Abstract Program

2024 Cell Biology and Physiology Research Day

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Chatham County Convention & Agriculture Center
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UNC SCHOOL OF MEDICINE
Cell Biology and Physiology

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Clathrin alternative splicing promotes proper skeletal muscle development

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The clathrin triskelion, composed of three clathrin heavy chain (CLTC) monomers with bound light chains, regulates clathrin-mediated endocytosis, cytoskeletal rearrangement, and intracellular trafficking. Alternative splicing generates two Cltc transcripts: a long form that includes a 21 nucleotide micro-exon (exon 31) and a short form that skips it. Inclusion of exon 31 is almost exclusive to striated muscles. In healthy skeletal muscles, the short CLTC isoform is predominant in fetal stages, whereas the long form is the primary isoform in adulthood. Cltc splicing is mis-regulated in skeletal muscle diseases such that the short form is predominant throughout development and adulthood. It was not known, however, whether Cltc exon 31 inclusion varies among different skeletal muscles and/or impacts skeletal muscle development. Here we show that the inclusion of exon 31 is more pronounced in skeletal muscles that contain a high proportion of fast-twitch fibers. To determine the functional consequences of Cltc mis-splicing on the development of skeletal muscles, we forced exon 31 skipping in mice using CRISPR/Cas9 gene editing. Adult homozygous (HO) mice, which express only the short CLTC isoform throughout development, exhibited decreased forelimb grip strength and muscle endurance capacity. HO mice displayed increased skeletal muscle mass due to hypertrophy of individual fibers. Both fast- and slow-twitch fibers were enlarged in HO mice, although fast-twitch fibers were enlarged to a greater extent than slow-twitch fibers. HO skeletal muscles also exhibited swollen mitochondria, whose morphology and ultrastructure were suggestive of impaired performance. Our data suggest that Cltc splicing is essential for the proper development of fast- and slow-twitch muscle fibers.

Decoding the molecular basis of Shank3-mediated behavioral outcomes in an early-life sleep disruption paradigm.

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The lifetime maximums of sleep amount and intensity occur in the first years of life. Early life is also a time of significant cortical development with implications for long term health. Sleep pathologies are a very common comorbidity of Autism Spectrum Disorders (ASDs); in the case of Phelan McDermid Syndrome (PMS), sleep disruption emerges early in life and persists into adulthood, and is accompanied by autism, intellectual disability, seizures, and other changes. Most cases of PMS are caused by mutations or deletions in Shank3, which encodes a prominent synaptic scaffolding protein that supports synaptic plasticity. We used a mouse model with C terminal truncation of Shank3 to determine whether the PMS-modeling Shank3+/ΔC mouse showed the same sleep disruption phenotype as the Shank3ΔC/ΔC mouse, and at what age these differences became detectable. We applied an early-life sleep disruption treatment to Shank3+/ΔC and discovered that ELSD interacted with genetic vulnerability to produce long-lasting, sex-specific behavioral changes. We intend to investigate the impact of ELSD on the synapse in this Shank3+/ΔC model. This will further lead to the discovery of molecular drivers of the behavioral changes we observed.

Deletion of Netrin-1from Renal Stromal Progenitors Results in Abnormal Vascular Patterning and Protection from Kidney Ischemia

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Netrin-1, an angiogenic guidance cue, is heavily expressed in the kidney. Our lab has previously reported that deletion of Netrin-1 from Foxd1-positive stromal progenitors (*Foxd1GC/+Netrin1fl/fl*) results in abnormal vascular patterning during kidney development, including a reduction in interlobar branching. However, it remains unknown how these changes in vascular patterning affect kidney function and physiology. In the current study, we hypothesized that changes in developmental patterning of the kidney vasculature would protect from renal ischemia-reperfusion injury in a sex-specific manner due to ischemic preconditioning to the kidney tubular cells. To test this hypothesis, we performed warm, bilateral ischemia-reperfusion by clamping the renal pedicles for 26-minutes in wild-type (*Netrin1fl/fl* and *Netrin1fl/+*) (4M, 4F), heterozygous (*Foxd1GC/+Netrin1fl/+*) (3M, 4F), and mutant (*Foxd1GC/+Netrin1fl/fl*) (6M, 6F) male (M) and female (F) mice. Following 24-hours of reperfusion, blood and kidneys were collected for analysis of kidney injury markers. We found that plasma creatinine (p2-wayANOVA=0.021) and blood urea nitrogen (p2-wayANOVA=0.009) were decreased in female mice compared to male mice regardless of genotype. In both male and female mutant mice, there was a decrease in these markers of injury compared to control, although this did not reach statistical significance. In agreement with these data, tubular injury scores (0-100%, PAS stain) indicate male and female mutant mice have significantly less injury than control mice (pGeno=0.009, psex=0.012). Immunofluorescent staining of kidney injury marker 1 (KIM-1) also suggests less tubular injury in mutant mice as staining was localized to the renal medulla in mutants but was observed in the renal cortex and medulla in control males. These data suggest that Netrin-1 mutant mice with abnormal vascular patterning are less susceptible to ischemic injury. Further, female mice, regardless of genotype, were protected compared to male mice. Future studies will investigate the underlying mechanisms which drive this injury phenotype between sexes. Understanding how renal vascular development impacts kidney injury is critical to the development of new therapeutic strategies to prevent the sustained injury following periods of ischemia.

ARAPas for a localized redox intervention at the atherosclerotic plaque

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Atherosclerosis is the underlying cause of most cardiovascular diseases, which represent the principal cause of death in the USA and worldwide. Atherosclerosis consists of the growing accumulation of lipids, immune cells, and cell debris in the subendothelial region, which causes impaired blood flow and could also end in triggering major complications such as stroke and myocardial infraction. Infiltration of immune cells into the atherosclerotic plaque consists in one of the pivotal steps that contribute to plaque progression, the secretion of cytokines and oxidant molecules provoke oxidative stress and perpetuate the inflammatory process. While numerous pre-clinical studies suggest a connection between oxidative stress and atherosclerosis, there is no effective translation of systemic redox-based therapies to clinical applications. Therefore, employing nano-encapsulated delivery approaches may be the key to successful clinical translations of redox therapies.

As cause of oxidative or electrophilic stress, the activation of the Nrf2 pathway induces the synthesis of several antioxidant and detoxifying enzymes. We hypothesized that localized activation of Nrf2 within the atherosclerotic plaque may prevent its progression. To achieve this, CDDO-me, an FDA approved Nrf2 activator, was encapsulated by flash nanoprecipitation in what we call “antioxidant response activating particles (ARAPas)” and was administered to atheroprone mice under a high fat diet.

We found that mice treated with ARAPas showed an increase in Nrf2 activation, reduced lipid oxidation and inflammation markers within the atherosclerotic plaque. Overall, our data indicate that this is a promising redox-based intervention that relies on the localized activation of Nrf2 at the atherosclerotic plaque.

Mapping the transition from senescence induction to a stable, SASP-producing phenotype

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Cellular senescence is a phenotypic state that contributes to the progression of age-related disease through the senescence associated secretory phenotype (SASP). Understanding how senescent cells emerge over time from populations of healthy cells under stress is critical for improving identification of senescent cells and understanding the heterogeneity of senescence. To explore how the duration of senescence modulates the SASP, we use Iterative Indirect Immunofluorescent Imaging (4i) to build a single-cell proteomics map of RPE cells at increasing durations of senescence. 4i allows for repeated immunofluorescence of the same sample, with washes between imaging rounds removing previous antibodies. Using 4i we can capture the expression of up to 60 different proteins at the single cell level. We can then map how the expression of SASP proteins increases alongside the expression of other known senescence markers such as $p16^{INK4a}$. Preliminary analysis of the data in bulk shows that RPE cells under long-term etoposide treatment follow a program that can be split into two primary phases. The induction phase, which occurs through day 11, and the stable phase that is largely consistent from day 11 through the end of our observations at 31 days. During the induction phase cells increase in size and begin increasing production of $p16^{INK4a}$, p53, phospho-p65, and SASP factors such as IL-6 and IL-8. The beginning of the stable phase is marked by an abrupt increase in several proteins, including PARP1 and GATA4. This increase coincides with a plateau in expression of the proteins that were upregulated during the induction phase. Single cell analysis is ongoing, but initial hierarchical clustering shows the emergence of a discrete highly senescent, SASP producing subset of cells that begin to accumulate in the stable phase. These data highlight the use of single-cell proteomics to identify the mechanistic pathways that govern the transition from senescence induction to a stable state of senescence characterized by the SASP.

Dissecting the Role of Folate-Driven One Carbon Metabolism in Tumor-Associated Macrophages

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The emergence of immune-checkpoint blockade cancer immunotherapies, such as anti-PD-1 (aPD-1), that activate a patient's adaptive immune response against their malignancy have revolutionized the cancer treatment landscape. Despite this success, a large portion of cancer patients remain refractory to immunotherapy. Resistance to aPD-1 has been associated with the expansion of a subset of suppressive immune cells that dampen the anti-tumor immune response, called tumor-associated macrophages (TAMs). Most TAMs phenotypically resemble anti-inflammatory, immunosuppressive M2-polarized macrophages that perform endogenous immunoregulatory functions, including wound-healing, tissue repair, and induction of peripheral tolerance for the prevention of autoimmunity. However, in the context of cancer, these M2-like TAMs are maladaptive and, through a variety of different mechanisms, prohibit an effective immune response against the tumor. Therefore, the development of novel strategies to therapeutically reprogram TAMs to an anti-tumor cell state is a clinically relevant approach to overcome cancer immunotherapy resistance and broadly improve patient outcomes. Using massively parallel single-cell RNA-seq analysis, our lab has identified a population of folate receptor β (FRβ)-expressing immunosuppressive TAMs that are enriched in the tumor microenvironment of aPD-1 resistant tumors. FRβ is a GPI-linked membrane protein exclusively expressed on the surface of activated macrophages that functionally mediates the uptake of folate and its reduced derivatives from the extracellular environment. Folate is a requisite cofactor for the one-carbon (1C) metabolism pathway, which facilitates the maintenance of cellular redox balance, nucleotide synthesis, and methylation of DNA, proteins and lipids. Despite correlative findings demonstrating the importance of 1C metabolism for M2 macrophage immunobiology, the mechanistic role of folate metabolism in driving TAM proliferation/survival, polarization, and immunosuppressive function in the tumor microenvironment is not known. Based on preliminary findings from our lab and others, we hypothesize that TAMs require FRβ for folate uptake and downstream 1C metabolism to sustain proliferation/survival in the tumor and functional suppression of the anti-tumor immune response. Furthermore, we hypothesize that disruption of FRβ-mediated 1C metabolism in TAMs will improve the efficacy of aPD-1 treatment in immunotherapy resistant cancer models.

The role of DDX3X in cardiac sex-differential protein expression

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Sex disparities exist in cardiac anatomy and physiology and the prevalence of types of heart disease. These differences are often attributed to sex hormones. Through a series of proteomics-based approaches, we demonstrated that in addition to sex hormones, genes on the X-chromosome are both necessary and sufficient to regulate cardiac sex-chromosome-derived protein differences (CSCDPs). Genetic screens in mice identified DEAD-Box Helicase 3 X- Linked (*Ddx3x*) as a candidate for regulating CSCDPs. Consistently, mutations in *Ddx3x* have been shown to cause congenital heart disease in females predominantly. To date, no investigation into the cardiac function of DDX3X has been performed. We have generated a *Ddx3x* cardiomyocyte conditional allele in mice. My preliminary data demonstrate that *Ddx3x* is essential for embryonic cardiac viability, with females but not males dying prior to E11.5. DDX3X has multiple proposed functions in RNA biology. Consequently, we have immunopurified the endogenous cardiac interactome under physiological conditions and demonstrated that DDX3X interacts with ribosomal proteins in the developing heart. To identify the E10.5 DDX3X cardiac target mRNAs, we performed Enhanced Crosslinking and Immunoprecipitation (eCLIP) in parallel with quantitative proteomic analyses of male and female *Ddx3x* mutant hearts. Our results imply that DDX3X functions to regulate translation initiation within the female but not the male mammalian heart at E10.5. To this end, our future studies will focus on determining the translationally dependent targets of DDX3X in the heart, thus providing insight into the basis for cardiac sex differences and potential clinical relevance for CHD patients.

Spatial modeling of the Wnt-associated planar polarity complex in collective migration of primary human intestinal epithelium

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The human intestinal epithelium (hIE) lines the entire inner surface of the intestine, handling all of the body's nutrient and fluid uptake while simultaneously serving as a barrier to toxic luminal contents and pathogens. To combat the constant damage human intestinal epithelial cells (hIECs) face, the hIE utilizes collective migration as both a rapid wound healing response, and as part of the homeostatic process of hIE renewal. Despite the importance of collective cell migration to intestinal function, little is known about how hIECs coordinate this process. The WNT-associated Planar Cell Polarity (WPCP) pathway, centered around the cell junction proteins CELSR, FZD, and VANGL2, has been increasingly implicated as a major player in the coordination of collective cell migration, but research is hampered due to both: 1) the lack of a comprehensive computational model of WPCP, and 2) a deficit in physiologically relevant model systems for studying WPCP in higher organisms. Here, I combine a compartmental ODE model that connects key WPCP-complex formation and phosphorylation events with an experimental system utilizing wound healing assays of novel genetically engineered primary human intestinal stem cell lines to test if the WPCP complex coordinates hIE migration during wound healing. My results demonstrate that: 1) WPCP-complex bistability strongly depends on negative feedback involving VANGL2 phosphorylation by FZD, 2) VANGL2 protein concentration and localization are dynamically regulated during both homeostasis and wound repair in the hIE, 3) sporadic overexpression of VANGL2 in an hIE monolayer is sufficient to repolarize the surrounding epithelial tissue and strongly impair wound healing. Together, these results demonstrate that the hIE utilizes the WPCP pathway to help coordinate collective cell migration during wound healing and provide a novel framework for studying the pathological roles of WPCP in other contexts.

