing cells through recruitment of Efc25. This in turn promotes activation of Cdc42, priming the old ends for growth immediately after cell division and allowing them to outcompete the new ends. These findings describe how cells self-organize the pattern of polarized growth in a growth-dependent manner.

B471/P0455

Metabolic-STAMP reveals spatiotemporal kinase signaling and phosphorylation node logic during GPCR-regulated insulin secretion in pancreatic ß-cells

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Pancreatic β-cells rely upon a diverse range of cues to coordinate glucose-stimulated insulin secretion (GSIS), including the sensing of paracrine and metabolic factors by extracellular-facing G protein–coupled receptors (GPCRs). To determine how stimulation of different receptors leads to context-specific mechanisms that modulate GSIS, we developed Metabolic-STAMP (Synchronized Temporal-Spatial Analysis via Microscopy and Phosphoproteomics), which enables time-resolved mapping of receptor-specific, compartmentalized signaling networks on the minutes time scale (GSIS timescale). Using mouse MIN6 cells and human islets as our model system, we have mapped key receptor-specific pathways of kinase signaling cascades that we predicted

coordinate differential cellular responses that all impinge on insulin secretion. As proof of principle of this pathway-specific regulation of GSIS, we focused on the differential and convergent signaling mediated by ciliary receptor FFAR4 versus GLP1R. Stimulation of each of these receptors in combination with high glucose led to increased insulin secretion, and combinatorial stimulation further amplified insulin secretion (suggesting pathway specificity). Using a combination of phosphoproteomics and imaging, we have identified key themes of convergence and divergence between the two receptor stimulations. First, we note differences in kinase signaling cascades. GLP1R stimulation, for example, exclusively activates the ERK pathway. Though both stimulate PKA, stimulation of each leads to differential phosphorylation of discrete PKA pathway machinery. While GLP1R stimulation promotes phosphorylation of PDE1C/8A, FFAR4 stimulation enhances AKAP1 phosphorylation and ciliary signaling. Together, these data highlight compartmentalized PKA signaling as a central logic gate—regulated by AKAP and PDE phosphorylation—that shapes local cAMP dynamics and tunes GSIS amplitude. We observe that FFAR4 versus GLP1R stimulation during GSIS leads to differential effects on key cellular compartments. For example, live/fixed imaging shows that FFAR4 lengthens cilia, while GLP1R has no effect. Stimulation of either GPCR reduces glucose-induced microtubule disassembly, though seemingly through different microtubule organizing centers (GLP1R through Golgi and FFAR4 through centrosomes). We observed increased microtubule acetylation in both conditions compared to high glucose, along with differential phosphorylation of HDAC6 and ATAT1 as key drivers. HDAC6 inhibition sharply decreased GPCR-driven GSIS, directly tying context-specific acetylation of microtubules to insulin release. Altogether, these data point to critical aspects of receptor pathway specificity that lie at the center of GSIS regulation.

B466/P0450

Pointing Shmoo North - Mapping the Polarized Proteome

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The study of polarized cell growth is significant to our understanding of cancer development, immune response, neuronal function and developmental defects. Growth in response to an external stimulus is orchestrated by the complex interactions of multiple systems, including the Rho GTPase polarity machinery, cell signaling proteins, exocytic and endocytic machinery, and the actin, microtubule, and septin cytoskeletons. Saccharomyces cerevisiae has been used to define the process of cell polarization as they form elongated mating projections, called shmoos, in response to pheromone treatment. This easily inducible system has been used to identify more than 100 proteins that polarize to the mating projection during shmoo formation. In order to fully understand cell polarity, we need to understand how each of these proteins interact and contribute to polarized cell growth. Previous approaches for mapping the polarized proteome, or shmoo-ome, have either been limited by low spatial resolution, or have relied on proteomics approaches that select for high-affinity interactions. We have generated a high-resolution spatial mapping of the shoo-ome that accounts for variability in cell shape, size, and the subcellular localization of proteins. The fluorescent intensity data collected for each protein is mapped into a common cell shape so that we can make an “apples to apples” comparison of protein localization. Our novel computational pipeline generates high-resolution composite images of polarized yeast scaled for orientation and size and has allowed us to classify the subcellular localization of polarized proteins from the yeast GFP-tagged collection. Our algorithm aligns all cells along the polarity axis, scales cell size, determines the average shape of the cells, reassigns the fluorescence from each cell into the average shape, and then averages the fluorescence data to produce high-resolution composite images. The algorithm we generated has recapitulated known biological interactions where endocytic and actin modeling proteins clustered together apart from exocytic proteins supporting the success of our experimental pipeline.

B469/P0453

Pre-stimulation Nuclear Morphology and Basal ERK Activity Predict Single-Cell Responses to PDGF

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The extracellular signal–regulated kinase (ERK) pathway is a central regulator of diverse cellular processes. Despite extensive study, single-cell analyses reveal substantial heterogeneity in ERK responses even within clonal populations, and the factors predicting such variability remain unclear. We sought to identify readily measurable pre-stimulation features that could predict individual cell responses to a defined stimulus. Using NIH-3T3 fibroblasts expressing a nuclear-targeted EKAR4 biosensor, we performed time-lapse fluorescence imaging before and after platelet-derived growth factor (PDGF) stimulation to monitor ERK activity dynamics. Each cell's response was quantified based on amplitude, timing, and persistence of ERK activation, enabling classification into distinct response types. We then measured pre-treatment cellular features, including nuclear size and shape, local cell density, and basal ERK activity, to test their relationships to response class. Individually, these features showed little correlation with response outcome. However, principal component analysis (PCA) revealed that specific combinations of pre-stimulation variables—particularly nuclear morphology and basal ERK activity—were significantly associated with whether a cell responded to PDGF. These findings indicate that subtle, pre-existing differences in nuclear structure and ERK signaling state jointly influence responsiveness. Integrating multiple pre-stimulation features thus improves prediction of single-cell signaling outcomes and provides insight into the sources of variability that shape cellular decision-making.

