FARE2024 WINNERS
Sorted By Study Section

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NLM
Artificial Intelligence - Machine Learning
A 3D Deep Convolutional Neural Network for Detecting Reticular Pseudo-Drusen in Spectral Domain Optical Coherence Tomography Scans
Age-related macular degeneration (AMD) is an eye disease that causes blurred central vision due to damage of the macula, which is part of the retina that controls sharp, straight-ahead vision. Losing central vision make it harder to see faces, read, and drive. AMD is a leading cause of legal vision loss for the elderly and is predicted to affect more than five million people worldwide. AMD is a progressive disease where its symptoms usually get worse over time from no symptoms at early stage to severe symptoms like waviness of straight lines, brightened colors, and trouble seeing in low lighting at late AMD. Reticular pseudodrusen (RPD) are subretinal drusenoid deposits, that are located above the retinal pigment epithelium, i.e., the pigmented layer of the retina. RPD are of particular interest because their highest rates of occurrence are predictors of end stages of AMD, i.e., choroidal neovascularization and geographic atrophy. RPD are found more frequently in females, with increased age and more commonly bilaterally than unilaterally. RPD have typical features visible on multimodal imaging and are particularly distinguishable using volumetric spectral domain optical coherence tomography (SDOCT). Therefore, the detection of RPD can help to manage AMD before its progress into late stages. In this work, we used the age-related eye diseases study 2 (AREDS2) Ancillary OCT Study dataset which was obtained at National Eye Institute (NEI) and other centers. SDOCT scans can provide detailed information about the changes in retinal layers associated with RPD. The dataset included 826 SDOCT scans with RPD present in 222 scans. We divided the dataset into independent training (70%), validation (10%), and test (20%) sets. There were many challenges in this work, which included creating ground truth labels, using a scan-level label (i.e., there are no image-level labels), and losing the RPD features when resizing the SDOCT volume due to low image quality. To address these challenges, we transferred labels from corresponding Fundus Autofluorescence (FAF) images to the SDOCT scans, developed a 3D deep convolutional neural network to process the input volume at one time, and cropped the input volume to remove irrelevant regions to RPD without sacrificing the size too much, respectively. Our results on the test set showed accuracy of 75%, area under receiver characteristic operating curve of 74%, and average precision of 73% on detecting RPD from SDOCT scans.

Benjamin Hou
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Artificial Intelligence - Machine Learning

Deep Learning Segmentation of Ascites on Abdominal CT Scans for Automatic Volume Quantification
Background: Ascites is the accumulation of fluid in the peritoneal cavity. It is an abnormal condition caused by diseases, such as; hepatic cirrhosis, cancer, heart failure, tuberculosis, and pancreatic disease. Accurate quantification of ascites volume on CT scans can be an indicator of disease severity. For example, in cases of ovarian cancer, ascites volume at initial diagnosis can be correlated with worse progression-free survival and overall survival. Manual quantification of ascites is time-consuming and tedious; it can take up to a day per scan depending on volume of ascites present. Automated quantification is equally challenging. First, unlike organ segmentation, ascites cannot be inferred by shape priors as it is a free-flowing liquid in the peritoneal cavity. Second, the intensity profile of ascites on CT is similar to other liquids in the abdomen such as gestational fluids, urine, and bile. Third, there are no existing publicly-available labeled datasets of ascites to train automatic methods. Methods: Two datasets were collected and annotated semi-automatically from scratch: the publicly available TCGA-OV dataset (285 scans) of women aged 38-82 years (avg: 61, s.d.:11), and an internal NIH private dataset for liver cirrhosis (25 scans) of mixed gender patients (11F, 13M, 1Unk) aged 29-85 years (avg: 59.4, s.d.:13.8). Active learning was used for TCGA-OV; the liver cirrhosis dataset was manually annotated due to small scan count. All final annotations were reviewed by an expert radiologist. nnU-Net, a deep learning model, was trained on the public dataset only and evaluated on the private dataset through five-fold cross validation. Results: Active learning accelerated the entire annotation process from potentially several months to just a few weeks. The model achieved an F1 Score of 0.85±0.06, precision of 0.94±0.08, recall (sensitivity) of 0.79±0.08 and specificity of 0.99±0.002. The avg predicted volume error was 0.44±0.38L, and avg percentage error of 17.8±8.8%. Upon visual inspection, many small pockets of localized ascites missed during annotation were caught by the model, but penalized by thick slices of 5mm. Conclusion: To the best of our knowledge, the proposed method represents the state-of-the-art for ascites segmentation with a high degree of accuracy. The method can therefore be used to accurately quantify ascites volume and may be a useful biomarker for diagnosing and treating patients with cancer and cirrhosis.

Bikash Santra
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Artificial Intelligence - Machine Learning
Deep learning for identifying the genetic mutations of pheochromocytoma and paraganglioma using CT scans
Background: Pheochromocytomas and paragangliomas (PPGLs) are neuroendocrine tumors whose pathogenesis and progression are greatly regulated by genetics. While most PPGLs are noncancerous, some are malignant and can metastasize. The intra-adrenal tumors are called pheochromocytomas while the extra-adrenal tumors are called paragangliomas. The genetic mutations of PPGLs can be broadly categorized into SDHx, VHL/EPAS1, Kinase Signaling, and Sporadic. The management of PPGLs depends critically on their genetic groups. Thus, identifying genetic groups is essential for treating patients with PPGLs. Objective: Genetic testing for PPGLs is currently very expensive and time-consuming. CT scans of PPGL patients are usually acquired at the very beginning of patient management. CT imaging is inexpensive compared to a genetic test. Thus, given a CT sub-image (patch) of the PPGL, our objective is to present a deep learning-based scheme that uses contrast-enhanced CT

(CE-CT) scans for identifying PPGLs' genetic mutations. Dataset and Challenges: We have curated a dataset patchPPGL (which will be made publicly available) with the 1010 patches of PPGLs extracted from the CE-CT scans of 285 human subjects. patchPPGL presents multiple challenges in machine learning paradigms such as the presence of class imbalance, intra-class variance, inter-class similarity, and a large variation from tiny to big patch sizes. Methods: We developed a two-branch vision transformer (PPGL-Transformer) to identify each tumor's genetic group. The standard of reference for each tumor included two items: its genetic group from clinical testing, and its anatomical location (head and neck, chest, adrenal, abdomen to pelvis but not adrenal). A supervised contrastive learning strategy was used to train the PPGL-Transformer by optimizing contrastive and classification losses for the genetic group and anatomic location of PPGLs. Results: Our PPGL-Transformer achieved 62.8±6.3% Balanced Accuracy (BA) and 46.5±8.1% F1-score on five-fold cross-validation of patchPPGL and outperformed competing methods (support vector machine and random forest with radiomics features, convolutional neural networks, and vision transformer) by 3-10% on BA, and 3-14% on F1. Sporadic and SDHX achieved higher performance on BA (68.3±0.1%) and F1 (74.8±0.1%) respectively. Conclusion: Our method and the dataset may lead to faster and more widely available genetic characterization of PPGLs.