Defining the role of MYC in KRAS-dependent pancreatic cancer

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Pancreatic ductal adenocarcinoma (PDAC) is the third leading cause of cancer-related deaths in the United States and standards-of-care are limited to ineffective cytotoxic chemotherapy. Mutationally activated KRAS is found in 95% of cases and drives PDAC growth predominantly through activation of the ERK mitogen-activated protein kinase (MAPK) cascade. Despite the key role of this signaling pathway, how ERK MAPK signaling supports KRAS-dependent PDAC growth remains to be established. While ERK regulates a complex phosphoproteome (>2000 direct/indirect substrates), we hypothesized that one substrate, the MYC oncoprotein and transcription factor, is the critical driver of KRAS- and ERK-dependent PDAC growth. We first applied reverse phase protein array (RPPA) analyses and determined that KRAS and MYC regulate significantly overlapping oncogenic signaling networks. Then, to delineate the contribution of MYC to KRAS-driven PDAC and establish a system-wide profile of the MYCdependent transcriptome, we applied RNA-sequencing of PDAC cells with acute siRNA suppression of MYC. Gene set enrichment analysis determined that MYC-regulated genes control diverse KRAS-driven cellular processes. We then used genetic suppression of KRAS or MYC to validate these processes, including RHO small GTPase activation, EMT, mitochondrial dynamics, and autophagy. Our ongoing analyses have profiled the KRAS- and MYC-regulated kinome to assess the involvement of MYC in KRAS-driven kinome remodeling in PDAC. In summary, our studies establish a significant role for MYC in diverse KRAS-driven cellular activities and support the provocative concept that inhibiting MYC function may be an effective strategy for targeting KRAS for PDAC treatment.

Melanoma cytoskeletal dynamics, adhesion, membrane remodeling, and motility are regulated by TRIM9

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Cell shape change and motility are essential in metastasis and involve remodeling of the actin cytoskeleton, cell adhesions, and plasma membrane. How these cytoskeletal and membrane remodeling are regulated and coordinated remains largely unknown. We previously identified TRIM9 as a regulator of actin dynamics and exocytosis in developing neurons. Deletion of murine *Trim9* impairs neuronal migration, netrin-induced axon turning, and axonal and dendritic branching, and increased exocytosis and filopodial stability. In addition, we have shown TRIM9 alters dynamics of the actin polymerase VASP at filopodia tips via non-degrative mono-ubiquitination. TRIM9 is expressed in other motile cells, but the non-neuronal role of TRIM9 remains unknown. TRIM9 was identified as a possible prognostic biomarker in melanoma and high expression correlates with low patient survival. Melanomas undergo phenotype switching, where three distinct phenotypes exist that are associated with differences in gene expression. The three phenotypes consist of a melanocytic proliferative state, a neural crest-like invasive state, an intermediate state. Single cell RNAseq (scRNASeq) data from patient-derived melanoma indicate TRIM9 is highly expressed in the proliferative melanocytic state, which correlates with poor prognosis. *We hypothesize that TRIM9 coordinates actin dynamics, adhesion, and exocytosis in melanoma.* We show that TRIM9 protein is enriched in several human melanoma lines. Here we examine the role of TRIM9 in regulation of focal adhesions, exocytosis, migration, and invasion in two of these lines. Genetic loss of TRIM9 increased random migration velocity but reduced directional persistence. Interestingly, we find that TRIM9 plays a role in promoting bleb-like morphology and inhibits the ability to durotax on shallow gradients on soft substrate. Fluorescence recovery after photobleaching (FRAP), Total internal reflection fluorescence microscopy (TIRF), and widefield microscopy revealed that loss of TRIM9 results in increased focal adhesions, cell size, and altered dynamics of focal adhesion proteins VASP, zyxin, and paxillin dynamics. In addition, TRIM9 knockout cells exhibit reduced filopodial length and density and altered filopodial localization of VASP. In-gel zymography indicates that TRIM9 knockout cells also display an increased degradative capacity. Current studies are investigating how loss of TRIM9 alters parameters of cell contractility, lamellipodia, invadopodia, and exocytosis to define the role of TRIM9 in melanoma motility. Together these findings suggest TRIM9 reduces adhesion and migration, increases proliferation and blebbing and may be an important regulator of phenotype switching in melanoma.

Proximity interactome of lymphatic VE-cadherin reveals mechanisms of junctional assembly and reelin secretion.

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The endothelial specific adhesion receptor VE-cadherin (CDH5) plays a critical role in vascular development and maintenance by controlling vessel permeability and function. Within lymphatics, VE-cadherin transduces an array of signals that modulate crucial lymphatic cell behaviors including cell growth restriction, lumen and valve formation, and cytoskeletal assembly. Consequently, VE-cadherin must interact with a multitude of intracellular proteins to exert these functions and undergo dynamic rearrangement at the plasma membrane to alter adherens junctional architecture and vessel permeability. Yet, the full protein interactome of VE-cadherin in endothelial cells remains a mystery. Here, we use proximity proteomics to illuminate the unique interactome of VE-cadherin in lymphatic endothelial cells. Furthermore, we characterize how the plasma membrane VE-cadherin interactome changes during junctional reorganization from permeable discontinuous junctions to less permeable continuous junctions, triggered by the lymphangiogenic factor adrenomedullin. These analyses identified both predicted and novel VE-cadherin interactors that reveal a critical role for ARF6 and the exocyst complex in VE-cadherin trafficking and recycling to the plasma membrane during adherens junction assembly. We also identify a requisite role for adrenomedullin-VE-cadherin signaling in controlling the secretion of reelin—a lymphangiocrine glycoprotein with recently appreciated roles in governing heart size and injury repair.

Understanding how actomyosin dynamics drive apical constriction

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Animal embryogenesis involves the bending of epithelia into specific shapes, and apical constriction is a key cell shape change that accomplishes this. How apical constriction is driven by precisely localized actomyosin regulators remains poorly understood. C. elegans gastrulation provides a genetically and optically amenable model to address this question. The Arp2/3 complex and one of its nucleation-promoting factors (NPFs), WAVE, have been shown to play important roles during C. elegans gastrulation. To understand the mechanisms through which they function, we first compared actin dynamics in wild-type and Arp2/3-depleted embryos. We found that the Arp2/3 complex controls F-actin density at specific locations. To understand how Arp2/3 is locally regulated within cells, we imaged embryos with endogenously tagged Arp2/3 complex and NPFs. We found that each of the three NPFs – WAVE, WASP, and WASH – colocalizes with Arp2/3 at distinct subcellular locations. Further, depleting each NPF led to Arp2/3 reduction at each of these subcellular locations. These results indicate that three NPFs modulate Arp2/3 activities at different subcellular locations, which we have exploited as a means to study the contributions of different populations of Arp2/3 in apical constriction. We found that depleting subsets of Arp2/3-rich structures by knocking down each NPF alone or in combination led to specific changes in F-actin localization. These changes are likely the cause of gastrulation defects that depend on specific NPFs disproportionately, with WAVE being the main contributor. We further showed that WAVE and Arp2/3 are recruited to cell-cell contacts by Rac. Our results pose hypotheses that Rac signaling activates WAVE and Arp2/3 at a subset of cell-cell contacts to produce branched actin networks, which play important roles in changing cell shape. We are currently investigating the downstream cell biological processes controlled by Rac, WAVE, and Arp2/3 that are important for apical constriction.

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 even greater inhibition of LPL. ANGPTL8 is only found in the bloodstream in complex with ANGPTL3. However, where this complex forms, and why ANGPTL8 requires ANGPTL3 for secretion remains a mystery. This proposal aims to understand why ANGPTL8 is unstable when it is not in a complex with ANGPTL3, as well as visualizing the trafficking dynamics of the ANGPTL3/8 complex.

Gigaxonin Kelch-3 and Kelch-4 motif deletions unmask intermediate filament phenotypes that mimic Giant Axonal Neuropathy (GAN) cells

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Gigaxonin is an intermediate filament (IF)-interacting partner that belongs to the 42-member family of Kelch-like (KLHL) proteins. KLHL proteins form diverse molecular interactions and regulate protein degradation and trafficking in different cell types. *KLHL16*, the gigaxonin-encoding gene, is mutated in the pediatric neurodegenerative disease Giant Axonal Neuropathy (GAN). The lack of functional gigaxonin in GAN patient cells impairs IF proteostasis, leading to localized IF protein accumulation and compromised cellular function, especially in neurons. Molecular level-understanding of gigaxonin-IF interactions can provide new insights into IF proteostasis regulation and GAN disease mechanisms. We hypothesized that gigaxonin forms molecular interactions via specific sequence motifs to regulate IF proteostasis. The goal of this project was to determine how deletion of each individual Kelch repeat on gigaxonin affects IF protein degradation and filament morphology. To accomplish our goal, we compared vimentin protein levels and filament organization in HEK293 cells over-expressing wild type (WT) gigaxonin, or gigaxonin lacking each of the six Kelch motifs: ΔK1 (aa274-326), ΔK2 (aa327-374), ΔK3 (aa376-421), ΔK4 (aa422-468), ΔK5 (aa470-522), and ΔK6 (aa528-574). We discovered that all gigaxonin deletion mutants (ΔK1-6) promoted the degradation of soluble vimentin to a similar extent as WT gigaxonin, suggesting that each individual Kelch motif is dispensable for this activity. However, comparison of vimentin filament organization revealed bundling and aggregation in cells expressing ΔK3- and ΔK4-gigaxonin compared to all other conditions, including untransfected and WT gigaxonin-transfected cells. Using immunoprecipitation of GFP-tagged gigaxonin followed by mass spectrometry analysis, we discovered that the ΔK3 and ΔK4 deletions eliminated the association between gigaxonin and NUDCD3, a molecular chaperone that is enriched in the nervous system, associates with the cytoplasmic dynein complex, and regulates mitochondria motility. Collectively, our cell biological data show the induction of a GAN-like IF phenotype in cells expressing ΔK3- and ΔK4-gigaxonin, while our mass spectrometry profiling links the loss of gigaxonin-NUDCD3 interactions with defective IF proteostasis.

Functional Impacts of Endoplasmic Reticulum Composition in CAR-T Cells

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While CAR-T cell therapy has revolutionized hematological cancer treatment, matching success in solid tumors remains elusive. Clinical trials of CAR-T therapies have utilized CD28 and 41bb signaling domains in the CAR-T cell design. CD28 CAR-T cells possess superior antitumor capacity with low persistence in patients, compared to 41bb CAR-T cells detected in patients up to 4 years later. Solid cancers possess a harsh tumor microenvironment that can cause endoplasmic reticulum (ER)-mediated deficiencies of T cell persistence and antitumor activity, and importantly, the ER has not been characterized in CAR-T cells previously. Here, we examine the ER between CD28 and 41bb CAR-T cells microscopically, biochemically, and by spectral flow cytometry. We show that ER composition reflects phenotypic indicators of T-cell memory and persistence, and our alteration of this composition increases markers of persistence. This work is the first to compare ER composition between CAR-T cells, and shows manipulation of the CAR-T cell's ER as a new target for the improvement of CAR-T cell therapy.

Tissue-layer specific analysis of palatogenesis and periderm development through cell adhesion signaling

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Cleft palate (CP) is among the leading congenital diseases in the US and across the globe. CP results from a failure to separate the oral and nasal cavities during embryogenesis, a process known as palatogenesis. One contributing factor to the widespread nature of CP is the over 200 genes in humans and mice that when mutated disrupt palatogenesis. Among the CP-linked genes, components of the adherens junction (AJ) complex expressed in the oral epithelia, have garnered recent attention. Mutations in genes that encode AJ receptors, like cadherins (*CDH1*) and nectins (*NECTIN1*), are associated with CP in humans. However, we still do not understand the mechanism by which AJ receptors facilitate palate closure. To address this, our lab has disrupted nectins and cadherins, as well as their downstream signaling effectors, to determine their effect on palate closure. Currently, we are focused on segregating the relative contribution of AJ signaling across the two oral epithelia layers, the progenitor basal keratinocytes and the protective periderm layer. To do so, we are knocking down and knocking out AJ proteins in both a periderm-specific manner as well as a whole oral epithelial loss. Our lab utilizes two genetic manipulation techniques: 1) traditional Cre/LoxP mediated gene knockout as well as 2) *in-utero* guided lentiviral injections that transduce the outer ectoderm with short-hairpin RNAs (shRNAs) and gene expression constructs. Preliminary evidence has suggested that periderm-specific loss of E-cadherin (*Cdh1*) demonstrates a similar palatal fusion defect as *Cdh1* knockdown in both oral epithelial layers. This suggests that E-cadherin signaling is at least in part required in the periderm. Future directions are focused on determining if periderm-specific loss of Afadin (*Afdn*), the downstream nectin binding partner, can drive CP at the same penetrance as whole epithelial *Afdn* loss does. These experiments aim to discern between two possible functions of the periderm. A passive function, where the periderm simply protects underlying basal keratinocytes from unwanted intraoral adhesions. Or an active role, where the periderm participates in palatal fusion through AJ-based cell adhesion programs. Moreover, this work continues to elucidate important signaling mechanisms that regulate palatogenesis with thepotential to generate novel models of CP in mice.