B470/P0454

Single-Molecule Tracking Reveals Amphiregulin Mediated Modulation of HER2

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ErbB2/HER2 is a receptor tyrosine kinase frequently amplified in cancer, especially breast cancer, where it preferentially heterodimerizes with other ErbB receptors to amplify MAPK and PI3K/AKT signaling. Unlike the epidermal growth factor receptor (EGFR), HER2 lacks a confirmed specific ligand. Amphiregulin (AREG), a known EGFR ligand, is synthesized as a transmembrane precursor and released as a soluble ectodomain containing an EGF-like domain and a heparin-binding domain (HBD) of unclear function. We asked whether soluble AREG can directly influence HER2 behavior, and whether the HBD and exogenous heparin modulate HER2 dynamics. Using single-molecule imaging and single-particle tracking of recombinant HER2 ectodomain (HER2-ECD) on supported lipid bilayers (SLBs), we find that soluble AREG markedly slows the lateral diffusion of HER2-ECD and subsequent addition of high-molecular-weight heparin further decreases mobility. Moreover, in live cells lacking endogenous EGFR expression, including CRISPR–Cas9 EGFR-knockout lines, treatment with 50 nM AREG reduces HER2 mobility at the plasma membrane. An AREG isoform lacking the HBD also decreases HER2 diffusion, but less so than wild-type AREG. These results support a model in which AREG may act as a putative HER2 ligand, with heparin/heparan-sulfate interactions mediated by the AREG HBD stabilizing HER2 complexes and extending their membrane residence. This putative AREG–HER2 engagement provides a potential mechanistic link between ligand availability, glycocalyx and extracellular matrix composition and HER2-driven signaling and may also suggest new strategies to modulate HER2 activity by targeting the AREG HBD or its heparan-sulfate interactions.

B467/P0451

Spontaneous Ca2+ flickering in single mitochondria prevents cellular Ca2+ overload via STIM1 sequestration

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Ca2+ signaling requires precise control of Ca2+ storage and buffering by endoplasmic reticulum (ER) and mitochondria. While STIM1-mediated store-operated Ca2+ entry (SOCE) regulates Ca2+ storage in ER, how mitochondria regulate global Ca2+ signaling has remained elusive. Here, utilizing mitochondrial Ca2+ sensors, we identified spontaneous Ca2+ flickering within individual mitochondria at ER-mitochondrial contacts (EMCs). We found that reducing EMCs by knocking down contact proteins such as mitofusin-2 and PDZD8 abolished mitochondrial Ca2+ flickering. Surprisingly, this EMCs reduction did not diminish SOCE activity but instead enhanced it, accompanied by increased ER-plasma membrane junctions and elevated STIM1-microtubule binding. These changes ultimately led to cellular Ca2+ overload, suggesting that mitochondrial flickering normally serves a protective function. Mechanistically, we revealed that mitochondrial Ca2+ flickers are driven by IP3R-VDAC1-mediated Ca2+ flow from the ER to mitochondria. This local Ca2+ transfer depletes Ca2+ in the peri-EMCs region of the ER, leading to STIM1 sequestration and suppression of excessive SOCE activation. Our findings demonstrate that ER, mitochondria, and microtubules constitute an integrated regulatory network wherein mitochondrial Ca2+ flickering serves as a protective mechanism against global Ca2+ overload by fine-tuning SOCE activity through local ER Ca2+ depletion.

B472/P1806

Temporal-spatial regulation of protein synthesis and degradation serves as a key checkpoint of G0 transition and fate change

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Every cell division requires daughter cells to either re-enter the cycle (G1) or exit the cell cycle to G0 and undergo fate change, the predominant state for cells in the body. G0 can manifest as terminally differentiated cells with tissue-specific functions or as stem-like cells poised to re-enter division upon stimulation. However, the sequence of events enabling G0 entry and fate specification remains poorly defined. Multiple processes must coordinate in time and space with internal and external cues, or the cell fails to exit cycle and differentiate. Understanding these transitions is crucial both for basic cell biology and for translational research into how disrupted G0 programs drives oncogenesis or other pathologies. Progress has been limited by the absence of homogeneous, non-cancerous G0 model systems, particularly since many transition steps are highly localized and transient. To dissect checkpoints controlling G0 entry, we developed Spatio-Temporal Analysis via Microscopy and Proteomics (STAMP), in which we synchronized retinal pigment epithelial (RPE) cells at metaphase, allowing them synchronously transition into G0 upon serum starvation. Over the course of 36 hours, we sampled at 1–2 hour intervals for live/fixed imaging and phosphoproteomics, focusing on the known stages of the formation of the primary cilium (a hallmark of non-cycling cells that is a critical, membrane-bound signaling hub) as temporal landmark. These studies, coupled with genetic and pharmacological perturbations, allowed us to track key patterns in the stages of G0 transition both molecularly and in the context of dynamic changes in different cell compartments. One key theme that emerged from our studies was the pivotal role of targeted protein synthesis and degradation (both autophagy and proteosome-mediated) that each occurred in two waves: one immediately after the cells have completed cytokinesis, and one

immediately after a fully-formed, GPCR-laden primary cilium has formed. We observed that blocking the first wave of either autophagy or protein synthesis led to failed ciliogenesis and defective establishment of cell fate. Using time-lapse SILAC proteomics and phosphoproteomics to observe what proteins are being newly synthesized and degraded over time, we observe key patterns of specific classes of proteins having time-dependent protein abundance and modification over time (e.g, a number of ciliary, vesicular traffic, and known tumor suppressors in the first wave, and cell fate specific factors in the second wave). Altogether, these findings set the stage for mapping key signaling cues and cellular functions in time and space, with future work to deconvolve how disrupting these processes in pathology ultimately hinders establishment of a healthy, fated cell program.