Brian Ondov
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Artificial Intelligence - Machine Learning
A benchmarking dataset for adapting biomedical text to plain language
Low health literacy has been associated with poorer outcomes and helps to drive education-related disparities. The unprecedented amounts of biomedical knowledge now available online could help to improve engagement, lifestyle choices, and truly informed consent. However, much of this knowledge is in the form of scientific literature and medical encyclopedias, which remain inaccessible to patients and caregivers due to the barrier of technical language and jargon. Recent advances in Deep Learning suggest the possibility of automatically adapting these texts to plain language for healthcare consumers. However, these methods still make errors and can invent facts using authoritative language. This means rigorous evaluation will be crucial for bridging the gap from capability to responsible deployment. A standard way to benchmark Machine Learning systems is to compare their output to gold standards, which represent the ideal outputs for a given set of inputs. Benchmarking data for generating plain language in the biomedical domain has thus far been largely automatically mined, consisting of either (1) pairs of similar documents from professional-facing and consumer-facing sources or (2) pairs of semantically similar sentences from such documents. Identical content is rare in both cases, and filtering for high-quality sentence pairs leads to loss of context and datasets that are too small for proper benchmarking. To address this issue, we created a large, gold standard corpus of manually adapted biomedical abstracts. Motivated by principles of plain language communication, we developed detailed guidelines for sentence-by-sentence adaptation of each abstract. The adaptations were written by biomedical literature experts from the NLM Index section and by contractors with science communication experience. To ensure quality, we conducted trainings for these workers and provided feedback on initial work. The resulting dataset contains 750 adapted abstracts, totaling 7,643 sentence pairs. The dataset is unique among high-quality, manually-created benchmarking data in the biomedical domain, both in its size and in the fact that it is both document- and sentence-aligned. This allows for

fine-grained comparisons while retaining valuable context from the surrounding document. We anticipate this dataset will be a valuable resource for ensuring high standards of quality as Deep Learning systems begin to be used to generate public-facing text in the biomedical domain.

Anushka Wickramaratne
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Biochemistry - General
Intermolecular interactions between J-domain proteins, Hsp90, and Hsp70
Protein folding and quality control are essential, well-regulated processes. Maintaining the complex phenomenon of proteostasis (protein homeostasis) involves highly conserved molecular chaperones, such as heat shock proteins Hsp90 and Hsp70, that participate in protein folding, unfolding, remodeling and activation. Dysfunctional protein folding is linked to Alzheimer's disease, Parkinson's disease, cancer, and many others. In cancer cells, Hsp90 is upregulated and promotes folding of many oncoproteins. Hsp90 and Hsp70 function collaboratively to remodel and activate many substrate proteins. Studies using E. coli and S. cerevisiae showed that the Hsp90 middle domain directly interacts with the Hsp70 nucleotide binding domain (NBD), where J-domain proteins (JDPs) also bind. Moreover, JDPs were shown to facilitate and stabilize the Hsp90-Hsp70 interaction. To explore the role of JDPs in the collaborative activity of Hsp90 and Hsp70, we tested the hypothesis that JDPs function in protein remodeling by directly interacting with Hsp90 and Hsp70. We addressed this biochemically via chemical crosslinking and pulldowns. Using purified cysteine mutants and tri-arm crosslinking, we discovered the formation of a ternary complex containing E. coli Hsp90, Hsp70 and JDP. This observation was validated by mass spectrometry. The Hsp90-Hsp70-JDP complex only occurred when all 3 proteins were present and was abrogated when any one of the cysteine mutants was substituted with the respective wildtype protein. The Hsp90 region that interacted with both Hsp70 and JDP localized to its middle domain, in the region where Hsp70 alone bound. The J-domain region of the JDP, to which Hsp70 binds, was necessary and sufficient for ternary complex formation. The region in the Hsp70 NBD where JDPs bind, simultaneously interacted with JDP and Hsp90. These results highlight a new Hsp90-Hsp70-JDP intermediate in the protein remodeling pathway. We also characterized an Hsp90-JDP interaction, conserved in both bacteria and yeast, by BioLayer Interferometry, pulldowns and crosslinking assays. Analysis of Hsp90 and JDP site-directed mutants showed novel Hsp90-JDP interaction sites. These data strengthen our understanding of how molecular chaperones function collaboratively during proteostasis. Elucidating the mechanistic workings of chaperone proteins provides more insight into how these proteins function and will pave the way for developing effective treatments for cancer and other pathologies.

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Biochemistry - General
Photochemical Regulation of Translation Via Molecular Recognition-Based Reversible Photo-Crosslinking