Coronin 1A and TRIM67 collaborate in netrin-dependent neuronal morphogenesis

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Neurons progress through several developmental stages during the establishment of neuronal circuitry. The dramatic neuronal shape changes during these morphological events depend on the cytoskeleton remodeling machinery. Previously, our lab discovered that the E3 ubiquitin ligase TRIM67 is highly enriched in the developing cortex and is essential for appropriate neuronal morphogenesis. We found that TRIM67 localizes to growth cone and actin-rich filopodia structures. Interestingly, neurons lacking TRIM67 exhibit aberrant axonal growth cone morphology and defects in netrin-dependent axon turning and branching, but the underlying molecular mechanisms by which TRIM67 regulates neuronal morphogenesis is unknown. As E3 ubiquitin ligases typically have multiple substrates, we conducted an unbiased proximity-dependent biotin identification assay to identify putative TRIM67 substrates. This identified Coronin 1A (Coro1A) as a potential TRIM67 binding partner. Coro1A is a conserved F-actin binding protein crucial for actin dynamics, yet its role in brain development remains elusive. We validated that Coro1A and TRIM67 are interacting partners and combining computational structural/docking analysis with biochemistry assays we show that the C-terminal coiled-coil domain of Coro1A is essential for Coro1A:TRIM67 interaction. We demonstrate that Coro1A protein level increases during neuronal maturation in vitro and in vivo. Through TIRF microscopy and immunofluorescence staining, we find that Coro1A is enriched in growth cones of developing cortical neurons and localizes to the base of filopodia structures, proximal to the cofilin-coated actin bundles known as cofilactin. We found loss of Coro1A or TRIM67 increases the length of cofilactin. Using cultured cortical neurons from *Coro1A*$^{+/+}$ and *Coro1A*$^{-/-}$ littermates, we show that Coro1A is crucial for netrin-dependent growth cone and axon branching morphology, similar to TRIM67. Additionally, by performing rescue experiments we show that Coro1A:TRIM67 interaction is important for neuronal shape change. Finally, we demonstrate that Coro1A is ubiquitinated by immunoprecipitating Coro1A under denaturing conditions. *These findings suggest a novel role for Coro1A in regulating neuronal morphogenesis, likely functioning downstream of TRIM67.*

Studying the Role of Brain Enriched E3 Ubiquitin Ligase TRIM9 in Alzheimer's Disease

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In the last decade, we have demonstrated that the brain-enriched E3 ubiquitin ligase TRIM9 regulates cytoskeletal dynamics, membrane remodeling, and netrin-dependent signaling pathways in all stages of neuron development, including the maturation of dendritic spines and electrophysiological activity. Moreover, TRIM9 protein levels increase in the adult brain and are maintained throughout adulthood. In the adult mouse brain TRIM9 is enriched within the postsynaptic density (PSD), a proteinaceous rich region in the post synapse, containing neurotransmitter receptors, scaffolding proteins, and cytoskeletal elements. The PSD proteome is significantly altered in adolescent mice when TRIM9 is lost, which is linked to changes in synaptic function. Our published proximity labelling experiments to characterize the TRIM9 interactome identified several proteins implicated in synaptic function and Alzheimer's disease (AD), including Tau.Cross-referencing this list with a published dataset of differentially expressed proteins (123) in the hippocampal PSD of 9 month old Tau P301S mice (PS19 AD model) versus non-transgenic control, revealed a significant percent (56 proteins, 45.5%) overlap.

Here we investigate the role of TRIM9 in tau-mediated neurodegeneration, since TRIM9 is poised to interact with many AD-associated proteins. Four genotypes were compared (*Trim9*$^{+/+}$, *Trim9*$^{-/-}$, *Trim9*$^{+/+}$*:PS19*, and *Trim9*$^{-/-}$*:PS19*), and 3-4 animals per genotype and sex were used in each experiment. We analyzed changes in dendritic spine density of six month old murine cortical and hippocampal neurons using the Golgi-Cox staining method. We also investigated changes in pathological tau accumulation, microglia and astrocytes activation using multiplexed immunofluorescence imaging and biochemical analysis. Iba1 positive microglia were significantly enriched in the hippocampus, entorhinal cortex, and amygdala of six month old *Trim9*$^{-/-}$*:PS19* mice compared to the other genotypes. Pathological tau accumulation had an increase trend in the hippocampus of *Trim9*$^{-/-}$*:PS19* mice compared to *PS19* only mice.

In conclusion, deletion of *Trim9* exacerbated the progression of Alzheimer's disease in six month old *PS19* mice. Future experiments will use primary cultured hippocampal neurons to further determine the mechanism through which *Trim9* deletion increases pathological tau accumulation.

CLK1 inhibition and mechanical stretch induce changes in the production of functional CLK1 and the phosphorylation of SR proteins in myotubes.

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Muscle cells use mechanotransduction to sense mechanical stimuli and respond to them via transcriptional and alternative splicing programs. Alternative splicing is an RNA processing mechanism that allows single genes to produce multiple transcripts. Striated muscle (cardiac and skeletal) has some of the most tissue-specific alternative splicing, due to their highly specialized myocytes. Numerous components of the sarcomere undergo alternative splicing regulation impacting contractility. Our lab has previously shown that cyclic mechanical stretch of skeletal muscle cells induces global changes in transcription and alternative splicing. Several differentially expressed and spliced transcripts were targets of the serine/arginine rich splicing factor 4 (SRSF4). CDC2-like kinase 1 (CLK1) regulates splice site regulation and subcellular localization of serine/arginine-rich (SR) proteins via phosphorylation. And Clk1 mRNA is itself a splicing target of SR proteins. We investigated the relationship between CLK1 kinase activity, SR protein phosphorylation, and mechanical stretch in muscle cells. After stretching or CLK1 kinase inhibitor (TG003) treatment, muscle cells increased the production of functional Clk1 transcripts. In stretched cells, SRSF4 phosphorylation increased. Treatment with TG003 resulted in increased CLK1 total protein while SRSF4 phosphorylation decreased. These findings show that CLK1 phosphorylation of SRSF4 impacts the production of functional CLK1 suggesting a feedback loop between mechanical stretching, CLK1 splicing, and SRSF4 phosphorylation. In summary, the function of SRSF4 as a mechanically sensitive RNA-binding protein that is regulated by CLK1 expands our current understanding of the interplay between mechanotransduction and alternative splicing in striated muscle.

Studies of the role of actin branching in organelle homeostasis and branch turnover by Coronin and GMFβ

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The actin cytoskeletal network is organized into branched and bundled structures. Arp2/3-mediated branched actin networks, well-studied in lamellipodia, are also present at endomembranes including sites of endocytosis, vesicular trafficking, and interorganelle contact, where branched actin polymerization contributes to the force production that enables membrane scission events. To illuminate the role of actin branching and debranching at endomembranes, I am studying (1) organelle morphology and distribution in the presence and absence of the Arp2/3 complex and (2) the interactions of the Arp2/3 complex with actin debranching factors, GMFβ and Coronin1B. Using Arpc2 conditional knockout mouse fibroblast cell lines, I observed alterations in the position and abundance of early and late endosomes, lysosomes, Golgi, and mitochondria, in the presence and absence of the Arp2/3 complex. In images acquired with confocal microscopy, I measured colocalization of Arpc2 and Coronin1B in cells with endogenously tagged fluorescent fusion proteins, and I quantified the dynamics of Arpc2 puncta in cells with endogenously tagged Arpc2 and overexpressed fluorescently tagged GMFβ. Point mutants of GMFβ illuminate the role of a phosphorylation site on GMFβ's debranching activity. While this initial work is done using overexpression models, it will be followed up with CRISPR knock-out and rescue lines, including taking a more targeted approach to disrupting endomembrane-localized branched actin by knocking out WASH (upstream Arp2/3 activator on endomembranes).

The intracellular role of APOE in glial cell lipid metabolism

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Alzheimer's Disease (AD) is a neurodegenerative disease that is the leading cause of dementia globally. The *APOE* gene is the strongest genetic risk factor for the development of AD. Relative to the common variant, *APOE3*, individuals who express *APOE4* display a marked increase in AD risk. APOE encodes for a secreted protein known as Apolipoprotein E. In the brain, APOE is primarily expressed by glial cells such as astrocytes and microglia and is important for the bidirectional transfer of lipids to and from neurons. This transfer of lipids is essential for the growth and maintenance of neuronal synapses. However, the intracellular function of APOE is not well characterized. Our lab has discovered an unexpected localization of APOE on the cytoplasmic surface of lipid droplets in astrocytes. This novel localization implicates a new role for APOE in lipid metabolism. To this effect, we have demonstrated that astrocytes expressing APOE4 display larger lipid droplets and increased levels of unsaturated triglycerides relative to APOE3 astrocytes. We hypothesize that APOE regulates lipid droplet turnover. There are two primary modes that the cell employs to turnover lipid droplets: lipolysis and lipophagy. In lipolysis, cytoplasmic lipases hydrolyze triglycerides in the droplets to fatty acids. Lipophagy is a selective mode of autophagy that enables the catabolism of neutral lipids from the lipid droplets via lysosomal lipases. Using pharmacological inhibitors of either adipose triglyceride lipase (ATGL), the most upstream lipolysis enzyme, or autophagy, we discovered that APOE primarily regulates lipid droplet turnover by lipolysis. We reasoned that lipid-droplet localized APOE might interact with proteins on the droplet surface to modulate lipolysis. To identify novel interacting partners of APOE on lipid droplets in an unbiased manner, we performed affinity-purification mass spectrometry. We identified multiple putative APOE-interacting proteins involved in lipid metabolism and signaling. We will continue to explore this mechanism by modulating the candidates identified by mass spectrometry. If targeting these proteins rescues the APOE4 lipid droplet phenotype, this could lead to novel targets for the treatment or prevention of Alzheimer's Disease.

The role of retrotransposons in pediatric epilepsy genetics

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Epilepsy is a chronic neurological disorder characterized by seizure activity that effects over 1% of the United States population including 470,000 children. Of this population, one third will be unable to control their seizures with currently available anti-seizure medication, which can contribute to detrimental developmental delays and lifelong disability. While genetic causes are thought to play a significant role in a subset of rare, heterogeneous disorders known as developmental and epileptic encephalopathies, over half of these patients still lack a genetic diagnosis, despite current clinical assessment including panel or exome sequencing for mutations in known epilepsy genes. Because these techniques only assess the tiny fraction of the human genome that encodes proteins, the answer could lie in intronic or regulatory regions outside of the exon. In contrast, whole genome sequencing (WGS) surveys these non-exonic regions, while long read sequencing (LRS) vastly expands the sequencing read length, enabling accurate mapping of highly repetitive and similar genetic regions. These methods offer the potential for insight into previously underappreciated sources of disease-associated genetic variants. One potential source of *de novo* causative genetic variants for epilepsy are retrotransposons. Retrotransposons are genetic sequences that are capable of transposing into new gene locations via a copy and paste mechanism and can disrupt function via direct gene insertion or downstream indirect mechanisms. Somatic mutations from retrotransposon activity have been linked to disease processes like neurodegeneration, autism, and various cancers. However, their role in generating *de novo* variants in pediatric epilepsy is still unknown. To investigate the role of retrotransposons, we will utilize the comprehensive transposable element analyzer (xTea) to accurately identify *Alu*, LINE1, and SVA retrotransposons in a cohort of epilepsy patients that have undergone WGS and LRS. The identified insertions will be compared to parental samples, if present, for the confirmation of *de novo* status, assessed for pathogenicity, and then stratified based on patient diagnosis and phenotype, known epilepsy genes, location of the insertion, and mechanism of gene disruption. Candidate insertions will then be confirmed in a neuronal cell culture model and assessed for functional disruption via multi-electrode array.

Plasma membrane expansion and remodeling during neuronal morphogenesis

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The plasma membrane (PM) of a developing neuron undergoes dramatic expansion and remodeling to establish the morphology necessary for neural network connectivity and function. The insertion of membrane material is facilitated by Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-mediated exocytosis, which is required for neuronal morphogenesis and axon guidance. Lipid transfer at endoplasmic reticulum (ER)–PM contact sites also contributes to membrane expansion and composition**.** How these two membrane remodeling pathways are regulated and their relative contribution to PM composition in developing neurons remains an open question. Here we investigate the molecular mechanisms regulating membrane dynamics during neuronal morphogenesis. Specifically, how focal adhesion kinase signaling regulates SNARE- mediated exocytosis and the interplay between Ca^{2+} signaling, SNARE-mediated exocytosis, and ER-PM contact sites during morphogenesis and axon guidance.