B465/P0449

The functional domains and downstream signaling of galvanin (TMEM154), a charged biosensor that steers electrotaxing cells

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Immune and epithelial cells migrate directionally in response to electric fields in a process termed “electrotaxis” that is hypothesized to contribute to wound-healing. When an intact epithelium is wounded, the transepithelial potential established by ion gradients is disrupted, generating an electric field with the cathode oriented toward the wound. The single-pass transmembrane protein galvanin (TMEM154) is required for the full electrotaxis response in many cell types from vertebrate animals. When an electric field is applied to individual cells expressing galvanin, the protein undergoes electrophoresis in the plasma membrane, depleting from the cathode-facing side of the cell and accumulating at the anode-facing side. This redistribution of the protein along the electric field axis is tightly temporally and spatially correlated with changes in cell motility, including rear retraction at the anodal side and front protrusion at the cathodal side. This suggests that galvanin performs direct signal transduction to the actin cytoskeleton, rather than influencing electrotaxis through a more indirect mechanism. Galvanin demonstrates a new signaling paradigm for cell motility, because its spatial relocalization in the membrane is the apparent driver of downstream signaling, contrasting with mechanisms of chemotaxis that rely on receptor occupancy by a ligand. Using a combination of truncations, internal in-frame deletions, and domain swaps, we have identified key functional domains of galvanin. The high net negative charge on the extracellular domain is necessary and sufficient for its relocalization in the presence of an electric field, while the intracellular domain is necessary for driving the reorientation of cell motility. Within the cytoplasmic domain, we identified a highly conserved sequence that is required for complete electrotaxis, while the remaining approximate two-thirds of the endodomain is dispensable. In order to determine the potential downstream signaling proteins and pathways, we have tagged galvanin’s endodomain with a promiscuous biotinylator to perform proximity labelling experiments and present preliminary candidate downstream proteins. This investigation strives to further illuminate the signaling mechanism of galvanin, a key contributor to electrotaxis with a novel signaling paradigm.

Systems and Quantitative Biology - Computational modeling of cellular dynamics and processes

B384/P1817

A Dynamical Systems Framework Applied to Live Cell Imaging of hiPSC-Derived Endothelial-Like Cells Under Flow Uncovers a First-Order Phase Transition Between Morphology-Based Cell States

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The Allen Institute for Cell Science aims to develop a theoretical understanding of cell state transitions during multicellular morphogenesis, including quantitatively defining cell states. Efforts to quantify a Waddington-like landscape from the perspective of dynamical systems, in which the valleys of the landscape correspond to the attractors of the governing cell state dynamics, have largely focused on describing cell state dynamics in terms of molecular mechanisms such as gene regulatory networks. Here we use morphological data to quantify stable cell states. We use the ability of human induced pluripotent stem cell-derived endothelial-like cells (hiPSC-ECs) to align, elongate, and migrate parallel or perpendicular to the direction of flow under low and high fluid shear stress (FSS), respectively, as a model of an inducible cell state change. We developed a segmentation-free unsupervised machine learning (ML) approach to extract features from transmitted light 2D standard deviation projections from 3D spinning disk confocal timelapse videos of hiPSC-ECs expressing GFP-tagged VE-Cadherin under different FSS. We combined these features with a dynamical systems framework to construct vector field representations of the cell state dynamics. From these vector fields we identified the stable fixed points of the dynamics at varying levels of FSS. We found these stable fixed points change with FSS in a way that suggests a first-order phase transition between low and high FSS, where a fixed point bifurcates into two at an intermediate FSS. We confirmed that these stable states represented qualitative characteristics of the observed biological cell states by using the ML model to reconstruct representative images of the VE-Cadherin channel at these fixed points. We also integrated segmentation-free features with segmentation-based metrics such as cell density and alignment, and individual cell trajectories obtained from tracked hiPSC-ECs. This analysis supported our earlier biological interpretation of the stable states and showed that cells take varied paths towards the fixed points. Our work complements existing approaches that characterize stable cell states using various types of single cell "omics" data to provide a proof-of-concept framework for quantitative identification of stable cell states from microscopy image-based data.

B376/P0952

Algebraic approach to representing mitochondrial dynamics leads to new similarity measure of mitochondrial morphology and understanding of potential regulatory mechanisms

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Mitochondria are cellular structures involved in metabolism that take the form of a network of membrane-based tubes that undergo continuous re-arrangement by a set of morphological processes, including fission and fusion, carried out by protein-based machinery. Because of their network structure, mitochondria can be

represented as graphs, and the morphological operations that take place in the cell, referred to as mitochondrial dynamics, can be represented by changes to the graphs. Prior studies have classified mitochondrial graphs based on graph-theoretic features, but an alternative approach is to focus not on the graphs themselves but on the set of morphological operations inducing mitochondrial dynamics, since this may provide a simpler representation. We derive a mathematical representation of mitochondrial network dynamics as a discrete vector space with an asymmetric distance metric. We then use estimates of this distance metric to develop a measure of similarity between mitochondria networks that is used to cluster pre-existing datasets, and to compare mitochondria graphs generated in silico by random walks to those observed in vivo. Our preliminary analysis suggests that some dynamical processes can not be modeled entirely stochastically, hinting at the potential existence of certain regulatory mechanisms. Beyond the potential immediate insights into the dynamics of mitochondrial networks, this work points to a general strategy for formulating a comprehensive mathematical representation of a cell structure state-space, based not on the shapes of cellular structures, but on relations between the dynamic operations that produce them.

B375/P0951

An agent-based model of lymph node-mediated abscopal immunity

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The abscopal effect, a “treat one, control many” response where local radiotherapy (RT) elicits distant tumor regression, reveals the immune system’s latent potential for systemic tumor control. Although RT reliably triggers antigen release and type-I interferon (IFN-I) signaling that license dendritic cells (DCs) to activate $CD8^+$ T cells, the priming events rarely extend beyond the irradiated site. This breakdown in propagation exposes a regulatory bottleneck within the tumor-draining lymph node (tdLN), where local activation must be translated into systemic control. Building on the experimental evidence that tdLNs coordinate systemic immune activation, we present a spatiotemporal agent-based model (ABM) that quantitatively formalizes tdLNs as an immune control architecture. This ABM framework recasts the lymph node from a passive priming site to an active decision-making hub, integrating DC licensing, $CD8^+$ T-cell scanning and differentiation, checkpoint saturation, and fibroblastic reticular cell (FRC)-guided topology that governs DC and T-cell encounter probabilities. The model reproduces four defining mechanistic gates of abscopal failure: (i) threshold-dependent amplification of early immune cues, (ii) temporal synchronization among DC influx, antigen flux, and T-cell availability, (iii) topology-driven spatial biasing of interactions by the FRC scaffold, and (iv) density-induced checkpoint dominance that silences otherwise antigen-rich contexts. Through this framework, we demonstrate that systemic amplification emerges only when DC licensing, antigen persistence, and IFN-I timing converge with FRC-guided topology to sustain productive DC-T-cell encounters. Additionally, we show how distinct immunotherapies (e.g., STING agonism, DC maturation, checkpoint blockade) engage discrete control gates within the tdLN, each reshaping immune trajectories through unique therapeutic mechanisms. Emergent patterns, including biphasic DC-T-cell contact distributions, effector egress kinetics, and cytokine diffusion ranges, recapitulate patterns observed in intravital and ex vivo studies. Together, these findings define the control logic of the tdLN and illustrate how coordinated modulation of its topology, timing, and feedback can recode rare abscopal events into reproducible systemic immunity.