Probe
The ability to control RNA structure and function has significant implications for understanding cellular processes and developing new therapeutics. Exploitation of RNA structures by chemical probes provide opportunities for the development of RNA-targeted drugs. Moreover, precise control of RNA function using small molecules or light-mediated techniques has opened new avenues for the development of RNA-based therapeutics and the study of complex biological systems. The present investigation showcases the capacity of CNV ligand to selectively recognize the PreQ1 aptamer through reversible covalent interaction and photochemical control of mRNA translation. This system relies exclusively on molecular recognition between a small molecule and RNA and is reversible, covalent and light-triggered. We demonstrate that the CNV probe specifically and covalently modifies PreQ1 aptamers using multiple biochemical and biophysical techniques. Importantly, both crosslinking and uncaging are highly selective and specifically dependent on both the chemical structure of the probe as well as the RNA sequence/structure. Synthesis of a biotinylated CNV probe facilitated the generation of a homogeneously modified caged mRNA containing a PreQ1 aptamer in the 5' UTR and a GFP coding sequence. In both in-vitro translation assays and when transfected into cells, the caged mRNA displayed repressed or no translation. However, upon irradiation with 302 nm light, the photocage was cleaved and translation was restored to levels equivalent to an unmodified mRNA. Thus, mRNAs containing the aptamer/CNV probe ligand pair can be used to enable direct, precise photochemical control over mRNA translation. Notably, this system provides a distinctive advantage of being precisely photochemically reversible using distinct UV wavelengths, thereby preventing any permanent chemical modifications in the RNA of interest. Consequently, this light-induced ON/OFF behavior in cells offers spatiotemporal regulation of gene expression without causing permanent changes in the cells, which can be extremely advantageous in situations where permanent alterations are not desirable. The approach outlined here is applicable to a wide range of complex RNAs and enables efficient photo-caging, thereby facilitating photochemical manipulation of RNA function. As a result, this technique holds considerable promise for regulating RNA function via photochemical means in diverse biological contexts.

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Biochemistry - General
Adipose tissue: a critical organ involved in ethanol metabolism and alcohol induced liver injury
Background: Liver is the major site of alcohol metabolism and a key target organ of alcohol-induced injury. Hepatic steatosis, characterized by lipid droplet accumulation in hepatocytes, develops in 90% of

alcoholic liver diseases (ALDs) patients, which may progress to steatohepatitis and cirrhosis. Despite some evidence suggesting the impaired capacity for fatty acid turnover, augmented NADH/NAD+ ratio and subsequently reduced β-oxidation of fatty acids in hepatocyte lead to steatosis, however, it is not well understood whether alcohol associated pathogenesis in adipose tissue contributes to hepatic steatosis, and the role of 'adipose-liver crosstalk' in alcohol induced liver injury is still obscure. Approach and Results: In the current study, to mimic long-term alcohol consumption in humans, first, 8-week plus multiple binges chronic ethanol feeding mouse model (EtOH-fed) and its control model were established. We compared the gene profiling of white adipose tissue (WAT) between control and EtOH-fed mice by performing RNA sequencing analysis, which suggested that ethanol feeding leads to a huge alteration of genes related to inflammation, cell cycle regulation, and fatty acid metabolism in WAT. Next, to explore the function and effects of acetaldehyde (AcH), a toxic metabolic byproduct of ethanol, in adipose tissue, we generated adipose specific aldehyde dehydrogenase 2 (ALDH2) deficient mice (Aldh2Adipo-/-) for EtOH-fed model establishment. Surprisingly, our data revealed that Aldh2Adipo-/- mice developed severer liver damage than WT mice, with higher serum ALT level, greater neutrophil infiltration and liver fibrosis, and more hepatic lipid droplet accumulation. Additionally, compared to the WT mice, Aldh2Adipo-/- mice showed decreased size of adipocytes, greater adipocyte death with more crown-like structures, less adipose/body weight ratio, and higher liver/body weight ratio after chronic ethanol feeding. Mechanistic study revealed that transcriptional co-activators YAP/TAZ, which has been known to protect against adipocyte cell death and control compensatory adipogenesis, were further downregulated in WAT of long-term EtOH-fed Aldh2Adipo-/- mice. Conclusion: Our study demonstrated that adipose tissue plays an important role in liver damage after alcohol consumption. Acetaldehyde produced in adipose tissue contributes to adipocyte death and lipid accumulation in liver, which promotes hepatic steatosis, liver inflammation and fibrosis.

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Biochemistry - Proteins, and Lipids
A small molecule inhibitor has a complex target profile including multiple ADP-ribosylation factor regulatory proteins
The ADP-ribosylation factor (Arf) GTPases are a subfamily of the Ras superfamily of small GTPases, functioning as regulators of membrane traffic and the actin cytoskeleton. Accumulating evidence indicates that Arf pathways might contribute to the pathological behaviors of cancers. An exciting discovery in this regard was the recent report of a small molecule, NAV-2729, that putatively bound to and inhibited Arf6 with high specificity and was effective against the presumed Arf6-dependent cancer uveal melanoma. We sought to determine if NAV-2729 could inhibit the proliferation of other cancer cell types that might depend on Arf pathways, including pediatric osteosarcomas and rhabdomyosarcomas. Different than other Arf pathway inhibitors, NAV-2729 exhibited similar cytotoxic potency in every cell line examined, but Arf6 expression levels did not correlate with the NAV-2729 potency of cell killing. Further, knockdown of Arf6 in two representative cell lines did not affect proliferation rates nor sensitivity to NAV-2729. Unexpectedly, NAV-2729 self-assembled into large structures in solution, but partitioned efficiently into phospholipid bilayers. NAV-2729 did not bind to native Arf proteins in the context of a membrane bilayer; however, NAV-2729 bound to Arf exchange factors including Brag2 and

ARNO, and Arf GTPase-activating proteins including ASAP1, ASAP3, and AGAP1, to inhibit their activity. Further biochemical and biophysical examination of ASAP1 revealed that NAV-2729 bound the ASAP1 Pleckstrin Homology (PH) domain at or near the site that the phospholipid phosphatidylinositol 4,5-bisphosphate binds to ASAP1. In addition, NAV-2729 had some cytoskeletal effects that were similar to the effect of reducing ASAP1, indicating that ASAP1 might be one target in cells. However, expression of neither Brag2 nor ASAP1 correlated with sensitivity to NAV-2729; similarly, knockdown of these proteins did not affect cell proliferation and only marginally affected sensitivity to NAV-2729, indicating neither was the sole nor primary target mediating the effects of NAV-2729. Unbiased screens identified at least 48 other potential NAV-2729 targets, including DNA-binding proteins. We conclude that NAV-2729 is not a specific inhibitor of Arf6 and that its cytotoxicity may be mediated by concurrent inhibition of multiple targets. In addition, we are using NAV-2729 as a tool to examine targeting of PH domain-dependent proteins, including both oncoproteins and their regulators.