Validating the embryonic chick model for Vcn and MVcn Studies

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Vinculin (Vcn) and Metavinculin (MVcn) are core adhesion proteins that link the actin cytoskeleton to the cell periphery to modulate adhesion formation, strength, and cell motility. Vcn, a well-characterized mechanosensory protein, tightly links filamentous actin (F-actin) into bundles or stress fibers, which are key to maintaining adhesion strength and force transmission. Studies characterizing Vcn's larger splice isoform MVcn have confirmed its co-expression and localization to adhesions in the heart and smooth muscle cells. Yet MVcn's role in force transmission, cell morphology, and adhesion formation is understudied. Notably, patients with MVcn-specific mutations show defects in actin organization, cell morphology, and development of the often-fatal group of cardiac diseases, cardiomyopathies (CM). MVcn CM mutations result in dilated or hypertrophic CM, which decreases blood flow efficiency due to altered ventricular wall morphology. While both Vcn and MVcn bind F-actin, MVcn lacks F-actin bundling capabilities. When the actin-binding tail domains of Vcn and MVcn are mixed *in vitro,* actin bundling is diminished. These findings suggest that MVcn may negatively regulate F-actin bundling when co-expressed with Vcn. Moreover, MVcn CM mutations promote higher order, yet more disordered F-actin assembles. **Thus, I hypothesize that MVcn acts as a negative regulator of Vcn to fine-tune cell morphology and force transmission**. However, most analyses of MVcn and Vcn co-regulation have been limited to *in vitro* studies and in nonbiologically relevant systems. Hence, I will utilize the embryonic chick heart to examine how expression of MVcn in the context of Vcn modulates actin organization, adherens junction (intercalated disc in the heart) and focal adhesion (costamere) formation, and traction force microscopy via immunofluorescent confocal microscopy. The embryonic chick heart provides a quick and cost-effective opportunity for direct genetic manipulation of MVcn, which is not expressed until incubation day 9 (E9). Using this biologically relevant system, I can perform tissue-specific mutagenesis while eliminating the need for animal husbandry or transgenic line maintenance. Moreover, our efforts will establish the chick model for *in vivo* experimentation to understand the complexity of MVcn and Vcn interactions. Results from our initial studies will be presented.

The role of tubulin code in organelle dynamics during neuronal differentiation

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A functional nervous system depends on the correct neuronal morphology established during neuronal differentiation. Microtubule (MT) cytoskeleton is required for neurons to successfully complete all the morphological transitions of differentiation such as initiating neurite formation, setting up local cues for organelle positioning, and serving as tracks for intracellular organelle transport. The dynamics and properties of MTs are regulated by a variety of evolutionarily conserved post-translational modifications (PTMs). Among all the modifications, acetylation of α-tubulin lysine 40 and polyglutamylation are the major modifications that link to neurodegeneration. Crosstalk between the modified MTs and membrane-bound organelles has been found in non-neuronal cells. However, how neurons rearrange organelles through modifying tubulin PTMs to meet their transition from stem cells to neurons remains to be fully delineated. We recently found that organelle contact increases during the stage neurons generate and elongate axons, when tubulin PTMs are also enriched. We hypothesize that neurons spatiotemporally coordinate the distribution of MT PTMs throughout neuronal differentiation to achieve organelle reprogramming during the differentiation process.

Here, we derived cortical neurons (iNeurons) from human induced pluripotent stem cells (hiPSCs) and found that α tubulin K40 acetylation and polyglutamylation display distinct changes in abundance and subcellular localization during neuronal differentiation. We found that acetylated MTs tend to accumulate around perinuclear regions while polyglutamylated MTs seem to locate uniformly in the cell body throughout differentiation. We successfully downregulated MT acetylation and polyglutamylation in day 7 iNeurons using lentiviral shRNA targeting the major α-tubulin acetyltransferase, ATAT1, or polyglutamylase, TTLL1. Next, multispectral imaging was implemented to study how tubulin K40 acetylation affects eight membrane-bound organelles simultaneously in day 7 iNeurons. We found that reducing MT acetylation affects the morphology and contacts among multiple pairs of organelles. Together, our results suggest that changes in the amount and distribution of MT PTMs are a mechanism of organelle remodeling throughout neuronal differentiation.

Defining a role for ER-LD contact sites beyond lipid droplet biogenesis and under stress

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Eukaryotic cells compartmentalize cellular functions into discrete membrane-bound compartments. However, organelles interact at membrane contact sites (MCS) to exchange proteins, lipids and metabolites to further these functions. Lipid droplets (LD) are intracellular organelles composed of neutral lipids surrounded by a phospholipid monolayer. LDs store neutral lipid species to meet the future energy or membrane-forming needs of the cell, while also sequestering toxic species. LDs interact with other organelles and have their own proteome. LDs originate by accumulation of lipids between the bilayers of the ER, the major site of lipid synthesis, that then bud off with the help of proteins to form discrete organelles. Mammalian cells have both ER-linked and cytoplasmic LD. I hypothesize that there exist two types of ER-LD contacts in human cells: those important for LD biogenesis and those that form or persist in response to cellular stressors.

Using a dimerization-dependent fluorescence system (ddFPs), we can visualize MCS usually indistinguishable using conventional confocal microscopy approaches due to the diffraction limit of light. The two halves, targeted to different organelles, only fluoresce when in closer proximity than the diffraction limit, allowing for differentiation of bona fide MCS (<30 nm) from close but not interacting organelles. With this system, we have been able to visualize ER-LD contact sites at steady state and during LD biogenesis. Future imaging of ER-LD ddFPs under conditions of cellular stress will reveal how these sites remodel to increase cell survival and maintain homeostasis through modulating lipid and protein trafficking to and from the LD and whether these stressors induce or maintain additional contacts over baseline.

Spatial proteomics allows for the labelling of proteins within a discrete radius (~10 nm) around a promiscuous biotinylating enzyme linked to a protein of interest. Biotinylated targets can be identified using mass spectrometry. We have created constructs of proximity labelling enzyme split-TurboID, linked to ER- and LD-specific targeting sequences similar to the ddFPs. We have demonstrated biotinylation of split-TurboID fragments targeted to LD and ER. Using these constructs will allow for the characterization of the ER-LD contact site proteome under both conditions of LD biogenesis and at steady state to differentiate proteins governing LD biogenesis from other ER-LD contact site proteins.

Phosphofrucktokinase-1 localization in the syncytiotrophoblast of healthy and preeclamptic placentas

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The placenta is an organ that is indispensable in maintaining mammalian species. Key to the placenta's development is the formation of a large multinucleate cell, the syncytiotrophoblast (STB). The STB acts as the barrier between maternal and fetal circulation, facilitating the exchange of essential nutrients and oxygen. Disruptions in STB development can lead to placental metabolic dysfunction and pregnancy-related complications such as preeclampsia and miscarriage. Here, we investigated the cellular localization of the metabolic enzyme phosphofructokinase-1 (PFK) within the STB of both healthy and preeclamptic patients' placentas. We demonstrate for the first time that PFK organizes into phase-separated droplets within placental tissue. PFK droplets were localized primarily in the cytoplasm as expected given the enzyme's function in glycolysis. Intriguingly, we also observed PFK droplets concentrated within some nuclei of the multinucleate STB, suggesting PFK may have functions beyond glycolysis in the placenta. There are also differences in nuclear localization of PFK in the STB of healthy and preeclamptic placentas. These observations also raise interesting questions concerning the degree to which nuclei sharing a common cytoplasm may have heterogeneous functions.

Identifying Regulators of Autoantigens in ANCA Vasculitis with Unbiased & Candidate Genes Approaches: A Search for Therapeutic Targets

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Anti-neutrophil cytoplasmic autoantibodies (ANCA) associated vasculitis, ANCA Vasculitis, is an autoimmune disease driven by an aberrant immune response to neutrophil/monocyte granule proteins. Of these proteins, Myeloperoxidase (*MPO,* MPO) & proteinase 3 (*PRTN3*, PR3) are the two main autoantigens targeted by ANCA. The ANCA bind to these proteins inducing neutrophil activation, degranulation, and cytokine release, which results in necrosis and apoptosis of the endothelial cells and vasculature.1 This pathogenic process is life-threatening if left untreated. While current therapies effectively induce remission, they involve non-specific immune suppression or B cell depletion and are associated with complications due to increased rates of infection. Thus, a major objective is the identification and development of targeted therapies. To identify potential specific therapeutic targets, we are investigating regulators of MPO expression as targets to suppress MPO. We conducted a genome-wide CRISPR-Cas9 knockout (KO) screen combined with FACs isolation of cells with MPO reduction to identify novel regulators of the MPO protein. Using a library of sgRNAs directing Cas9 to ~18,000 genes, the screen individually knocked down each gene. The library population yielded an enrichment of cells with lower MPO protein which were sorted by FACs, sgRNAs were isolated and sequenced. From the screen analysis, three of the top “hits” that were enriched in the low MPO cells will be validated via targeted knockdown approaches. In addition, we identified 12 candidates based on prior studies on transcriptional and post-translational control of MPO. We will test the ability of these candidates to suppress MPO expression in a myeloid cell using CRISPR-dCas9 mediated knock-down and flow cytometry to measure MPO expression. These strategies to identify factors controlling autoantigen expression will inform potential biomarkers, allow patient screening to predict disease severity, and reveal candidates for targeted therapy.

Elucidating an Unexpected Requirement of Cytokines for Axon Pruning

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Axon degeneration can occur by both apoptosis and axon pruning. During apoptosis, in response to global trophic factor deprivation (e.g., NGF), the entire neuron, both axons and soma, are degraded. In the context of axon pruning, triggered by axonal NGF deprivation, the neuron has a unique capability of degenerating only targeted axon segments while the structural integrity of the rest of the neuron remains intact. Although the outcomes of apoptosis and axon pruning are distinct, both pathways utilize the same degenerative machinery, caspases. Interestingly, while caspase activation in the context of apoptosis requires the protein Apaf-1, caspase activation during axon pruning occurs independently of Apaf-1. How caspases are activated independently of Apaf-1 in the context of pruning remains incompletely understood.

To study axon pruning, our lab uses microfluidic chambers that enable both the spatial and fluidic isolation of neuronal cell bodies and their axons. While using microfluidic chambers to explore how caspases are activated in the context of pruning, our lab recently discovered that Caspase-1 is required for axon pruning but not apoptosis. Strikingly, we also found that downstream targets of Casp1, specifically IL-1β/IL-18, are also required for axon pruning. These results are unexpected as the only known functions of IL-1β/IL-18 are to serve as secreted pro-inflammatory cytokines. However, our microfluidic models of developmental axon pruning are void of immune molecules and pathogenic stimuli. In ongoing projects, we are examining the novel role of these cytokines in axon pruning.

Characterization of Pancreatic Ductal Adenocarcinoma Fibroblasts

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Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease with a 5-year survival rate of just 12%. Tumors are composed of tumor cells and a dense fibrotic stroma made up of cancer associated fibroblasts (CAFs), immune cells, and extracellular matrix (ECM). CAFs have been shown to promote tumor growth however ablation or broad targeting of CAFs can have a similar eUect – suggesting that these cells may play both positive and negative roles in the tumor microenvironment. Through transcriptomic analysis of patient tumors, we have identified two prognostic molecular subtypes of CAFs: tumor restrictive (restCAFs) and tumor permissive perm(CAFs), the latter of which shows poorer clinical outcomes. We hypothesized that permCAFs promote more aggressive tumor phenotypes through paracrine interactions. Interestingly, tumor cells stimulated with restCAF conditioned media displayed an increased invasive phenotype. As CAFs are major contributors to ECM deposition, we decided to derived matrix from both CAF subtypes. We found the permCAF ECM promoted a pro-migratory tumor phenotype. Overall, we find that secreted factors and matrix may play opposing roles in the tumor microenvironment and merits further study.

CD73 is a novel repressor of mutant β-catenin oncogenic activity in endometrial cancer

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β-catenin is an important oncogene commonly (20-30%) activated by somatic missense exon 3 CTNNB1 mutations in low grade, early stage endometrial cancer (EC). Although exon 3 CTNNB1 mutation associates with increased risk of recurrence in many (~50%) patients with low grade, early stage EC, an equal number of patients with β-catenin mutant tumors will not recur. It remains unclear why patient outcomes are so variable. The variability suggests there are unknown determinants of β-catenin mutant tumor aggressiveness. The purpose of this study was to evaluate CD73, a cell surface 5'-nucleotidase, as a critical factor controlling mutant β-catenin oncogenic activity in EC. We previously identified CD73 downregulation in exon 3 CTNNB1 mutant EC predicts recurrence and reported, using a highly homologous Xenopus exon 3 β-catenin mutant, that CD73 restrains mutant β-catenin to the membrane. For this study, we interrogated patient-relevant exon 3 CTNNB1 mutation frequencies in 5 publicly available databases and developed 7 (D32N, S33F, S33Y, G34R, S37C, S37F, and S45F) β-catenin mutant vectors for expression in EC cells in which we applied CRISPR-Cas9 deletion or the re-expression of CD73. With CD73 loss, transcriptional activity for all mutants increased significantly in HEC-1-A EC cells (normally CD73+/+). With re-expression of CD73 in Ishikawa EC cells (normally CD73-/-), transcriptional activity of several but not all β-catenin mutants decreased, which provides evidence for the first time that β-catenin mutants are differentially controlled in endometrial cancer. Using RNA-seq data from TCGA, we assessed whether β-catenin mutants in ECs lacking CD73 exhibited unique tumorigenic transcriptomes. Contrary to current paradigms for exon 3 mutant β-catenin, a dependency on expression of canonical Wnt/β-catenin gene targets was not observed in CD73-deficient β-catenin mutant tumors. In contrast, unique gene signatures (e.g., neuron biology and alcohol metabolic process) that have not previously been linked to β-catenin mutant EC were identified. Ongoing computational studies of pooled EC datasets are assessing the unique transcriptomes of individual patient-specific β-catenin mutants in the context of CD73 loss. Our results describe CD73 as a novel determinant controlling mutant β-catenin in EEC. Furthermore, we provide mechanistic rationale to explain the variability in outcomes in endometrial cancer patients with exon 3 β-catenin mutant tumors.