B377/P0953

From nucleus to migration: linking nuclear mechanics with predictive models of cell motility

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Cancer progression is tightly governed by cell and nuclear mechanics: mechanical properties of the extracellular matrix (ECM) together with cell-matrix adhesions, and cytoskeletal architecture set the forces and deformations experienced by the nucleus—the cell's largest and stiffest organelle—thereby tuning transcriptional programs, epigenetic state, and invasive behavior. Here, we tackle this question of nucleus-cytoskeleton connectivity and whole-cell mechanics, using a combined, experimental, modeling, and data-driven approach. Our experimental efforts employ a model system, where a study by Vahabikashi et al. uncovers that A- and B-type lamins distinctively interact with the linker of nucleoskeletal and cytoskeleton (LINC) complexes that bind to actin and vimentin filaments to differentially modulate whole-cell rheology in mouse embryonic fibroblasts. Specifically, they find that A-type lamins engage with both F-actin and vimentin and knockouts of A-type lamin promote nuclear expansion, whereas B-type lamins predominantly engage with perinuclear vimentin and its shielding absence compresses the nucleus. These findings suggest a complex link between mechanics of the F-actin cytoskeleton, vimentin intermediate filaments, and the nuclear envelope via LINC complexes. To further investigate the mechanisms and enable prediction, we develop a mathematical model of the nucleus-cytoskeleton using a 2D elastic, deformable cable-network description. Model simulations reproduce the morphological shape changes of the nucleus in response to A- and B-type lamin knockouts and recapitulate traction force trends. Inspired by recent experiments on spreading cells, we transition from the static adherent geometry to an unconstrained geometry to account for dynamic cell spreading. This modeling of nucleus-cytoskeleton dynamics in the context of a live cell allows us to probe dynamic nuclear and cellular deformation and ultimately, understand the mechanics of the initial stages of cell movement. By grounding predictions in nuclear dynamics, this framework highlights the nucleus as a central integrator of cellular mechanics. Such insight is critical for uncovering novel modes of cellular mechanical adaptation and identifying aberrant behaviors that underlie motility-dependent diseases such as cancer, fibrosis, and chronic inflammation.

B373/P0949

Interrogation of signaling and metabolism through systems biology reveals functional phosphosites that determine cellular metabolic reprogramming in liver tissue

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Phosphorylation functions as a tunable regulatory mechanism that dynamically reprograms metabolic networks in response to environmental and cellular stress. Unlike genetic mutations, phosphorylation is reversible and context dependent, enabling plastic modulation of enzyme activity, localization, and interactions. This flexibility coordinates pathway-wide changes that adapt cells to fluctuating nutrients and redox states, producing metabolic flux patterns that cannot be inferred from pathway stoichiometry alone. By treating phosphorylation as a systems-level input, we uncover emergent properties such as altered flux, substrate specificity, and allosteric regulation that govern cellular redox homeostasis. Using mass spectrometry-based phosphoproteomics, metabolomics, and lipidomics in obese mouse liver, we defined phosphorylation sites on metabolic enzymes that predict shifts in lipid metabolism. Integration of supervised and unsupervised computational models revealed features of redox stress linked to functional consequences of the metabolic phosphoproteome. Together, this systems biology framework shifts metabolic networks from static maps to dynamic, phospho-regulated landscapes, opening new opportunities for predictive modeling and therapeutic targeting.

B382/P1815

Investigating cytoskeletal and cellular dynamics during morphogenesis in the zebrafish

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The first coordinated morphogenetic cell movement during zebrafish development is a process called epiboly, wherein the cells of the embryo spread over and envelop the yolk. These morphogenetic movements are essential to the transition from a group of individual cells to the formation of a body plan during development. Thus, the dynamics and regulation of epiboly remains a fundamental question in biology. Epiboly is initiated by an expansion of an epithelial enveloping cell layer (EVL) coupled with EVL planar cell divisions that leads to yolk cell doming and progression to 50% epiboly. However, the mechanism/s of epiboly progression beyond 50% remain less clear. Here we use live and fixed imaging of actin to investigate cytoskeletal and cellular dynamics in mediating epiboly progression, together with computational modeling. Specifically, we investigated straightening of the leading edge of the EVL margin, which is an emergent property of our mathematical model of epiboly. We show that the embryo EVL margin starts uneven, but by the end of epiboly, the margin straightens. Additionally, we investigate cell shape changes using CellPose and a custom algorithm to track cells during the morphogenetic process. We show that cells initially do not elongate as epiboly progresses and rather cells rearrange at the EVL leading edge margin, while nearer the end of epiboly, cell elongation dominates. We quantified these changes by an increasing cell shape index as epiboly progresses. Additionally, we show that the number of junctions per cell does not change dramatically as epiboly progresses. These and other results set the stage for further investigation of epiboly dynamics in mutants and fluorescent reporter lines of the cytoskeleton and adhesion factors, as well as comparison to our mathematical models.