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Biochemistry - Proteins, and Lipids

Generation of neurokinin receptor ligands with specialized signaling properties via nanobody conjugation

Nanobodies (the smallest antibody fragments that retain full antigen-binding capacity) are rapidly gaining traction as promising tools for pharmacologic undertakings. Nanobody-ligand conjugates can generate G protein-coupled receptor (GPCR) agonists with substantially greater activity and selectivity than prototype ligands from which they were derived. Biased agonism, the ability of a receptor to differentially activate downstream signaling pathways, has emerged as a powerful strategy for developing safer and more effective medications. The neurokinin-1 receptor (NK1R) is a GPCR from the tachykinin receptor family that is targeted by peptide agonists. Substance P (SP) and other endogenous agonists, such as neurokinin A (NKA), bind to NK1R and induce signaling via multiple pathways. In this work, we hypothesized that the conjugation of nanobodies to GPCR ligands could yield biased ligands. To test our hypothesis, we generated several NKA and SP6-11 (a truncated version of SP) nanobody conjugates that activate NK1R using sortase A-mediated ligation. Then we evaluated if they have signaling properties that diverge from that of the natural ligands. We found that NKA conjugation to nanobodies results in reduced efficacy but prolonged cAMP production, while Nb-SP6-11 conjugates failed to elicit cAMP. In addition, NKA and SP6-11 nanobody conjugation decreased β-arrestin2 recruitment, however, had little impact on Gq signaling. These findings demonstrate that the conjugation of Nbs with peptide ligands for GPCRs offers new avenues for the discovery of compounds with useful properties such as biased agonism or prolonged duration of action. Such biased ligands provide an unprecedented level of control of receptor functions, allowing for fundamental research into GPCR signal transduction mechanisms. Furthermore, it can translate into more efficacious and long-lasting therapeutic effects, potentially reducing side effects in clinical applications.

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Biochemistry - Proteins, and Lipids
Quantitative analysis of sterol modulated monomer-dimer equilibrium of β1-adrenergic receptor by DEER spectroscopy
G protein-coupled receptors (GCPR) play a vital role in intracellular signaling pathways and control various physiological processes in eukaryotes. The oligomerization properties of GPCRs, and hence their cellular functions, may be modulated by various components within the cell membrane (such as the presence of cholesterol). Modulation may occur directly via specific interaction with the GPCR or indirectly by affecting the physical properties of the membrane. Despite extensive investigations on structure-function relationships of membrane G protein-coupled receptors (GPCR), the effects of membrane components, such as cholesterol and its derivatives, on oligomerization preferences remains to be clarified. Using double electron electron resonance (DEER) spectroscopy, we demonstrate different effects of soluble cholesterol analog cholesteryl hemisuccinate (CHS) and cholesterol derivative bile salt sodium cholate on the oligomerization propensities of β1-adrenergic receptor (β1-AR) in DDM micelles. β1-AR is mostly expressed in cardiac tissue, where failure of cholesterol regulation can develop into various heart diseases. Global fitting of DEER echo curves for spin-labeled β1-AR upon titration with sodium cholate and CHS demonstrates that saturation of micelles with the former induces receptor dimerization, while specific binding of the latter to β1AR inhibits dimerization and stabilizes the monomeric form. The more novel technique for mimicking the natural membrane conditions are nanodiscs. Nanodiscs are self-assembled phospholipid bilayer enclosed in two helical membrane scaffold proteins. To investigate the dimerisation process we incorporated two β1-adrenergic receptor molecules into a single nanodisc. It appeared that unlike micelles, which restrict GPCR motion, the native-like environment of nanodiscs provides more degrees of freedom, which is important for recognition by different binding partners. Knowledge about the role of membrane composition in modulating the GPCR oligomerization process has far-reaching pharmaceutical application. Our results illustrate how quantitative analysis of DEER data can contribute to studies of GPCR oligomerization.

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Biochemistry - Proteins, and Lipids
Cryo-EM of Magnesium Transporter MgtA reveals a Dimeric P-type ATPase
Magnesium (Mg2+) uptake systems are present in all domains of life given the vital role of this ion. Bacteria acquire Mg2+ via conserved Mg2+ channels and Mg2+ transporters. The transporters are required for growth when Mg2+ is limiting or during bacterial pathogenesis, but, despite their significance, there are currently no known structures for the transporters. Here we report the first structure of the Escherichia coli transporter MgtA as solved by cryo-electron microscopy (cryo-EM). We obtained high resolution structures of both a homodimeric form (2.9 Å), the first for a P-type ATPase, and the standard monomeric form (3.6 Å). Each monomer unit of MgtA displays a similar overall structural architecture as other P-type ATPases. The dimer interface consists of contacts between residues in adjacent nucleotide binding and phosphorylation domains. The ATP binding site and consequences of nucleotide binding were characterized by a combination of cryo-EM, molecular dynamics simulations, and hydrogen deuterium exchange mass spectrometry. Finally, our structure

revealed a Mg2+ ion in the transmembrane segments, which, when combined with sequence conservation, allowed us to propose a model for Mg2+ transport across the lipid bilayer. Our dimeric structures of MgtA have functional implications for related P-type ATPases such as the calcium pump SERCA.

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Bioinformatics - algorithms, packages and tools
De novo assembly of Macropodus opercularis genome and annotation
There is a tendency in biomedical research to limit model organism research to a handful of the most used models such as yeast, drosophila, zebrafish, and mouse. One of the key reasons for this is that until very recently, a high-quality genomic assembly was costing millions of dollars. This has changed dramatically in the last few years as “long-read” sequencing technologies have both advanced in robustness and dropped in price. The result of this is that many more organisms with interesting biological features can realistically become “model” organisms. The Macropodus opercularis, has a repertoire of physical morphology and interesting behaviors that can be leveraged for understanding behavioral genetics and the evolution of traits. These behaviors and drastic changes physiology simply cannot be studied using zebrafish as the species simply does not display the strong behaviors or sexual dimorphism displayed by paradise fish. Currently the zebrafish genome remains the most highly annotated and referenced fish genome. Alternative fish genome assemblies and annotation are urgently needed to increase range of possible scientific inquiry. In this project, we assembled and annotated the paradisefish genome. We used 160X coverage of PacBio HiFi long reads and Hifiasm software to assemble a high-quality genomic assembly with 90% of all sequence incorporated into 23 contigs which matches the number of chromosomes in the paradise fish. We then used deep transcriptome sequence data and the Trinity RNA assembler to create a comprehensive transcriptome. We used both GeneMark-ES and Augustus to train gene models on the genome and then used the models to annotate the genome using the MAKER gene annotation pipeline. Our analysis demonstrated that paradisefish genome had low heterozygosity (0.07%) and the genome repeats content was predicted to be ~10.4%. We produced a highly contiguous genome assembly with an NG50 of 20mbs and > 99% canonical k-mers composition being 1x copy number. The genome and transcriptome assemblies had BUSCO scores of 98.5% and 99.6%, respectively. We showed that more that 99% of the genes had annotation edit distance (AED) value <= 0.5. The Ex90N50 was 4.4kbs and the transcriptome mapping rate was 98.4%. By doing genome assembly and annotation of paradisefish genome, we produced a highly quality assembly and annotation file (GFF) which will be freely shared with the scientific community to help advance the species as a useful model organism