Investigating the role of enteroendocrine cells in epithelial barrier function in the gastrointestinal tract

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Enteroendocrine cells (EECs) are rare nutrient-sensing cells in the intestinal epithelium that synthesize and secrete hormones, metabolites, and small molecules in response to nutrient and microbial cues. EECs and the hormones they produce are often dysregulated in gastrointestinal (GI) diseases such as inflammatory bowel disease (IBD). However, little is known about how EECs regulate their local intestinal environment, and how they may act in important roles in the development and pathophysiology of GI disease. Studies in our lab have previously indicated that EECs are essential regulators of intestinal cell biology and function. Through single-cell RNA sequencing and lipidomics analysis on intestinal tissues lacking EECs, our lab found that lipid metabolic processes and inflammatory pathways were altered in the absence of EECs. Furthermore, we discovered that several species of ceramides and glucosylceramides were reduced or absent without EECs. Ceramides are sphingolipids that are important for maintaining barrier integrity and function in epithelial tissues, including the intestine. Misregulation of ceramides has been implicated in various GI disease states, including IBD, in which the intestinal barrier is frequently more permeable than in healthy intestine. To investigate the mechanism of function between EECs, ceramides, and barrier function, we analyzed intestinal tissue from EEC-deficient mice. Junctional proteins that promote a tight epithelial barrier are decreased and Claudin-2, a protein that promotes a leaky barrier, is increased. Additionally, Cers4, a ceramide synthase that synthesizes long-chain ceramides, is decreased in the intestinal crypts, supporting our lipidomic analysis. Using EEC-deficient human intestinal organoids, we observed a significant decrease in barrier integrity compared to control. Our future studies will define the mechanism of EECs in regulating the synthesis of ceramides integral for proper barrier function in the intestinal epithelium.

Pharmacological approaches to rescue premature termination codon variants of *CFTR*

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Cystic fibrosis (**CF**) is a life-limiting, autosomal recessive disease caused by mutations to the cystic fibrosis transmembrane conductance regulator (***CFTR***) gene, which encodes the CFTR epithelial chloride channel. Together, CFTR and the epithelial sodium channel (**ENaC**) maintain hydration of the respiratory mucus layer. In CF, decreased or absent CFTR activity and increased ENaC activity result in dehydrated mucus, leading to obstructive pathology throughout the body and especially in the lungs. Approximately 90% of people with CF are eligible for recently approved small-molecule CFTR modulator therapies, which have revolutionized the treatment of CF. The remaining 10% of people with CF have *CFTR* variants such as premature termination codons (**PTC**s) that result in no druggable CFTR mRNA or protein and are not eligible for CFTR modulators. There is an unmet need for novel therapies to treat cystic fibrosis in this 10% of the patient population. **CC-90009** is a small-molecule cereblon E3 ubiquitin ligase-modulating drug that specifically induces the degradation of the translation termination factor **eRF3a** (eukaryotic release factor 3a). We have previously demonstrated that in two CF PTC variants (G542X and W1282X), CC-90009 both rescues CFTR activity and reduces ENaC activity. Jointly, this dual mechanism of action is therapeutically promising in the context of CF. However, it is unknown which other *CFTR* PTC variants CC-90009 rescues and how CC-90009 reduces ENaC activity. Here, we examine the electrophysiologic responses of nine *CFTR* PTC variants to CC-90009 alone and in combination with other potentially synergistic drugs, and present preliminary insight into the mechanism by which CC-90009 reduces ENaC activity.

Measurement of cellular traction forces in customizable three-dimensional geometries

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Cells migrate by exerting physical forces against the surrounding extracellular matrix. These forces are generated by actomyosin contractility and are responsive to both biochemical and physical stimuli. Traction force microscopy, which quantitatively measures these forces, is an indispensable tool to study cell migration. However, the majority of traction force microscopy applications have utilized flat planar geometries or homogeneous 3D hydrogels. To better understand how cells migrate through complex three-dimensional geometries, similar to those they might encounter invivo, we have developed a versatile traction force microscopy platform that enables the generation of compliant substrates in a variety of micron-scale, user-defined 3D geometries. Using this system, we have measured cellular traction force, F-actin distribution, and nuclear geometry as cells navigate through complex 3D structures, including narrow confinements. Our findings suggest cells exert multipolar patterns of traction forces to navigate through confining regions and further, that traction magnitude significantly increases as the nucleus transits the confinement. These results are consistent with previous work suggesting the size of the nucleus and the degree it must deform to navigate a confinement is critically important for navigation of tight constrictions. Future work will probe the interplay between cytoskeletal and adhesive structures and biochemical signaling pathways as cells migrate through complex substrate geometries.

Endoplasmic Reticulum Dysmorphia Underlies CD8+ T Cell Efficacy in Solid Tumors

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The challenges encountered by tumor immunotherapies stem from immune suppression mechanisms present in the solid tumor microenvironment (TME), which include persistent antigen exposure, nutrient shortages, and hypoxic stress. The endoplasmic reticulum (ER) assumes a pivotal role in responding to cellular stress, influencing protein and lipid biosynthesis. These ER functions are ascribed to specific structural components—sheets, responsible for proper protein synthesis and folding, and tubules, which maintain lipid synthesis and organellar contacts. Under prolonged protein stress in the TME, chronic triggering of the unfolded protein response (UPR) occurs in the ER of tumor-infiltrating lymphocytes (TILs), undermining T cell efficacy in tumors. Given that one major ER stress sensor axis, IRE1a -XBP1s, regulates ER structure in multiple cell types, we hypothesized that chronic activation of the IRE1a-XBP1s axis in CD8+ tumor-infiltrating T cells (TILs) could dysregulate ER structural homeostasis, impairing ER function and tumor immunity. Utilizing confocal microscopy and flow cytometry, we examined ER morphology in CD8+ TILs from various tumors, comparing it with CD8+ T cells from non-tumor tissues. We observed significant ER distention in CD8+ TILs across diverse tumor models and patient samples, correlating with CD8+ TIL dysfunction and exhaustion, as well as the expression of IRE1α-regulated transcription factor XBP1s. *In vivo* models of viral infection demonstrated that tumor infiltration was necessary for ER structural dysmorphia, and *in vitro* assays revealed that this dysregulation occurs specifically under hypoxic stress. Genetic interventions, combined with imaging and spectral flow cytometry, indicated that restriction of the IRE1α XBP1s axis rectified ER structural dysmorphia under hypoxic stress but did not fully restore tumor control. We aimed to identify downstream targets for intervention, focusing on ER structural targets affected by TME stress. Our investigation highlighted the critical role of the ER sheet protein CKAP4 in TILs. By targeting CKAP4 through CRISPR/Cas9-mediated gene editing and a novel CKAPfl/fl CD8cre mouse model, we investigated the influence of ER sheet proliferation on T cell immunity against solid cancer. Genetic ablation of CKAP4 restored ER structural homeostasis in tumor stress, enhancing control of solid cancers. Our findings highlight the impact of chronic IRE1α-XBP1s signaling on ER morphology in CD8+ TILs within solid cancers. Furthermore, CKAP4-mediated ER sheet proliferation emerged as a crucial factor in ER structural dysmorphia, offering a novel avenue for enhancing the effectiveness of cancer immunotherapies.

Gastrokine 2's emerging role in pancreatic cancer progression

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Pancreatic ductal adenocarcinoma (PDAC) s one of the deadliest adult cancers with a 5-year survival rate of only 13% and is one of the top five leading causes of cancer-related deaths for men and women in the United States. These dire statistics underscore the need for a better understanding of the mechanisms that promote pancreatic cancer initiation and progression. Recently, the gastric epithelial genes Gastrokine (Gkn) 1 and 2 have been shown to be *de novo* upregulated in metaplastic epithelial cells in pancreatic tumorigenesis. Gkns are abundantly expressed by normal stomach epithelial cells and are considered tumor suppressor proteins in gastric cancer. However, the functional roles of gastrokines in pancreatic neoplasia and cancer progression remain unclear. Here, we investigate the etiology of gastrokine expression and identify a role for Gkn2 in pancreatic cancer development. Using shRNA-mediated knockdown of Gkn2 in murine pancreatic cancer cells, we've identified a possible regulatory role for Gkn2 over axonal guidance factors, which have been implicated in pancreatic cancer subtype and prognosis. Expanding our knowledge of Gkn2's upstream and downstream regulatory networks will help illuminate how a gastric identity gene program emerges early in transformed pancreatic epithelium and acts as a potential impediment to the progression of this aggressive cancer.

Inhibition of Neointimal Hyperplasia by Cinnamic Aldehyde through Nrf2 Signaling: Insights from a Preclinical Study

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Restenosis poses a significant challenge in vascular interventions due to Neointimal hyperplasia, primarily characterized by abnormal proliferation/migration of vascular smooth muscle cells (VSMC). Activation of the Nrf2 pathway has shown promise in inhibiting neointimal hyperplasia by maintaining redox homeostasis. This study investigates the causal relationship of Nrf2 activation and inhibition of neointimal hyperplasia through Nrf2 signaling. CA treatment significantly reduced Neointimal hyperplasia and minimized vessel occlusion in Nrf2 WT rats after carotid artery balloon injury, but not in Nrf2 KO rats, indicating the Nrf2 dependency of CA's effect. *In vitro*, CA inhibited VSMC proliferation and migration in Nrf2 WT VSMC, with increased expression of Nrf2 downstream targets including HO-1, NQO1, and PGC-1α, but not in Nrf2 KO VSMC. Additionally, CA treatment reduced MMP-2 levels, crucial for VSMC migration, in an Nrf2-dependent manner.

These findings underscore the potential of Nrf2 activators as a novel therapeutic strategy for preventing restenosis, highlighting the importance of Nrf2 signaling in mediating its effects. The observed effects of CA on VSMC function and the underlying mechanisms involving Nrf2 target proteins and MMP-2 provide valuable insights into its therapeutic potential. The findings suggest CA's potential in restenosis therapy by modulating VSMC behavior without compromising endothelial function. Future investigations should focus on optimizing drug delivery methods for clinical translation, highlighting the translational potential of CA in combating restenosis.

Identifying novel regulators of platelet adhesion

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Platelets facilitate hemostatic and thrombotic plug formation via integrin-mediated adhesion, which is controlled by the small GTPase, Rap1. Activation of Rap1 proceeds through two phases: initial activation by the guanine nucleotide exchange factor (GEF), CalDAG-GEFI, followed by sustained activation mediated by protein kinase C (PKC) signaling. Classical PKC isoforms are important for granule secretion, and thus provide the signaling required for feedback platelet activation via the secreted platelet agonist, ADP. However, there are also reports suggesting that PKC signaling can directly affect platelet integrin activation. We here investigate the hypothesis that novel PKC isoforms, particularly PKCθ, contribute to Rap1 activation in platelets via phosphorylation of RapGEF2, similar to what was shown for T cells. Mice deficient in RapGEF2 (megakaryocyte-specific, *Rapgef2mKO*), CalDAG-GEFI (*Caldaggef1-/-*), PKCθ (*Pkcθ-/-*) or both RapGEF2 and CalDAG-GEFI (dKO) are used to investigate PKCθ and RapGEF2 signaling in platelet function. Both Rap1 activation and aggregation of *Caldaggef1-/-* platelets were diminished by inhibition of PKC or additional deletion of RapGEF2. *Rapgef2mKO* platelets showed a normal aggregation response to most agonists, but not the PKC agonist, phorbol 12-myristate 13-acetate (PMA). Additionally, Rapgef2mKO platelets exhibited impaired adhesion to collagen under high but not low shear stress conditions, and Rapgef2mKO mice were partially protected from arterial thrombosis. Studies with *Pkcθ-/-* mice and platelets activated in the presence of isoform-selective PKC inhibitors are ongoing. Our studies are highly relevant to our understanding of platelet function in health and disease, especially considering that genome-wide association (GWAS) studies suggest a significant role for RapGEF2 in platelet integrin inside-out activation and coronary artery disease (CAD).