B378/P0954

Mechanisms of VEGF receptor trafficking dynamics in endothelial cells

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Vascular endothelial growth factor receptors (VEGFRs) bind to cognate ligands to facilitate signaling pathways critical for angiogenesis, the growth of new capillaries from existing vasculature. Intracellular trafficking regulates the availability of receptors on the cell surface to bind ligands, which regulate activation, and the movement of activated receptors between the surface and intracellular pools, where they can initiate different signaling pathways. Targeting VEGF receptors to disrupt blood vessel growth in cancer and retinopathy has achieved limited success and over a dozen human clinical trials of VEGF delivery to increase vascular growth in patients with peripheral artery disease (PAD) and coronary artery disease (CAD) have failed. Thus, we need to better understand how VEGF works, including the stability and trafficking dynamics of membrane-bound receptors (VEGFRs), ligands (VEGFs and PLGFs), and co-receptors expressed on endothelial cells. We have developed and validated a molecularly detailed computational model of VEGFR1, VEGFR2, and co-receptor Neuropilin1 (NRP1) trafficking in endothelial cells to quantify how the binding of different VEGF ligands alters the trafficking of these receptors. The model uses coupled, nonlinear, deterministic ordinary differential equations simulating biophysical and biochemical reactions of receptor dimerization, ligand-receptor binding,

and trafficking processes. We have integrated careful experimental quantification of receptor protein localization by subcellular location in the presence of ligands and trafficking perturbations with the model. Our model aims to demonstrate the consequences of receptor localization and ligand binding on the localization and dynamics of signal initiation complexes. We show that VEGFR1, and VEGFR2 trafficking is not the same and that VEGFR2 trafficking is altered in response to ligand binding, but that trafficking of VEGFR1 is not. We also show that the altered trafficking can be explained by a single mechanistic process, increased internalization of the VEGFR2 receptor when bound to ligand. We further show that even though the canonical view of receptor tyrosine kinases is of activation on the cell surface, most of the ligand-receptor complexes for both VEGFR1 and VEGFR2 are intracellular. We also explore the competition between the receptors for ligand binding, the so-called 'decoy effect', and show that while in vitro on the cell surface minimal such effect would be observed, inside the cell the effect can be substantial and influence signaling. These results expand our understanding of receptor-ligand trafficking dynamics and are critical for the design of therapeutic agents to regulate ligand availability to VEGFRs and hence VEGFR signaling in angiogenesis.

B379/P1812

Reactive Oxygen Species Counteract Zebrafish Wound Contraction and Promote Wound Healing

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Reactive oxygen species (ROS) are second messengers that drive wound closure. However, the mechanism by which ROS regulate wound contraction to facilitate wound healing remains unclear. Here, we report that ROS counteract wound contraction by inhibiting the phosphorylation of myosin regulatory light chain. Acute ROS inhibition, through pharmacological perturbations, disturbs wound relaxation, delays wound closure, and impairs regrowth following amputation. Moreover, actomyosin inhibition relaxes tailfin contraction without impairing wound closure or regrowth. Over-contraction, on the other hand, impedes wound closure. Meanwhile, chronic depletion of epithelial ROS during embryonic development, achieved through morpholino-mediated knockdown of the duox gene, alters tissue stiffness, as measured using atomic force microscopy-based nanoindentation. Despite a reduced contraction force, the wound also appears to be over-contracted, with delayed healing and regrowth. An in silico linear elasticity simulation to calculate the second principal stress based on node-wise prescribed displacement recapitulated the contraction dynamics during acute and chronic ROS inhibition. Together, our results provide a novel understanding of how reactive oxygen species (ROS) facilitate wound closure, a process instrumental in restoring tissue integrity and maintaining homeostasis.

B381/P1814

Reverse engineering organ size and shape control through the integration of quantitative experiments and multi-scale computational modeling

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Predicting how an organ's size and shape emerge from complex biomolecular interactions remains one of the most significant challenges in systems biology and tissue engineering. Our group's research integrates experimental and computational approaches to elucidate the underlying principles of morphogenesis. To do so, we use the larval wing disc of the fruit fly (Drosophila melanogaster) as a powerful model system for

discovering the mechanisms regulating a growing organ's shape and size. I describe the formulation and experimental validation of a multi-scale computational model that has generated new mechanistic insights into the control logic of morphogenesis. This work relies on the integrative efforts of multiple collaborative projects. Using tools that create spatially defined perturbations to growth or mechanotransduction pathways, we demonstrated that increasing cell proliferation through different pathways leads to distinct outcomes in the regulation of organ shape. For example, increased insulin signaling enhances the tissue's basal curvature while stimulating growth through Bone morphogenetic protein (BMP/Dpp) signaling or elevated Myc expression has the opposite effect and flattens the central tissue region. These variations in outcomes arise from differences in how each growth pathway regulates actomyosin contractility, cell-ECM adhesion, and ECM stiffness. In summary, we propose that organ size control relies on orchestrating tissue morphogenesis through the balancing of the differential effects of intrinsic, extrinsic, and mechanotransduction regulators of growth and cell mechanics.

B374/P0950
Screening for mutants defective in biomass-volume coordination
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The coordination between cellular biomass and volume maintains macromolecular concentration homeostasis, thereby defining the chemical environment for biochemical reactions. Disruptions to this biomass-volume coordination are linked to senescence and aging. However, the underlying mechanisms are poorly understood, and no specific regulators have been identified. Here, we used genome-wide screening and directed evolution in budding yeast to identify mutants with an altered mass-volume balance. Intriguingly, cells with defective mass-volume coordination frequently carried mutations in ion transporters and genes related to protein synthesis, suggesting that the balance of small and large molecules influences cellular density. Additionally, several density mutants exhibited extra copies of chromosomes, implying that gene dosage also helps regulate cell volume and biomass. Taken together, our study provides insights into how protein concentration homeostasis is achieved and the causal relationship between this homeostasis and cellular senescence.