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Bioinformatics - algorithms, packages and tools

Large-scale Pre-trained Language Models for Dense Information Retrieval and Beyond
Background: Most literature search engines only return articles with exact mentions of the input queries. As a result, relevant articles that do not contain the exact search terms will be missed. Recent progress in information retrieval (IR) and deep learning has shown that dense vectors generated by language models can perform better text retrieval by matching semantically related terms (e.g., “kidney” and “renal”). However, training such a dense retrieval model requires abundant query-article annotations, which are difficult to obtain in biomedicine as manually annotating large-scale data is prohibitively expensive. Objective: We aim to investigate a first-of-its-kind dense retrieval model with large-scale training data from literature search logs, and systematically evaluate it on various biomedical IR tasks with respect to both model accuracy and generalizability. Methods: We collected 15 million query-article pairs from the click logs of a widely used biomedical literature search engine. Using such data, we then trained transformer-based query and article encoders with a novel loss function, which is a combination of in-batch cross-entropy loss and contrastive triplet loss. As such, the training makes the query vectors close to their clicked/relevant article vectors and far away from other irrelevant article vectors. Results: Experimental results show that our method achieves better mean average precision (MAP) than the classic term-matching BM25 method on an independent test set (76.9% v.s. 66.4%) and surpasses previous state-of-the-art (SOTA) MAP on BioASQ (34.1% v.s. 17.0%), a task on retrieving relevant articles for biomedical questions. We also assessed the query and article encoders on their respective tasks: the query encoder achieves SOTA MAP on the COVID-FAQ similar question retrieval task (39.9% v.s. 37.3% by previous SOTA); Our article encoder achieves superior F1 on the MeSH classification task of the SciDocs benchmark (89.9% v.s. 89.4% by previous SOTA) and a better average score on the RELISH similar article evaluation (91.9 v.s. 89.9 by previous SOTA). Conclusion: We show that our novel language model encoders pre-trained by large-scale search logs not only perform well on query-article retrieval tasks, but also generate better query and article embeddings for tasks that are related to information-seeking behaviors.

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Bioinformatics - algorithms, packages and tools
Shotgun metagenomics sequencing reveals novel insights of indoor dust microbiota compared with 16S rRNA technology
Due to advances in high-throughput sequencing technologies, microbiome has been identified as a key regulator of human health. A common method, 16S rRNA gene amplicon sequencing (16S), amplifies regions of highly conserved bacterial genes. Despite its low cost and well-established analysis pipelines, 16S is typically restricted to genus level taxonomic resolution. A different approach is metagenomic whole genome shotgun sequencing (WGS), which sequences all sample DNA, allows for the higher resolution to species and even strain levels, as well as identification of viruses, providing a more comprehensive description of the microbial community and improved ability to identify novel signatures. However, analysis pipelines are less established. Further, evaluation of WGS on microbial community characterization in low microbial biomass samples, such as dust, remains unexplored. To this end, we analyzed indoor dust samples from 781 participant homes in the Agricultural Lung Health Study using both 16S and WGS, to investigate their reliability in depicting dust microbiota profiles and capacity

for novel signals retrieved exclusively by WGS. First, we built a bioinformatics pipeline for processing WGS dust sample data including raw-read quality control, removal of host-derived DNA contaminants, and profiling and quantification of microbial composition. We then utilized generalized linear regression model (ANCOM-BC) to investigate associations between individual microbe abundance and environmental risk factors, including participants' farming status and home conditions. The Benjamini-Hochberg method was used to control the false discovery rate at 0.05. Finally, results were contrasted with results from the same samples using 16S. Overall, WGS identified more microbes than 16S (6526 vs. 1346), including 615 viruses. Specifically, 16S detected only part of the phyla (17) revealed by WGS (59) in dust microbiota, where most WGS-unique microbes are not rare. Moreover, additional associations were observed by WGS. Notably, abundance of a unique phylum (Ascomycota) was associated with crop farming activity and the unique species pseudintermedius and felis were associated with the presence of indoor pets (p<0.05). We showed WGS is more powerful to survey microbial community in low biomass samples. WGS-only taxa are biologically meaningful, revealing novel insights between indoor dust microbiota and environmental exposures, which are critical for studying human health.

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Biophysics and Biomedical Engineering

Divergent systemic T cell response to injury and biomaterial implantation

The current framework of immune cell infiltration, tissue regeneration and fibrotic encapsulation after injury and biomaterial implantation in the muscle and skin is largely focused on innate immune responses. As such, the role of T cells in shaping host response to wound healing and tissue regeneration is less understood. Utilizing a mouse model of volumetric muscle loss (VML), we surgically removed a 3x3x3 mm portion of quadriceps muscle allowing for material implantation to examine the local and systemic responses of T cells to injury and implanted biomaterials. Clinically, both synthetic and biological scaffolds are used for providing structural support, as filler materials and to promote wound healing. We utilized decellularized extracellular matrix (ECM) derived from porcine small intestinal submucosa and polyethylene (PE) as representative experimental implants. Furthermore, mice which received saline after VML and quadriceps from uninjured mice served as controls. Inguinal lymph nodes (ILN) which drain the quadriceps, axillary and brachial (ABLN) as well as mandibular lymph nodes (MaLN) which are distal to quadriceps, mesenteric lymph nodes (MeLN) which do not drain quadriceps, and spleens were collected from mice 1 week after VML. Using a 19-parameter panel comprising lymphoid markers, we performed spectral flow cytometry to quantify the abundance of activated T cells at secondary lymphoid sites. In ILN (p<0.001) and ABLN (p<0.05), with saline and ECM treatments but not PE, we observed ~3-fold higher CD62L- CD44+ activated CD4+ T cells as compared to uninjured controls. In spleens (p<0.001) and ABLN (Saline:p=0.0001; ECM:p=0.01; PE:p=0.002) activated CD4+ T cell abundance increased significantly with injury across different material treatments. In contrast, CD8+ T cell activation was promoted by PE and saline but not ECM treatment in ILN (PE:p= 0.002) and ABLN (PE:p= 0.02). However, activated CD8+ T cell abundance converged across all treatments in MaLN (p<0.05) while activation was promoted by PE treatment alone in spleen (p=0.01) and MeLN (p=0.002). Taken together, our data suggests that firstly, T cells are systemically activated in response to traumatic muscle injury and biomaterial implantation. Secondly, T cell response can be modulated using