Development of an in vitro model system to study IBD

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Inflammatory Bowel Disease (IBD) is a chronic inflammatory disease of the gastrointestinal epithelium with no cure. Symptom management is through anti-inflammatory drugs, but 30% of patients don't respond to treatments. Thus, new preclinical models are required to efficiently screen drug efficacy to manage inflammation. While primary human gut epithelium can now be cultured indefinitely, the other key cell type in IBD is the gut-associated immune cell. These cells are poorly studied in part due to challenges in isolation, cryopreservation, and long-term culture. Our lab developed methods to isolate gut-associated lymphocytes (GAL) from human intestine and colon, but the lack of effective cryopreservation methods limits downstream studies including developing an epithelial-immune cell co-culture. The two main components in standard cryopreservation solutions are DMSO, which keeps ice crystals from killing cells upon freezing, and FBS, which provides nutrients to the cells and prevents dehydration. A third common component is cell culture medium, like RPMI 1640 to provide the cells vitamins. Previous studies indicate an optimal range of 10-15% DMSO, with 20% or greater being toxic to cells. However, the literature varies on optimal concentrations of FBS (40-90%), and if RPMI 1640 is necessary. The literature is also lacking on how the freeze-thaw cycle affects immune cell subtypes like T cells, B cells, and monocytes. Therefore, I hypothesize that a freeze solution composed of 50% RPMI 1640, 40% FBS, and 10% DMSO will confer high immune cell viabilities after a freeze-thaw cycle. As a proxy for intestinal immune cells, human peripheral blood mononuclear cells (PBMCs) were isolated and frozen in different media conditions to test viabilities post thaw (Condition A: 90% FBS, 10% DMSO; Condition B: 47.5% RPMI, 40% FBS,12.5% DMSO; Condition C: 50% RPMI, 40% FBS, 10% DMSO; Condition D: 40% RPMI, 50% FBS, 10% DMSO). Flow cytometry using a live/dead stain on PBMCs stained for various individual immune cell lineage markers was used to assess viability. Across three PBMC donors the lineage ratios were 82% T cells (SD = 2.4%), 10.6% B cells (SD = 1.1%), and 2.5% monocytes (SD = 0.5%) . PBMC viabilities for the four conditions tested were Condition A: 93.5%, B: 91.5%, C: 92%, D: 92%. Immune cell lineage viabilities were Condition A – T cells: 90% B cells: 83.3% Monocytes: 90.9% B - T cells: 84.5% B cells: 81.2% Monocytes: 86.8%, C - T cells: 89.3% B cells: 82.1% Monocytes: 93.6%, D - T cells: 89% B cells: 80.7% Monocytes: 91.7%. These data demonstrate that there is not a significant difference in immune cell viabilities post thaw when including RPMI 1640 in the cryopreservation media. It also confirms that there isn't a significant difference in cell viabilities if the DMSO concentration is between 10-15%. These findings show that T cells are the most abundant immune cell type isolated, and that they retain high viability after thawing. This is crucial, as T cells are a prominent cell type in IBD so one that will be necessary in our *in vitro* IBD platform. I intend to use the optimized freeze media tested on PBMCs to intestinal immune cells, and coculture them with epithelial cells to have a physiologically relevant model that can accurately screen disease phenotypes

Age-Dependent Characterization of Fusiform Cells

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Background:
The cochlear nucleus is considered the sole target of auditory nerve input, and the first site of central auditory processing. The cochlear nucleus is comprised of 3 main compartments, one being the dorsal cochlear nucleus (DCN). The DCN integrates auditory information with somatosensory, vestibular, and proprioceptive signals for external sound localization and inhibition of self-generated sounds. This function is orchestrated by excitatory and inhibitory neurons in the DCN. The primary excitatory output of the DCN are fusiform cells. Fusiform cells are known to change their electrophysiology during the onset of hearing and are thought to reach stable maturity by day 15-16 in murine models. However, other excitatory cell types in the cochlear nucleus change physiologically after 6-7 months, with a possible mechanism coinciding with morphological alterations. Therefore, this study seeks to characterize morphological and physiological properties of DCN fusiform cells beyond juvenile age.
Methods:
CBA/CAJ mice at 4 age points: preweaning (p<21), pubescent (p21-49),young adult (p50-180), and mature adult (p>180) were used for experiments. Fusiform cells in the DCN were subjected to whole-cell recording and filled with lucifer yellow pre-fixation. Post-fixation, brain slices were imaged on the Zeiss 780 confocal laser scanning microscope. Z-stack images were imported into IMARIS for 3D reconstruction of filled fusiform cells.
Results:
Preliminary results indicate no change in fusiform cell membrane or spike properties with age. Interestingly, we observed significant and trending differences in fusiform cell firing properties with age. Specifically, we observed the maximum “gain” of fusiform cells (denoted by the max Hill slope) decreased in the young adult and pubescent groups, compared the preweaning and mature adult groups. The amount of current needed to achieve the fusiform cell maximum “gain” also differed with age, with the young adult and pubescent animals showing noticeable increase in needed current, compared to the preweaning and mature adult groups. Alongside these findings are observational differences in fusiform cell dendritic complexity. Preliminary Sholl analysis indicates possible dendritic pruning occurring after initial hearing onset.
Discussion:
In this study, we provide novel characterization of fusiform cell morphology and physiology across a wide age range. The decrease in maximum firing “gain” and subsequent increase in current needed to achieve this maximum “gain” coincides with the dendritic pruning we observe. We hypothesize that once hearing onset is established, fusiform cells adapt and attenuate their firing properties and dendritic connections within the DCN. This work will give critical insights into understanding the development of key auditory neurons, and their potential role in age-related auditory processing.

Spatiotemporal mapping of renal innervation and impact of *in utero* fetal denervation on kidney development

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Proper kidney function is intricately linked to cardiovascular health. To help maintain homeostasis, the kidney is innervated by peripheral nerves which play significant roles in the regulation of renal function. Our lab has recently established that peripheral neurons composed of both sympathetic and sensory fibers innervate the kidney concomitantly during arterial differentiation, following guidance cues released by renal stromal cells. Yet, it remains unknown whether renal nerves actively direct organogenesis as they establish neuroeffector junctions to begin mediating physiology. In this study, we hypothesize that renal nerves release spatially and temporally important signaling factors which regulate kidney development. To test this hypothesis, we genetically ablated renal nerves in utero utilizing a whole animal *Ntrk1* knockout (TrkA) which is required for neuronal survival. Using 3D light-sheet imaging and IMARIS analysis on wild-type (*TrkA+/+*) (n=6), heterozygous (*TrkA+/-*) (n=6), and mutant (*TrkA-/-*) (n=6) mice, we assessed morphology and number of renal structures in at P0. We found total glomeruli number was decreased (P1-way Anova <0.001) and glomeruli diameter was increased (P1-way Anova <0.001) in heterozygous *TrkA+/-* and further in mutant *TrkA-/-* denervated kidneys compared to *TrkA+/+* wild-type kidneys at P0. Using immunofluorescence (IF) on kidney section, we observed that proximal tubule diameter was increased (PT-test < 0.0001) in mutant *TrkA-/-* denervated kidneys compared to *TrkA+/+* wild-type kidneys at P0. These data suggest that renal nerves are poised to impact nephron morphology and/or function during kidney development. We further established that renal nerves trafficked Synapsin 1+ vesicles showing that they establish neuro-effector junctions with developing kidney structures like glomeruli and tubules suggesting potential cellular crosstalk important for development. Future efforts will aim to conditionally delete sensory or sympathetic renal nerves independently and investigate the developmental and resulting functional phenotypes. Taken together our findings will significantly bridge the gap in knowledge concerning the establishment of renal innervation and the role of nerves in kidney development and disease.

IKKγ is required for CD8 T cell persistence in chronic infection and cancer

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Chronic antigen stimulation in persistent infection or cancer drives CD8 T cell differentiation into a dysfunctional or exhausted cell state. Although the molecular drivers of exhaustion have been heavily investigated, the precise mechanisms dictating the development and maintenance of exhausted CD8 T cells remain unclear. Inhibitor of nuclear factor kappa-B kinase subunit gamma (IKKγ) is involved in the potentiation of NFkB activity and T-cell receptor (TCR) signaling. As CD8 T cell exhaustion is induced primarily through prolonged or aberrant TCR signaling, we sought to clarify the role of IKKγ in regulating CD8 T cell fate and persistence in settings of exhaustion. We employed genetic mouse models to deplete *Ikbkg* (encoding IKKγ) in CD8 T cells in the tumor microenvironment or in chronic Lymphocytic Choriomeningitis Virus (LCMV). We found that IKKγ maintains the long-term persistence of exhausted CD8 T cells in cancer and chronic infection. IKKγ may also enforce distinct gene expression programs in CD8 T cells during chronic infection and cancer as we detected discrete phenotypes in each disease setting in *Ikbkg* deficient CD8 T cells. Therapeutic modulating of the TCR-IKKγ axis may present a promising path for effectively manipulating T cell differentiation and durability in chronic infection or cancer.

The Role of Endothelial Twist1 in Atherosclerosis

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Coronary artery disease (CAD) is the leading cause of mortality in the United States. Multiple cell types contribute to the atherosclerotic plaques causing CAD, influencing its growth and propensity to rupture. Endothelial-to-mesenchymal transition (EndMT), the process by which endothelial cells (ECs) acquire characteristics of mesenchymal cells and loose canonical endothelial features, is correlated with an unstable plaque phenotype, although this transition remains poorly characterized. We hypothesize that a specific population of ECs may undergo EndMT, potentially contributing to the progression of atherosclerosis. Endothelial-specific lineage-tracing was induced in *TgCdh5-CreERT2*, *ApoE-/-* mice, followed by 16 weeks of high-fat diet (HFD) to produce atherosclerosis. Aortic roots were collected from disease-condition animals and a baseline cohort. Single cell RNA sequencing was performed and analyzed using the Seurat package. Transverse aortic root sections were analyzed for tdTomato signal to characterize the lesion. Multiple endothelial subtypes were identified within the aortic root. One population, characterized by the marker Klk10, appears to give rise to a disease-specific population characterized by expression of Edn1. Upstream regulator analysis of differentially expressed genes between Edn1+ and Klk10+ populations predict this EC transition to be driven by EndMT-associated transcription regulators including SNAI2, TWIST1, STAT3, SNAI1, YAP1, and TEAD2. This analysis also predicted upregulation of pulmonary fibrosis idiopathic signaling, wound healing signaling, pathogen induced cytokine storm signaling, and hepatic fibrosis signaling pathways.

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 large flat lattice structures, alternatively known as clathrin plaques. Clathrin plaques have been proposed to serve as a receptor hub, increasing signaling by increasing the lifetime of cargo and provide a structural role in straited muscle cells and osteoclasts. Recently, Arp2/3 branched actin has been shown to be involved in the breakdown of plaques. Consistent with this finding, we can induce accumulation of clathrin plaques in mouse fibroblasts (JR20 cells) via conditional knockout of *Arpc2*. This plaque accumulation is accompanied by a decrease in endocytosis of fluorescently tagged transferrin, a CME-dependent cargo. Identifying factors involved in the regulation of clathrin plaques is an area of ongoing research. Our JR20 cells present an interesting model to study this pathway because parental JR20 cells do not readily form plaques under typical experimental conditions, but form large, stable plaques upon *Arpc2* deletion. 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. 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, either small molecule inhibition of ACK or overexpression of a kinase dead mutant results in increased plaque accumulation in parental JR20 cells. Overexpression of ACK^{WT} in GD25 cells is not sufficient to resolve plaques. 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.

TNIK is essential for the classical subtype PDAC organoid phenotype

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Introduction: Pancreatic ductal adenocarcinoma (PDAC) is a deadly cancer with dismal longterm survival. There are two molecular subtypes of PDAC, basal and classical; classical tumors are more differentiated and patients have better outcomes and response to first line therapy FOLFIRINOX. Organoid models recapitulate the glandular organization noted on histology, and may be used to evaluate changes in differentiation. We've identified distinct kinase expression profiles between the two molecular subtypes with TNIK as the most highly differentially expressed kinase. The aim of this project is to evaluate the essentiality of TNIK in classical subtype PDAC differentiation through patient derived xenograft (PDX) organoids.
Methods: Using PDX tumors, multiplexed kinase inhibitor beads, and mass spectrometry we identified distinct kinase expression between the subtypes and found TNIK as the highest expressed classical kinase compared to basal. We derived a classical organoid line from PDAC PDX mouse tumors. We transduced in an inducible Cas9 system and either a TNIK targeting short guide RNA (sgRNA) or a control, non-targeting sgRNA. Organoids were then treated with doxycycline to induce Cas9.
Results: Classical tumors and organoids exhibit glandular organization, whereas the basal tumors show apparent loss of polarity and differentiation with disorganized structures and no clear glands. Knocking out TNIK caused the classical organoid to lose its maintained architecture and hollow lumen, and shift to appear phenotypically more basal with a filled lumen and cellular disorganization seen in Figure 1. TNIK knockdown was confirmed using western blot.
Conclusions: TNIK expression is essential to the organization and structure of the classical organoid. TNIK has previously been shown to be involved in the regulation of cell spreading and cytoskeletal rearrangement and is also an essential activator of the Wnt signaling pathway, a highly conserved signaling pathway involved in cell differentiation, proliferation, migration, and survival. Phenotypically, the TNIK knockout organoids appear more basal-like with a disorganized structure. It is unknown if the destruction of organoid structure through TNIK inhibition will improve or decrease the viability of tumor cells and invasion potential. The exact role TNIK plays in PDAC is unclear, and while it may be a potential therapeutic target, knocking TNIK out may result in a subtype shift. This has important implications as TNIK inhibitors are being studied in clinical trials for other types of TNIK expressing cancers, such as lung and colorectal cancer, and could be detrimental as a therapy to PDAC patients.