B383/P1816
Space Negotiation Strategies of Cells Invading Extracellular Matrix: Lessons from a Physics-Based Model
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Cell migration through extracellular matrix (ECM) presents a fundamental biophysical paradox: cells require ECM fibers as adhesive substrates for traction force generation via filopodia, yet these same structures form mechanical barriers that impede movement. To navigate this trade-off, cells employ three distinct space negotiation strategies—shape adaptation through deformability, mechanical remodeling of elastic ECM networks, and proteolytic degradation via matrix metalloproteinases (MMPs). While each mechanism has been studied in isolation, their integrated dynamics and relative contributions to invasion success remain poorly understood. We developed a computational physics-based model that captures the interplay between these space negotiation strategies and force-driven migration. Our agent-based framework represents cells as deformable entities with explicit filopodia that mechanically interact with an elastic ECM network through a two-component contractile model, capturing force-velocity relationships observed in actomyosin systems. Cells can both mechanically displace ECM nodes through traction forces and enzymatically degrade network

components. By systematically varying ECM density, fiber stiffness, and cellular parameters, we identify emergent migration regimes where different strategies dominate. Our simulations reveal that optimal migration occurs in intermediate-density environments where mechanical remodeling through filopodial traction alone suffices, while high-density matrices necessitate coordinated deployment of MMP activity and enhanced deformability. The detailed filopodia model demonstrates how the spatial distribution and dynamics of cell-ECM adhesions critically influence migration efficiency across different microenvironmental contexts. This work provides a computational sandbox for investigating the complex interplay between cellular mechanics, ECM properties, and enzymatic degradation in 3D invasion. By explicitly modeling filopodial force generation alongside cell deformability and ECM remodeling, our framework enables systematic exploration of how perturbations to specific space negotiation mechanisms—such as MMP inhibition or cytoskeletal disruption—alter invasion dynamics, offering testable predictions for experimental validation.

B380/P1813
Yeast growth is controlled by the proportional scaling of mRNA and ribosome concentrations
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Despite growth being fundamental to all aspects of cell biology, we do not yet know its organizing principles in eukaryotic cells. Classic models derived from the bacteria E. coli posit that protein-synthesis rates are set by mass-action collisions between charged tRNAs produced by metabolic enzymes and mRNA-bound ribosomes. These models show that faster growth is achieved by simultaneously raising both ribosome content and peptide elongation speed. Here, we test if these models are valid for eukaryotes by combining single-molecule tracking, spike-in RNA sequencing, and proteomics in 15 carbon- and nitrogen-limited conditions using the budding yeast S. cerevisiae. Ribosome concentration increases linearly with growth rate, as in bacteria, but the peptide elongation speed remains constant (≈9 amino acids s^{-1}) and charged tRNAs are not limiting. Total mRNA concentration rises in direct proportion to ribosomes, driven by enhanced RNA polymerase II occupancy of the genome. We show that a simple kinetic model of mRNA–ribosome binding predicts both the fraction of active ribosomes, the growth rate, and responses to transcriptional perturbations. Yeast accelerate growth by coordinately and proportionally co-up-regulating total mRNA and ribosome concentrations, not by speeding elongation. Taken together, our work establishes a new framework for eukaryotic growth control and resource allocation.

Systems and Quantitative Biology - Multi-scale computational models

B385/P1818
Signal processing and robustness of the BMP network during zebrafish embryogenesis
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In developing tissues, signal transduction from morphogen gradients conveys positional information to cells, resulting in cell specification and differentiation. One such morphogen is bone morphogenetic protein (BMP), of the TGF-β superfamily, whose network is highly conserved across many species. In zebrafish species Danio rerio, this signaling pathway directs dorsoventral axis formation during early embryogenesis. Many of the molecules that play a role in this network are known; however, the mechanisms through which they achieve noise attenuation and gradient robustness have not been defined. Specifically, the heterodimer-heterotetramer complex has been shown to be required for signal transduction, but deterministic modeling of

the BMP membrane receptors at this stage has not given any insight into evolutionary drivers of the requirement. Building and developing a stochastic, multiscale model of this process allows us to mechanistically assess zebrafish phenotype variability related to the distributions of noise and stochasticity. We can also analyze time-dependent signaling and frequency metrics that are not available in traditional, deterministic modeling. The stochastic receptor model has been constructed using the open-source python wrapper GillesPy2 and the framework StochKit2. This framework employs tau-leaping and Gillespie's stochastic simulation algorithm to rapidly simulate stochastic trajectories in biochemical systems with facilitated cluster-implementation. Fast Fourier Transform and cumulative energy spectral density visualization show that the heterodimer-heterotetramer complex functions as part of a low-pass mechanism in the dorsal-ventral axis formation process. Reliance on the heterodimer-heterotetramer complex for signal transduction through the few hours of embryogenesis may drive higher accuracy in downstream cell differentiation by way of fewer low frequency fluctuations. To further probe this system, we are working to develop an experimental optogenetics protocol to genetically manipulate dorsoventral signal transduction in vivo and collect and analyze fluorescence intensity of the downstream pSmad gradient. Through these multiscale modeling efforts and experimental coupling, we hope to further understand the noise origins and signal processing of this network. As the BMP signaling pathway is highly conserved and has been implicated in human bone growth and wound healing, its study in simpler systems stands to accelerate our comprehension of BMP network structure and molecular mechanisms with application in regenerative medical studies.

Systems and Quantitative Biology - Single-cell systems and cellular heterogeneity

B370/P0946

Functional mapping of KOLF2.1J hiPSCs via genome-scale CRISPRi Perturb-Seq

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This study aims to systematically map genotype–phenotype relationships underlying human induced pluripotent stem cell identity by generating an expressed genome-wide CRISPRi Perturb-Seq atlas in KOLF2.1J hiPSCs. We designed a CRISPRi library targeting 11,739 expressed genes in KOLF2.1J, profiled over 2.5 million cells by single-cell RNA-Seq, and conducted parallel bulk fitness screens. We performed differential expression analysis across all perturbations. Orthogonal validation included size-exclusion chromatography mass spectrometry (SEC-MS), BioID interactome profiling, stable-isotope metabolic tracing, qPCR, immunofluorescence, western blot, and bulk RNA-Seq. We identified 1,665 perturbations that strongly alter the transcriptional state of hiPSCs. Clustering of these perturbation profiles enabled reconstruction of a functional complex map—encompassing known protein assemblies and genes with redundant functions—thereby constituting a comprehensive functional atlas of KOLF2.1J hiPSCs. This analysis recapitulated established complexes (e.g., mitochondrial ribosomes, Mediator complex, SAGA complex) and uncovered novel regulators of pluripotency and metabolism. ZBTB41 emerged as a key regulator of mitochondrial metabolism, with its knockdown impairing TCA cycle flux shown via isotopologue tracing. In addition, we identified RNF7 co-clustered tightly with POU5F1 (OCT4), and its depletion led to a marked reduction in POU5F1 transcript and protein levels, phenocopying OCT4 knockdown, as confirmed by immunofluorescence, western blot, and bulk RNA-Seq. This genome-scale Perturb-Seq atlas constitutes a comprehensive resource for dissecting

transcriptional and post-transcriptional networks in human iPSCs, enabling discovery of novel regulators and informing strategies for stem cell state engineering.