biomaterials and lastly, we provide a basis for future studies to evaluate mechanisms of T cell-biomaterial interaction using single cell RNA sequencing and T-cell receptor sequencing.

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Biophysics and Biomedical Engineering

Experimental evidence for millisecond structural annealing of the ultra-fast folding villin headpiece subdomain HP35

Protein folding is a complicated structural conversion process which lies at the heart of many biophysical and biological systems of interest. Protein folding kinetics, however, remain poorly understood while being of crucial importance for a range of pathologies associated with misfolded or aggregation-prone proteins. Kinetic experiments carried out after triggering a structural conversion can provide invaluable insights into folding kinetics, including the possibility of directly accessing short-lived intermediate or off-pathway states; however, combining sufficiently fast time resolution with techniques that provide detailed, atomic-scale spatial resolution is challenging. Time-resolved solid-state nuclear magnetic resonance (ssNMR) spectroscopy combines site-specific, atomistic structural information with millisecond time resolution by first triggering a conversion process, then taking "snapshots" of structural changes by rapidly freezing after a variable evolution delay. In this work we use sub-millisecond negative temperature jumps to trigger folding of the 35-residue villin headpiece subdomain (HP35), an ultra-fast folding protein studied extensively and widely used as a folding benchmark. Using a specially constructed temperature-jump apparatus utilizing copper capillary tubes anchored to temperature-controlled copper plates, solutions containing HP35 are first heated to 95 °C, causing HP35 to unfold, then rapidly cooled to 30 °C in 0.6 ms to trigger folding. After a variable evolution time at 30 °C, HP35 is frozen in ~0.1 ms in a -145 °C isopentane bath. Frozen ensembles are subsequently studied using low-temperature ssNMR enhanced with dynamic nuclear polarization (DNP). 1D and 2D ssNMR spectra acquired as a function of the variable evolution time show signals consistent with native secondary structure forming on the sub-millisecond timescale, as expected from previous studies, but crucially provide evidence that full structural order, including the alignment of native sidechain configurations, forms much slower through a millisecond structural annealing process. ssNMR provides the quantitative, site-specific, and atomistic information necessary to characterize this annealing process, which has not previously been observed in ultra-fast folding proteins. Time-resolved ssNMR triggered by rapid temperature jumps offers direct access to short-lived structures and complexes and is applicable to diverse biomolecular and biophysical systems.

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Biophysics and Biomedical Engineering

Pressure Jump NMR: a new technique to study on-pathway protein folding intermediates

Most proteins are believed to transiently populate partially folded species, called folding intermediates

along their pathway to the native state. These folding intermediates play a key role in protein folding and function and also are key to misfolding and aggregation. Yet, due to their short lifetime, high energy, and low population, they are not easily accessible to most structural techniques, and very few folding intermediates have been described in atomic detail. Hydrostatic pressure can reversibly shift the thermodynamic equilibrium between folded and unfolded states, enabling experimental control over folding and unfolding. We utilize spectrometer-controlled hardware that performs rapid and repeatable pressure switching within a sample cell to record the residue-specific NMR signals immediately after a drop in hydrostatic pressure. This provides repeatable method for probing the transient, short-lived intermediates that occur when shifting conditions from those that favor the unfolded state (high pressure) to those that favor the folded state (low pressure). Repeating the pressure jump process many thousands of times allows the study of the folding processes on timescales much shorter than the time needed to acquire a full NMR spectrum. Several studies have proposed that ubiquitin can fold through a meta-stable folding intermediate. Here, by employing a pressure-sensitive mutant of ubiquitin (L50A) and our pressure-jump apparatus, we could confirm the presence of a functionally important on-pathway folding intermediate with a lifetime of ~250 ms at 5°C. In addition to this known folding intermediate, we also detected another on-pathway folding intermediate with shorter lifetime and lower population which had not been observed by other methods before. This work enables the first detailed structural folding study of a protein with multiple on-pathway intermediates and provides new insights into folding dynamics and kinetics.

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Water exchange rates measure tissue viability and homeostasis

For its size, the brain is the most metabolically active organ in the body. Most of its energy demand is used to maintain stable homeostatic physiological conditions. Altered homeostasis and active states are hallmarks of many diseases and disorders. Yet current reliable non-invasive methods used to characterize basal activity in the central nervous system either require exogenous tracers or contrast agents (e.g., Positron Emission Tomography requires radioactive tracers) or are indirect and sensitive only to metabolic changes (e.g., functional Magnetic Resonance Imaging relies on an indirect hemodynamic coupling effect on relative signal changes). Steady-state water transport across plasma membranes of the dense network of neurons and glia could provide an endogenous marker for brain activity, but it occurs too rapidly to be observed by current techniques. We report the development of a low-field, high-gradient diffusion exchange Magnetic Resonance (MR) method able to “see” these rapidly exchanging water molecules. Exchange rates are 140 +/- 16 1/s under normal conditions in viable ex vivo neonatal mouse spinal cords. High repeatability across samples suggest that values are absolute and intrinsic to the tissue. Using temperature and drug (ouabain) perturbations, we find that most of the water exchange is metabolically active and coupled to active transport by the sodium–potassium pump. Simultaneous microscopy imaging and MR measurements are performed during Oxygen and Glucose Deprivation (OGD) to understand how water exchange is affected by energetic failure during stroke. Simultaneous measurements of Apparent Diffusion Coefficient (ADC, from MR) and Intrinsic Optical Signal (IOS, from microscopy) monitor cellular swelling and shrinking, while membrane voltage and

intracellular calcium (from fluorescent microscopy) monitor cell function. Results show that exchange is not directly linked to structural changes, as it is unconnected from ADC and IOS observations of a first cell swelling response to OGD and a recovery of extracellular space after OGD. Rather, the exchange is linked to a loss of tissue homeostasis and viability, as it drops simultaneously with a second rise in the IOS, a drop in voltage, and a massive increase in intracellular calcium. This method can potentially be translated to clinical MRI applications for measuring cell function, activity, and viability in the human brain and body.