Meningeal lymphatic CGRP signaling governs pain, neuroinflammation, and cerebrospinal fluid efflux during migraine

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Migraine is a highly prevalent neurovascular condition characterized by elevated calcitonin-gene related-peptide (CGRP) in the cerebral circulation leading to debilitating pain, vasodilation, and inflammation in approximately 15% of the general population. CGRP signaling also plays a critical role in lymphatic function leading to decreased lymphatic vessel permeability. The CGRP receptor, calcitonin receptor-like receptor (Gene: Calcrl Protein: CLR) and its receptor activity modifying protein (Gene: Ramp1 Protein: Ramp1) are both highly expressed in lymphatic endothelial cells and are pharmacologically tractable targets for novel, FDA approved, therapeutics that are highly effective in migraine. Leveraging an inducible, lymphatic specific Calcrl knockout (Calcrlfl/fl;VEGFR3-Cre-ERT2) and the nitroglycerin (NTG) mediated model of migraine, we have demonstrated that Calcrlfl/fl;VEGFR3-Cre-ERT2 mice exhibit significantly reduced pain and light avoidance behavior. NTG mediated migraine induced increased translation of immune cell interaction genes such as Madcam1 in the meningeal lymphatic vessels (MLVs). Multispectral flow cytometry revealed an elevated relative abundance of Madcam1 interacting T cells in the meninges draining lymph nodes during migraine that was abrogated in Calcrlfl/fl;VEGFR3-Cre-ERT2 mice. Direct CGRP stimulation of LECs with CGRP in vitro favors the formation of continuous vascular endothelial cadherin (VE-Cadherin) endothelial junctions and reduced LEC monolayer permeability to fluorescent streptavidin which could be reduced with the small molecule receptor antagonist Olcegepant. Consistent with these findings, NTG mediated migraine also induced VE-Cadherin rearrangement in MLV endpoints which was not reproduced in Calcrlfl/fl;VEGFR3-Cre-ERT2 mice. Moreover, direct CGRP stimulus via intra cisterna magna injection dramatically reduced dye efflux to the draining deep cervical lymph nodes. Injected dye efflux was not impacted by direct MLV stimulus with CGRP in Calcrlfl/fl;VEGFR3-Cre-ERT2 mice. Collectively, these data indicate that there is reduced cerebrospinal fluid efflux through the MLVs during migraine, and that the MLV response to CGRP governs migraine associated pain, pathophysiology, and immune responses. Therefore, meningeal lymphatic dysfunction likely contributes to migraine pathophysiology.

Prepronociceptin-expressing neurons in the extended amygdala regulate arousal a signal darting responses to an aversive odor

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Atypical arousal responses are a core symptom in neuropsychiatric disorders and occur in parallel to maladaptive behaviors. However, arousal encoding within the context of motivated behavior is unknown. The bed nucleus of the stria terminalis (BNST), a subnuclei within the extended amygdala, is prominently studied for its role in neuropsychiatric disorders, and motivated behavior. In addition, previous research identified that neurons expressing the *prepronociceptin* gene in the BNST (*Pnoc*BNST*)* encode rapid arousal responses to a motivationally salient odor (TMT; a derivative of fox urine and feces), when performing two-photon microscopy simultaneously with pupillometry in head-fixed mice (Rodriguez-Romaguera et al., 2020). To test if *Pnoc*BNST neurons also regulate distinct aspects of motivated behavior, we leveraged miniaturized head-mounted one-photon microscopes to image *Pnoc*BNST neurons while mice freely explore TMT. We found that the population activity of *Pnoc*BNST neurons selectively increased when mice approached TMT but not a control odor (water). Analysis of individual *Pnoc*BNST neuronal activity dynamics showed that neurons that were more excited to TMT correlated with proximity to odor and the action to dart away from it. However, inhibition of *Pnoc*BNST neurons did not modulate the time mice spent near TMT, distance to TMT, or darting behavior, indicating that they do not regulate these behaviors. Previously, we found that driving the activity of *Pnoc*BNST neurons was sufficient to induce rapid arousal responses in head-fix mice (Rodriguez-Romaguera et al., 2020). Therefore, we tested if inhibition of *Pnoc*BNST neurons was necessary for arousal responses to TMT using pupillometry in head-fixed mice. Interestingly, we found that inhibition of *Pnoc*BNST maintained heightened arousal responses to TMT. Our results demonstrate that *Pnoc*BNST neurons signal the location of an aversive odor and the action to dart away from it, but do not regulate such behaviors. In addition, our findings suggest that the role of *Pnoc*BNST neurons is specific to regulating arousal responses to an aversive odor.

Social threat recognition induces unique behavioral syllables and reshapes functional brain connectivity

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Social cues guide a significant portion of mammalian behavior, informing motivated action in response to positive or negative valence social stimuli to optimize an individual's survival. This can be seen through recognition and subsequent approach of positive valence social stimuli, such as potential mates, or by recognition and subsequent avoidance of negative valence social stimuli, such as aggressive or threatening individuals. While the neuronal representations of positive valence social cues are relatively well characterized, there is a substantial lack of research on the recognition and response to threatening social stimuli. To model recognition of social threats in rodents, we employed a modified social fear conditioning task that automates the pairing of interaction with a same-sex, novel mouse with a mild foot-shock. With this task, we successfully drove defensive behavioral responses toward a social stimulus on the day of training. Furthermore, stimulus-specific aversion persisted over time, as shown by place avoidance in the three-chamber sociability test on the day following SFC. These classic behavioral phenotypes were supported by sub-second behavioral classification using the Keypoint-MoSeq analysis pipeline to detect stereotyped, fine movement patterns using unsupervised machine learning. This unbiased approach allowed for the detection and analysis of differences in behavioral syllables across conditioning and recall of social threats. We subsequently sought to identify changes in brain wide functional connectivity evoked by social threat recognition through quantification and analysis of cFos protein expression across hundreds of brain regions. This was done using semi-automated atlas registration using the analysis pipeline Aligning Big Brains and Atlases and automated cFos-positive cell quantification using QuPath. Though we observed modest changes in single region cFos expression following SFC, we observed notable changes in correlations in cFos expression between these regions as a measure of functional brain connectivity. Accordingly, these data support the use of SFC to study the acquisition and recall of negative valence social cues to drive alterations in the brain and in behavior. The results described here open many avenues of investigation into potential neural circuits that may underlie recognition and subsequent behavioral responses toward social threats.

Biometric ocular photometry to track pupil size, respiration, and heart rate in real time.

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Physiological signals such as pupil size, respiration, and heart rate provide access to internal brain states. Dysregulation of these signals is known to occur in multiple neuropsychiatric disorders such as PTSD, depression, or social anxiety, and they fluctuate even in the absence of externally observable behavioral outputs. Despite their relevance in neuroscience research, current biometric monitoring technology for research in animal models is bulky, impractical, or unreliable, and falls short of the current needs of experimental neuroscience. To address these shortcomings, and study the the relationship between neural activity, behavior, and physiological arousal biometrics, we have developed Biometric Ocular Photometry (BOP), a biometric monitoring system that is capable of tracking multiple physiological measures of arousal (pupil/respiration/heart) simultaneously with a single compact device that measures photons traveling through the eye. Our experimental results show that our device can measure these biometrics with the same accuracy as current technology, in awake animals, and with minimal disturbance to behavior. We present several implementations of our device, with experiments that track the effects of pharmacological manipulations, with freely moving behavioral paradigms, and with closed-loop optogenetic stimulation based on biometric output from the device. We anticipate that our technology will facilitate new experiments to reverse engineer the relationships between neural activity, behavior, and fundamental physiology, and will pave the way for new experimental implementations and novel therapeutic interventions.

Elucidating Gene Functions Responsible for Sensitizing Stem Cells for Apoptosis

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Stem cells are notorious for their ability to quickly undergo apoptosis in response to adverse stimuli. This mechanistic response is necessary to avoid any aberrant mutations that could have potentially catastrophic consequences. Stem cells are already primed for apoptosis because of their readiness of active BAX. BAX is located within the golgi of stem cells and is a gene known to permeabilize the mitochondria outer membrane; a key factor in the apoptotic pathway. NOXA, a BH3-only protein known for initiating apoptotic events, is also constitutively high, natively within stem cells. It is unsurprising that there are multiple pathways for stem cells to commence a caspase cascade, thus we are interested in learning more about the undefined mechanisms of stem cell apoptosis.

To gather a better understanding of what we know about apoptosis in stem cells, our lab conducted a human genome wide screen. 20,000 genes were screened and 30 of them appeared to be relevant to the role of increasing sensitivity to apoptosis. To validate their functions, our lab is conducting knockout and knock down of the 30 candidate genes utilizing CRISPR and CRISPRi technologies. Additionally, our lab is using cell death assays to elucidate their importance in stem cell apoptotic sensitivity. Our lab would expect that knocking out or knocking down the sensitizer gene to apoptosis would result in a prolonged life expectancy for these cells.

An open-source system for Calcium-imaging, Pupillometry, and Locomotion-estimated Tracking (CaPuLeT)

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A wide selection of behavioral assays in systems neuroscience rely on head-fixation protocols for *in vivo* experimentation. Specifically, the emergence of multi-photon imaging techniques that cannot be miniaturized has prompted researchers to seek out and integrate a variety of head-fixed experimentation procedures. Locomotion wheels are popular devices to measure behavioral responses in mice. In addition, pupillary dynamics are also a key measurement performed in head-fixed studies on awake ambulatory mice. Here, we present an open-source protocol for constructing a head-fixation setup that integrates locomotion and pupillometry measurements with high temporal resolution. Our design is customizable for use with major tools in monitoring or modulating neural activity, such as multi-photon calcium imaging and optogenetics. Instructions for assembling an entire system are provided, including details on the underlying circuitry and a custom-made graphic user interface for user-friendly implementation. We also detail surgical implantation procedures necessary for head-fixation and the habituation procedures needed to begin collecting data.

RNA Binding Proteins Regulate Alternative Splicing Networks During Postnatal Atrial Development

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Alternative splicing is a mechanism that allows cells to produce multiple transcripts from a single gene. Alternative splicing plays a significant role in development and is regulated by RNA binding proteins (RBPs), which thus act as main contributors to splicing outcomes. RBP-regulated splicing abnormalities have previously been implicated in heart diseases. Furthermore, abnormal expression of certain ventricular RBPs can lead to a reversion to fetal splicing patterns, which is evidence of the role of RBPs in cardiac maturation. Similar research has yet to be performed in the atria, which has a unique cellular composition. We hypothesize that differentially expressed atrial RBPs may be vital for proper postnatal maturation of the atria through the regulation of age-specific splicing networks.

We performed RNA-sequencing studies on atrial RNA samples isolated from FVB mice of various developmental stages: postnatal day 4.5 (PN 4.5), PN10, PN28, and PN90. After DEseq analysis, we identified Rbfox1 and Fmr1 as candidate RBPs differentially expressed across atrial development. To validate these findings, we performed quantitative real time PCR (qPCR) assays to observe mRNA expression and western blotting to observe protein expression. We also performed reverse transcriptase PCR (RT-PCR) assays to evaluate splicing patterns throughout atria development. To further explore the role of FMR1, an Fmr1 knockdown was performed in the HL-1 atrial cardiomyocyte cell line to observe splicing patterns and protein expression changes.

We found that Rbfox1 (mRNA and protein) is upregulated during atrial development while Fmr1 is downregulated. We also found that Fmr1 itself is alternatively spliced in both atria and ventricle tissues with increased long isoform inclusion throughout development. Additionally, we observed that expression of the FMR1 homolog, FXR1, is unaffected in the absence of FMR1. Our next step is to investigate downstream Fmr1 splicing targets and protein interactions. Similar to FMR1, we also aim to explore the splicing targets of RBFOX1 using si-Rbfox1 HL-1 cells. Finally, we will observe changes in atria morphology and function within Fmr1flox and Rbfox1flox Cre-lox mouse lines using atrial-specific gene delivery to analyze the functional impact of these RBPs.

Genetic factors underlying susceptibility to prenatal alcohol and cannabinoid exposure

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Genetic factors play a significant role in determining an individual's susceptibility to developmental teratogen exposure, in particular abnormalities involving the early development of the brain and craniofacies. In our mouse model, both alcohol and cannabinoids induce craniofacial and brain abnormalities similar to fetal alcohol syndrome. Both drugs inhibit the sonic hedgehog (Shh) pathway, which is involved in cell proliferation and migration for craniofacial development, through different mechanisms. In the same mouse model, we have identified numerous candidate genes that modify susceptibility to alcohol by comparing various strains of mice with a spectrum of abnormalities, namely the C57BL/6J strain (highly susceptible) and the closely related C57BL/6N strain (moderately susceptible). This current work describes the discovery of a mouse strain (129S1) that is completely resistant to developing the characteristic brain and craniofacial abnormalities associated with early embryonic alcohol exposure. Surprisingly, this 129S1 strain was resistant in spite of the fact that it presented with a significantly higher blood alcohol concentration following early embryonic alcohol exposure as compared to the C57 strains. We also discovered that the 129S1 stain is resistant to cannabinoids and other Shh pathway antagonists, which are also highly teratogenic to the brain and face. To explore potential differences between strains accounting for this differential susceptibility, we performed qRT-PCR on 192S1 and C57 embryos. As one example, preliminary data suggests that Efcab7, involved in the Shh pathway and primary cilia function, is more highly expressed in the 129S1 strain. In order to identify more candidate genes that may modify susceptibility to prenatal alcohol exposure, we are in the process of performing transcriptomic comparison (RNA-seq) of embryos from the 129S1 strain to the C57BL/6J strain. In addition to identifying individual candidate genes that can be tested in human populations, these data identify cellular and molecular pathways that are possibly involved in prenatal drug-related pathogenic mechanisms of action as well as potential druggable pathways that may serve to help ameliorate the effects of prenatal teratogen exposure.