B372/P0948
Heterogeneous Escape From X Chromosome Inactivation in Lymphocytes Revealed by Quantitative Allele-Specific FISH
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Females (XX) often mount stronger immune responses than males (XY), resulting in better outcomes from infection but also a higher risk of autoimmunity. Because the X chromosome encodes many immune-related genes, differences in gene dosage may underlie these sex biases. However, in therian mammals, dosage balance is accomplished by X chromosome inactivation (XCI), which hinders transcription from all but one randomly chosen active X chromosome (Xa), through a process regulated by the long noncoding RNA Xist. Notably, in mouse B and T cells, the recruitment of Xist to the inactive X (Xi) is stimulation-dependent and heterogeneous. Genes that continue to be transcribed from the Xi, called “escapees”, have the potential to accomplish the imbalance that multiple X chromosomes portend. The mechanism by which genes escape is poorly understood, but the stimulation-dependent heterogeneity of Xist localization suggests that escape from XCI may behave similarly. Immune effector genes themselves are expressed heterogeneously across lymphocyte populations in response to stimulation. These diverse expression patterns enable flexible responses to stimuli, with certain cells playing outsized roles in activation. Whether increased dosage from escape contributes similarly to heterogeneous regulation remains unknown, as most studies have relied on bulk RNA-seq to assess expression from the Xi. This work intends to deepen the understanding of heterogenous XCI by applying sequential RNA and DNA fluorescent in situ hybridizations (FISH) to measure allele-specific nascent transcription in naïve and stimulated lymphocytes. Single molecule intronic probes allow nascent RNA to be quantified, while Xa and Xi specific DNA probes are used to label the origin of each transcription burst. Analysis of two constitutive escapees in thousands of cells, the demethylase Kdm6a and the RNA helicase Ddx3x, revealed that both the intensity and frequency of escape increases in response to stimulation in splenic B220+ and T cells. Although escape occurs in a subset of cells, it accounts for a significant portion of total gene expression in both naïve and stimulated lymphocytes, suggesting heterogeneous regulation. Heterogeneous XCI escape represents a potential source of functional diversity in lymphocytes with implications for sex differences in immunity.

B368/P0944
Intestinal Epithelial Zonation Emerges Through Local Cell-Cell Communication
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The intestinal epithelium exhibits zonated gene expression along the crypt-villus axis, with distinct transcriptional programs in enterocytes located at the villus top versus villus bottom. However, the mechanisms establishing these spatial patterns remain unclear. Three models have been proposed: specification by external gradients, cell-intrinsic temporal programs, or local self-organization. Here, using spatial transcriptomics and spatial perturbations of two-dimensional intestinal organoids, we show that zonation emerges via spontaneous self-organization in the absence of extrinsic mesenchymal, neural, or

vascular inputs. Purely cell-intrinsic models of zonation were eliminated by spatial perturbation experiments: we transplanted cells into established monolayers and showed that these transplanted cells progressively adopted both individual gene expression patterns and coordinated zonation profiles matching their new location, with strongly zonated genes showing the greatest adaptive responses. Single-cell RNA-seq receptor-ligand analysis identified Ephrin signaling as a candidate mediator. Pharmacological Ephrin inhibition dramatically disrupted zonation, causing aberrant co-expression of villus top and bottom markers in edge cells. These findings identify Ephrin-mediated local cellular interactions as a driver of intestinal zonation, demonstrating that self-organization through cell-cell communication can generate spatial patterns independently of external positional cues or predetermined programs.

B371/P0947

Multiplexed single-cell proteomics using DNA-barcoded readout

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Cell functional states fluctuate rapidly during development and disease, yet traditional assays average out crucial cell-to-cell variability, limiting our ability to capture cell decision events at the single cell resolution. Transcriptomics is typically used to infer cell state, and nucleotide sequences can be readily amplified. However, transcriptomics cannot reliably measure the functional state of these cells because a cell's state is more proximally set by its proteomic profile; protein abundance and post-translational modifications (PTMs) are not captured through transcriptomics. Single-cell proteomics are a robust means to infer a cell's activity; however they are less scalable, and proteins present in low copy numbers remain challenging to quantify. Additionally, existing fluorescence-based imaging limits the ability to distinguish between isoforms or isolate PTMs. Here, we present single-cell Western (scWestern) blotting with DNA-barcoding (scProteome-seq), which enables the quantification of protein expression at the individual cell level, providing insights into cell-to-cell heterogeneity in protein abundance and PTMs. scProteome-seq includes a DNA barcode step that contains spatial information for individual cells, which removes reliance on the optical detection of antibodies and enables multiplexed readout of single-cell proteomic state. Following a standard scWestern, individual cells are lysed and proteins are electrophoretically separated and immobilized onto nitrocellulose. Oligonucleotide-conjugated antibodies that bind to protein analytes are captured onto a DNA microarray chip to assess protein amounts and positions on single-cell blots. Preliminary evidence suggests compatibility of this high-throughput DNA barcoding technique with next-generation sequencing (NGS). We demonstrate antibody-mediated transfer of signal from spotted purified proteins onto nitrocellulose and subsequent transfer onto the DNA microarray. Then, we reconstruct this transfer on the DNA microarray from NGS readout by mapping the location IDs from the DNA library to a coordinate matrix in Python. This produces a 16-bit representative image of the spatial map of NGS reads. Future advances in scProteome-seq will advance the scalability of single-cell proteomics, enabling multiplexed detection of protein states to advance our understanding of mechanisms that govern cell state in development, homeostasis, and disease.