Kyle Schwab
Other
NEI
Cell Biology - Cell Cycle and Metabolism
A Novel 3D-Printed Stirred Bioreactor to Improve Retinal Organoid Cultures
Retinal organoids (ROs), derived from pluripotent stem cells, closely replicate in vivo development and retinal morphology, providing a platform to study and develop treatments for hereditary degenerative retinal diseases, the leading causes of vision loss. However, researchers have encountered difficulty cultivating ROs that fully recapitulate in vivo retinogenesis. One possible explanation could be insufficient oxygen availability during differentiation due to static culture methods. During static culture of retinal tissues, the oxygen consumption rate may exceed the rate at which atmospheric oxygen can diffuse through media volume. To address this limitation, bioreactors have been shown to significantly improve organoid culture outcomes by increasing oxygen availability. We have developed a novel, 3D-printed stirred bioreactor (SBR), which can be easily implemented in most labs, requiring only a 100mm Petri dish, magnetic stir plate, and small stir bar. Our SBR system provides a widely applicable and straightforward platform to increase and maintain dissolved oxygen concentrations leading to the generation of ROs with greater size, yield, and improved differentiation. We evaluated our SBR using an adherent culture protocol with three human pluripotent stem cells (hPSCs) lines, NRL-L75pfs, NEI901, and PEN8E. Once hPSCs reached 20-30% confluency in a 100mm Petri dish, differentiation was begun using neural-induction media. At D6, SBRs fitted with small stir bars were inserted and returned to incubation on a magnetic stir plate set to 300RPM. Culture proceeded until days 18–21, when sufficient maturation of optic vesicles was observed. Cultures were then scraped and transferred to low-adhesion dishes for continued free-floating culture. Measurements of the partial pressure of oxygen in cell culture media were taken using contactless optical sensor spots placed on the bottom of the dish. Dishes containing SBRs maintained an average of 28.05 mmHg, while static conditions averaged 2.34mmHg, representing a greater than 10-fold increase in available oxygen. Analysis of the number and size of hPSC-derived ROs performed on D24 demonstrated significantly higher yield and larger size in all cell lines cultured using SBRs (p<0.05) compared to static conditions. Our research suggests that the SBR platform maintains high oxygen concentrations during differentiation leading to greater size and yield of ROs compared to traditional static culture methods.

Yingbiao Zhang
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Cell Biology - Cell Cycle and Metabolism
Wdr59 promotes or inhibits TORC1 activity depending on cellular context
Target of Rapamycin Complex I (TORC1) is a central regulator of metabolism in eukaryotes that responds to a wide array of negative and positive inputs. The GATOR signaling pathway acts upstream of TORC1 and is comprised of two subcomplexes. The trimeric GATOR1 complex inhibits TORC1 activity in response to amino acid limitation by serving as a GTPase activating protein (GAP) for the TORC1 activator RagA/B, a component of the lysosomally located Rag GTPase. The multi-protein GATOR2 complex inhibits the activity of GATOR1 and thus promotes TORC1 activation. Because of lack of in vivo study tools, we constructed a knockin toolbox of transgenic flies includes molecules up/downstream of TORC1 by using CRISPR/CAS9 method, aim to uncover the in vivo regulation mechanisms in GATOR/TSC/TORC1 pathways in an endogenous level. All the target proteins are tagged in small tags which can be used in immunolocalization and biochemistry assay, furthermore, we also tagged in a small GFP11 which allows to perform a live imaging assay to track molecules dynamic exchange by combining a GFP1-10 fragment genetic method. By using the knockin toolbox of transgenic flies, we report that Wdr59, originally assigned to the GATOR2 complex based on studies performed in tissue culture cells, unexpectedly has a dual function in TORC1 regulation in Drosophila. We find that in the ovary and the eye imaginal disc brain complex, Wdr59 inhibits TORC1 activity by opposing the GATOR2 dependent inhibition of GATOR1. Conversely, in the Drosophila fat body, Wdr59 promotes the accumulation of the GATOR2 component Mio and is required for TORC1 activation. Similarly, in mammalian HeLa cells, Wdr59 prevents the proteolytic destruction of GATOR2 proteins Mio and Wdr24. Consistent with the reduced levels of the TORC1 activating GATOR2 complex, Wdr59KOs HeLa cells have reduced TORC1 activity which is restored along with GATOR2 protein levels upon proteasome inhibition. Taken together, our data support the model that the Wdr59 component of the GATOR2 complex functions to promote or inhibit TORC1 activity depending on cellular context.

Zenia Kaul
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Cell Biology - Cell Cycle and Metabolism
Itk is essential for metabolic reprogramming, protein translation and effector functions in CD4+ T lymphocytes
The Tec family non-receptor tyrosine kinase, IL-2 inducible T cell kinase (Itk), is a critical component of T cell receptor (TCR) signaling, required for full activation of phospholipase-Cg and effective TCR signaling. However, downstream consequences of loss of Itk are still not fully understood. Activation of T cells is characterized by promotion of gene expression, effector differentiation, IL-2 signaling, nutrient uptake and reprogramming of metabolism. We previously reported that Itk-/- CD4 T cells have impaired differentiation to multiple T helper lineages with the most severe defects in Th9 cells. We linked this defect to impaired production of IL-2, a cytokine having immune activation functions. I found that Itk-/- Th9 cells had decreased expression of Myc and multiple nutrient transporters—including the Glucose transporter 1 (GLUT1), transferrin receptor (CD71), amino acid co-transporter (CD98) and arginine transporter (SLC7A1) compared to WT. To look at metabolism in these cells, we used SCENITH—a flow cytometry-based technique that monitors effects of metabolic pathways on protein translation. We