Unveiling the Impact of *Pseudomonas* Infection on CFTR Rescue

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People with cystic fibrosis (pwCF) suffer from chronic and recurring bacterial lung infections that begin very early in life and contribute to progressive lung failure. CF is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, which encodes an ion channel important for maintaining proper hydration of pulmonary surfaces. When CFTR function is compromised, airways develop thick and sticky mucus, initiating a destructive cycle of infection and inflammation. CFTR modulators, a category of therapeutics for pwCF, aim to rectify the CFTR defect directly, reinstating normal airway hydration and mucociliary clearance. Despite the application of highly effective CFTR modulator therapies (HEMT), bacterial infections persist. To create an applicable model of diseased airway epithelia, we established a primary human airway epithelial culture system afflicted with persistent *Pseudomonas aeruginosa* infection. Utilizing this model, we investigated the impact of *P. aeruginosa* infection on rescue of F508del CFTR by the HEMT, ETI (elexacaftor/tezacaftor/ivacaftor). Our findings revealed that the presence of *P. aeruginosa* heightened CFTR mRNA, protein levels, and activity. We also investigated the impact of ETI on bacterial persistence. Although CFTR modulators did not exhibit direct antimicrobial activity, we observed a reduced bacterial burden in the presence of these therapeutic agents, possibly due to increased CFTR function. In addition, bulk RNAseq demonstrated significant changes in expression of genes involved in immune response and ion transport upon bacterial infection. This research approach serves three key purposes: 1) elucidating changes in airway host pathways due to bacterial infections, 2) identifying potential mitigating effects of CFTR modulators on changes induced by bacterial infection, and 3) revealing the effects of CFTR modulators on bacterial burden. Our study underscores the importance of incorporating live bacteria to faithfully replicate the CF lung, emphasizing that understanding the effects of infection on CFTR modulator efficacy is crucial for the assessment and enhancement of drug therapies for all pwCF.

Discovery of genetic loci underlying daily patterns of sleep behavior using collaborative cross mice

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Sleep is an essential physiological process seen in all members of the animal kingdom. It is widely believed that sleep behavior has both genetic and environmental determinants. Our project set out to investigate the genetic basis underlying the daily expression of sleep behavior. We examined baseline sleep behavior in 12 strains of mice from the Collaborative Cross, a genetically diverse collection of recombinant inbred mouse strains, and observed a wide range of sleep phenotypes and daily patterns. We selected two mouse strains based on their distinct sleep phenotypes (CC036, “Rudolph” - highly regulated sleep with strong daily rhythmicity and separation of sleep between the light and dark phases; CC057, “Run” - highly divergent sleep patterns with seemingly no daily rhythmicity). We then generated F1 and subsequent F2 hybrid generations from the parental Rudolph and Run strains, to collect a robust population of mice (F2: N=271; all females) with a wide range of sleep phenotypes to enable genomic mapping of quantitative trait loci (QTLs) based on sleep measures. Sleep/wake behavior for each mouse was recorded for a 5-day period using PiezoSleep recording technology from Signal Solutions. We collected a wide range of sleep phenotypes, including percent time and mean bout lengths for REM, NREM, and wake and daily rhythmic behavior for each mouse. These phenotypes were then used for QTL mapping, to potentially identify a chromosomal region likely to contribute to the differences in sleep phenotypes between mice in this population. QTL mapping results identified a region of Chromosome 7 as a likely genetic locus responsible for the variation in many of the examined sleep phenotypes, such as light cycle and NREM-specific phenotypes. This suggests that one or multiple genes in this chromosome could play a role in the differential expression of sleep behavior across members of this population. Furthermore, we found evidence for maternal effects on the sleep patterns of the F1 generation, suggesting that environmental factors may also be involved in the observed phenotypical variation.

Assessing Sleep Behavior Across Alzheimer's Disease Model Mice

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Alzheimer's disease (AD) is a progressive neurodegenerative disease that leads to a marked loss of cortical and hippocampal synapses, presenting as a progressive loss of memories, motor function, changes in personality, and many other functions. Additionally, many patients report changes in their sleep patterns which may lead to the well-known night wandering phenotype.

Due to the severity of AD worldwide, transgenic mouse models have been created to study the effects of known mutations in human amyloid precursor protein (APP), presenilin proteins (PSEN), and Tau protein. Based on preliminary data, a mouse model of Tauopathy was found to exhibit a breakdown of dark cycle sleep; therefore, we analyzed synaptic changes in PS19 animals in response to sleep disruption via Western Blotting. We also analyzed 5xFAD sleep behavior at 3-, 6-, and 11-month-old mice to understand amyloidal effects on sleep. To do this, we analyzed baseline sleep at all ages, along with two windows of sleep recovery after a four- hour sleep deprivation period, of male and female 5xFAD mice compared to their wildtype littermates. We found there to be a decrease in dark cycle sleep in older males, with no differences in sleep behavior after deprivation. Females exhibited within- and between-genotype differences in light cycle sleep at 3- and 6-months of age, while males did not. Following sleep recording via the Piezosleep system, we analyzed hippocampal inflammation, marked by TNFα and ILβ-1, of mice from each age group via quantitative PCR.

The E3 ubiquitin ligase TRIM9 regulates synaptic function and actin dynamics in response to netrin-1

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During neuronal development, dynamic filopodia emerge from dendrites and mature into functional dendritic spines during synaptogenesis. Dendritic filopodia and spines respond to extracellular cues, influencing dendritic spine shape and size as well as synaptic function. Previously, the E3 ubiquitin ligase TRIM9 was shown to regulate filopodia in early stages of neuronal development, including netrin-1 dependent axon guidance and branching. Here we demonstrate TRIM9 also localizes to dendritic filopodia and spines of murine cortical and hippocampal neurons during synaptogenesis and is required for synaptic responses to netrin. In particular, TRIM9 is enriched in the post-synaptic density (PSD) within dendritic spines and loss of *Trim9* alters the PSD proteome, including the actin cytoskeleton landscape. While netrin exposure induces accumulation of the Arp2/3 complex and filamentous actin in dendritic spine heads, this response is disrupted by genetic deletion of *Trim9.* In addition, we document changes in the synaptic receptors associated with loss of *Trim9.* These defects converge on a loss of netrin-dependent increases in neuronal firing rates, indicating TRIM9 is required downstream of synaptic netrin-1 signaling. We propose TRIM9 regulates cytoskeletal dynamics in dendritic spines and is required for the proper response to synaptic stimuli.

Development of Strategies to Purify Filopodia and Related Organelles

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Filopodia are dynamic, finger-like cellular protrusions with important roles in many biological processes, including synapse formation, angiogenesis, and cancer. Although purification procedures have been developed for many other cellular organelles such as mitochondria, primary cilia, and inner ear stereocilia, little has been done to purify filopodia or determine their composition using modern proteomics approaches. We report here preliminary efforts to purify filopodia using two different approaches—one using classical subcellular fractionation in conjunction with the actin stabilizing probe SIR-actin and the other taking advantage of nanoporous alumina to separate filopodia that have extended into pores from the rest of the cell.

To test if the ~1000 filopodia typically present on a HeLa cell can be removed by hydrodynamic shearing, we sheared cells 10-15x through a 20-gauge needle. After low-speed centrifugation to remove the sheared cells, imaging of the supernatant showed large numbers of small membranous structures, including some with a cylindrical morphology and lengths of 3-4 um. The cylindrical structures could also be stained with plasma membrane probes and with the F-actin probe SIR-actin, indicating they exhibit key criteria of filopodia. We fractionated the sheared supernatant on a sucrose step gradient and found the putative filopodia eluted at approximately ~27-34% sucrose. These results indicate that, in the presence of the actin stabilizing agent SIR-actin, many putative filopodia are stable enough to survive sucrose gradient fractionation.

We are also testing if filopodia can be purified by allowing cells to insert their filopodia into 0.2 um diameter pores in wafers of nanoporous alumina. When such wafers are blasted with a stream of buffer to remove cells and then stained for F-actin, the wafers are covered with cell “footprints”. Each footprint consists of tens to hundreds of F-actin-stained protrusions extending into the wafer for several um, in some cases 10-20 um. In cells transfected with GFP-Myo10, a marker of filopodial tips, puncta of green can often be observed at the ends of the actin-stained protrusions. We can detect as many as ~4500 actin-stained spots per 100x field, corresponding to over 30 million per 13 mm disk. Our current efforts are focused on optimizing this approach so we can use proteomics to determine the composition of filopodia and related protrusions.

Gene expression studies analyzing the effect of *Pseudomonas aeruginosa* infection in primary cystic fibrosis airway epithelial cultures.

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Background: Cystic fibrosis (CF) is caused due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The major pathological consequence of impaired CFTR function is recurring bacterial lung infections, resulting in respiratory failure. Although highly effective CFTR modulator therapies are widely studied, their impact on bacterial infections in primary cell culture is not well characterized.
Aims: The goals of this study are to 1) establish a model of diseased airway epithelium by co-culturing *P. aeruginosa* with primary human bronchial epithelial (HBE) cultures and 2) analyze the effects of bacterial infection and CFTR modulators on epithelial gene expression.
Methods: Fully differentiated HBE cells were infected with *P. aeruginosa* strain PA01. Tobramycin was added 3h after bacterial infection and cultures were treated with CFTR modulators VX-661, VX-445 and VX-770. RNA isolated from these epithelial cultures with and without bacterial infection was used for gene expression analysis.
Results: We established a bacteria-epithelia co-culture model in primary airway epithelial cells to mimic persistent bacterial infection. Bulk RNAseq demonstrated significant changes in expression of genes involved in immune response, ion transport, and cell surface glycoprotein secretion upon bacterial infection. Although CFTR modulators did not exhibit direct antimicrobial activity, we observed a reduced bacterial burden in presence of these therapeutic agents, potentially due to increased CFTR function.
Conclusions: Our study underscores the importance of investigating how bacterial infections and CFTR modulators impact gene expression in a physiologically relevant model. This research approach serves three key purposes: 1) elucidating changes in airway host pathways due to bacterial infections, 2) identifying potential mitigating effects of CFTR modulators on changes induced by bacterial infection and 3) enhancing our comprehension of the side effects associated with current CF therapies.

Analysis of metabolomic, proteomic and mitochondrial dysfunction in *rd10* retinas

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Purpose: Mitochondrial dysfunction and metabolomic and proteomic profiles were compared in wild type (WT) and *rd10* mice to identify early-stage events in cellular dysfunction in a model of autosomal recessive retinitis pigmentosa.
Methods: Mice were raised under normal cyclic light. A Seahorse XFe96 Flux Analyzer was used to measure the oxygen consumption rate (mitochondrial respiration) and extracellular acidification (glycolysis) in WT and *rd10* retinas from P14 to P18. Untargeted LCMS metabolomics at P15 was performed using flash-frozen retinas that were analyzed by Ultra-High Performance Liquid Chromatography (UHPLC) followed by high resolution mass spectrometry (HRMS). Metabolite peaks were matched to compounds in an in-house library and pathway analysis was performed. Proteomics was performed on crude mitochondrial fractions at P15 and P18 using High Performance Liquid Chromatography (HPLC) followed by protein identification and quantification by mass spectrometry.
Results: Maximal respiration was significantly reduced in *rd10* mice at P16. Both basal and maximal respiration were significantly reduced at P16 and P18 indicating progressive loss of mitochondrial function. At P18, basal glycolytic activity was also significantly reduced. At P15, both metabolomics and proteomics revealed significant differences between WT and *rd10* mice. Eleven pathways were significantly enriched based on analysis of the metabolites. These pathways include several linked to mitochondrial function, including Pantothenate and Coenzyme A biosynthesis, β-alanine metabolism and Purine metabolism. The Primary Bile Acid Synthesis pathway was also enriched consistent with a critical role for the amino acid taurine in the regulation of oxidative stress, cholesterol metabolism and apoptosis. Proteomic analysis identified 49 proteins with significant fold-changes at P15 and 35 proteins with significant fold-changes at P18. Among the differences detected were proteins involved in mitochondrial transport, electron transport, fission, apoptosis and glycolysis.
Conclusions: Cellular pathways altered in the *rd10* mouse were identified prior to significant degeneration. These pathways are involved in mitochondrial function and other cellular functions such as DNA synthesis and cholesterol metabolism. Additional analyses such as western blot analysis, immunohistochemistry and electron microscopy will be performed to validate these affected pathways.

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