B365/P1811

Organelle packing interactions and cell anatomy patterns revealed by whole-cell imaging: a two-pronged approach

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The past decade has uncovered the complex interconnectivity of organelles through contact sites and functional crosstalk. The next frontier is how the whole organelle network spatially arranges at the meso- and whole-cell scales. What are the structural patterns and physical rules defining the state of the organelle network? To what extent is cell anatomy a reflection of organelle confinement in the crowded cytoplasm? Addressing these questions requires minimal-artifact, high-resolution whole cell multi-organelle reconstructions, with sufficient throughput to analyze morphological variation and perform robust comparisons of cell populations. Such data are challenging to generate, typically limiting single-study sample size. We address this with two complementary approaches: 1) a high-throughput study in budding yeast, and 2) a synthesis of whole-cell imaging literature. We have leveraged auto-segmentation to create the largest-yet dataset of whole-cell images of S. cerevisiae using soft X-ray tomography, featuring nucleus, vacuole, and lipid droplets for 490 cells across 3 strains at 30-35nm isotropic resolution. Statistical 3D morphometrics show that vacuole enlargement progressively disrupts nuclear scaling, position, and shape. Spherical harmonics-based shape modeling points to key morphological features driving anatomical variation in wild-type and large-vacuole populations. Quantitative whole-organelle shape analysis and biophysical simulations suggest that asymmetric deformations may arise due to vacuole-nucleus packing, with directionality dependent on differential organelle rigidity. Ongoing work extends our statistical and biophysical modeling to incorporate bulk high-resolution shape metrics. We also adapted evidence synthesis methods to comprehensively curate the whole-cell imaging literature between 2004-2024. Using a “scoping review” methodology, we assembled a corpus of 89 studies representing 13 imaging modalities and a large range of cell types, biological contexts, and organelle combinations. This establishes a knowledge base for cell anatomy research and secondary analysis, and motivates a call for standardized study design, reporting, and data sharing. Ongoing work merges organelle metrics to identify common and context-specific anatomical patterns and organelle relationships. Complementing our yeast SXT study, we find additional evidence that physical packing of organelles in the limited cytoplasmic volume plays an underappreciated role in establishing organelle morphology and global cell patterning. Future outlooks include establishing a community resource dedicated to cell anatomy data, and expanding evidence synthesis methods to drive meta-science work and novel discovery in cell biology.

B369/P0945

Preferential Host Cell Targeting by Toxoplasma gondii: Disentangling Stochasticity from Selection

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The single-celled eukaryotic parasite Toxoplasma gondii is able to invade almost any vertebrate cell using an active process driven by the parasite’s actomyosin cytoskeleton. This allows it to infect a wide variety of hosts and aids its ability to disseminate through host tissue, including traversal of critical barriers such as the blood–brain barrier. Given this remarkable ability, we ask: to what extent do T. gondii “choose” their host cells? Is their wide tropism coupled to an indiscriminate invasion strategy, or can they preferentially select a certain cell over its neighbor? We test whether host cell invasion is purely stochastic or shaped by host cell heterogeneity and parasite communication. Using infections of human umbilical vein endothelial cells (HUVECs), we quantify parasite distributions per host cell by analysis of immunofluorescence images. We use statistical modeling to predict stochastic infection distributions, and we establish an experimental baseline through control assays with myosin-inhibited parasites. These results provide a null model against which to compare invasion outcomes in normal infections. Our preliminary analyses suggest that T. gondii invasion is not stochastic, but instead reflects preferential targeting of certain host cells. We explore whether heterogeneous susceptibility

arises from host cell phenotypic states or from “priming” by prior invasion events, and we aim to quantify the contribution of these factors to invasion outcomes using statistical analyses of invasion distributions. This approach allows us to disentangle the role of host cell heterogeneity and the role of parasite manipulation in shaping susceptibility to T. gondii infection.

B367/P0943

Spatial represention of microglia functional programs as correlated transcriptional modules with MERFISH

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Microglia present a spectrum of functional states to support development, maintain homeostasis, and respond to disease. While neuroimmune function is necessarily molecular and supported by transcription, microglial functional states remain largely defined in terms of “associated” context. Protein biomarkers offer only a coarse view of activation and single-cell transcriptomics analyzed by manifold learning rarely return cluster-specific genes. The lack of definitive molecular markers for neuroimmune states, suggests a different model of microglial function and method of analysis. Here we posit a concurrent network model of microglial function, wherein observed states are a result of overlapping transcriptional programs, with no one program uniquely expressed in any state. We explore the validity of weighted gene correlation network analysis (WGCNA) as a representation of cell function using the Allen Brain Cell Atlas (ABCA), then build a network model from mouse scRNA-seq data and finally experimentally test model validity with spatial transcriptomics using Multiplexed Error Robust Fluorescence In Situ Hybridization (MERFISH). Using ABCA, we compared a standard cell state model based on differential expression (DE), compared accuracy metrics in cell types with ground truth labels, and evaluated informativeness in microglia based on GO enrichment. This was compared against high-dimensional WGCNA to identify and evaluate modules of highly co-expressed genes. While hdWGCNA was highly informative compared to DE, this was at the cost of cell specificity. To represent candidate networks in single cells, hdWGCNA modules were represented as eigengenes, projected onto UMAP, then spatially mapped using the ABCA MERFISH dataset. We then moved to a publicly available scRNA-seq dataset of mouse microglia generated by Hammond and Stevens 2019, taking a subset of only healthy adult mice vs a lysolecithin injury model. To validate our candidate transcriptional networks in their biological context, we used module eigengenes to generate a custom probe list for MERFISH. Replicate MERFISH datasets were acquired for naïve and injury model mice and gene modules were represented as eigengene expression projected onto a spatial mapping of microglia in the mouse brain. Our early data suggests module eigengenes can represent transcriptional networks, both in scRNA-seq data, and spatially, using MERFISH. The concurrent network model of neuroimmune function offers a useful means to molecularly define functional states of microglia when unique biomarkers are lacking and better infer neuroimmune biology from histological samples.

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