found that WT Th9 cells were primarily glycolytic but Itk-/- T cells exhibited marked defects in protein synthesis and were more dependent on mitochondrial respiration. Itk-/- T cells had decreased expression of key genes regulating glycolysis compared to WT cells, yet Itk-/- mitochondria were also less polarized and functional measured by Mito-tracker Red and mitochondrial superoxide production. All these defects could be rescued by adding IL-2. Arginine catabolism product spermidine is required for the post-translational modification hypusination of eukaryotic translation initiation factor 5A (eIF5A), which is known to regulate protein translation and mitochondrial function. I found that Itk-/- cells had less hypusinated eIF5A than WT, which was improved by addition of IL-2 to similar levels seen in WT. Our results suggest that Itk and IL-2 provide a metabolic checkpoint, regulating nutrient transport, metabolism and perhaps protein translation through hypusination of eIF5A, which are all required for T cell effector function, providing insight into the underlying cellular and metabolic programs that regulate T cell activation that may be key for developing novel approaches to modulate T cell responses in disease. We will further investigate pathways/targets affected by Itk by RNASeq, protein translation, and metabolomic studies.

Alexandra Harris
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Cell Biology - General
Chromatin accessibility landscape of human triple-negative breast cancer cell lines reveals variation by patient donor ancestry
African American (AA) women are at an increased risk of developing and dying from Triple-Negative Breast Cancer (TNBC), an aggressive breast cancer subtype, compared to European American (EA) women in the United States. In addition to social determinants, further investigation into biologic factors that contribute to these disparities is needed to fully understand this multi-factorial problem. Taking a reductionist approach, we employed ATAC-sequencing to characterize differences in chromatin accessibility between 9 commonly used TNBC cell lines derived from patients of European (n=4) vs. African American (n=5) ancestry. Principal component analysis of the top 50,000 peaks with the most variance revealed separation of chromatin profiles by genetic ancestry. Motif enrichment and digital footprinting analysis of differentially accessible chromatin regions identified increased binding of 62 transcription factors (TFs) in AA TNBC cell lines associated with aggressive tumor features, such as epithelial-to-mesenchymal transition (ZEB1, SNAI1, SNAI2, GRHL2), cancer stemness/chemotherapeutic resistance (TFAP2C, NRF1), and proliferation (E2F, E2F1, E2F2), as well as KAISO, which is linked to poor outcomes in AA breast and prostate cancer patients. Differential ATAC signal analysis identified 1831 genes located within promoters of differentially open chromatin regions in AA-derived TNBC, with DNA methyltransferase 1 (DNMT1) as the top upregulated gene associated with African ancestry (|log2FC|=6.73, FDR=4.9x10^-12). Pathway analyses of differential genes associated with African ancestry revealed significant enrichment of hypoxia, cancer metabolism, and inflammatory pathways. Culturing cells under hypoxia (1% O2) revealed ancestry-specific stress responses that led to the identification of a core set of AA-associated TFs related to TP53, cell cycle, and cell stress that formed a significantly enriched protein-protein interaction network (p<1x10^-16; N-nodes=55; N-edges=148). Together, these data reveal a differential chromatin landscape associated with the aberrant activity of critical TFs and downstream gene expression changes that may contribute to worsened TNBC biology in

women of African ancestry. Further, as many of these cell lines are used routinely in biomedical research, these findings also indicate that the ancestral origin of patient derived cell lines matters and the inclusion of diversely sourced cell lines should be considered in experimental design.

Inderjeet Inderjeet
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Cell Biology - General
A novel large conductance ion channel involved in malaria parasite transmission via mosquitoes
Plasmodium spp. cause malaria and have complex life cycles in their vertebrate hosts and mosquito vectors. In the mosquito, immature sexual forms termed gametocytes must differentiate into male and female gametes, undergo fertilization, traffic across several membranes, and achieve large-scale replication to produce infectious sporozoites for transmission in the vector saliva. Each of these steps requires transport of ions and nutrients across parasite membranes, but these transport activities remain largely unexplored. We have now devised a protocol to isolate and study membrane transport on single Plasmodium female gametes. Using a transgenic rodent Plasmodium berghei pathogen with a female-specific fluorescent reporter (RFP) and anti-TER119 to stain erythrocyte membranes, we excluded parasites still trapped in their host erythrocytes and isolated xanthurenic acid-activated female gametes. Pbs21 antibody staining further confirmed successful recovery of female gametes free of mouse membranes. This approach allowed the first patch-clamp study of transport at the female gamete plasma membrane. Under our recording conditions, a single reproducible ion channel was detected on the parasite surface. The channel exhibited complex voltage-dependent behavior and a large single channel conductance (1550 pS) in molar salt solutions. Replacement of the pipette salt solution with uncharged sorbitol yielded a reduced conductance of 630 pS with an unchanged reversal potential, suggesting high permeability to both cations and anions and weak saturation at molar salt concentrations. We propose that this channel is critical for gamete ion homeostasis and are testing an essential role in fertilization by male gametes. The channel's stage-specific activation and surface location suggest it is an ideal target for transmission-blocking vaccines or insecticide-like drugs that prevent spread of malaria. The methods developed here will enable discovery of other ion channels and transporters on parasite membranes in the mosquito vector. Our findings provide foundational insights into mosquito-stage parasite biology.

Jorge Romo Tena
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Cell Biology - General
PAD2-mediated citrullination of nuclear factor-kappa B regulates endothelial-to-mesenchymal transition.
Endothelial-to-mesenchymal transition (EndMT) is a process where endothelial cells (ECs) trans-differentiate into mesenchymal cells. This process can be triggered by proinflammatory cytokines such as tumor necrosis factor (TNF)-alpha. EndMT has been implicated in rheumatic diseases including Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis (RA). Citrullination is a posttranslational

modification where arginine residues are converted into citrulline and is catalyzed by peptidylarginine deiminases (PADs). PAD2 and PAD4 are activated during inflammation in myeloid cells and have been implicated in pathogenic responses and end-organ damage in SLE and RA. However, the role of citrullination has not been investigated during EndMT. Therefore, we hypothesized that protein citrullination regulates TNF-mediated EndMT through citrullination of NFkB-p65. EC lines were used to study EndMT. qPCR, Western blot and rhodamine-citrulline probes were employed to assess the expression and citrullination capabilities of PADs. Transfection of recombinant proteins combined with co-immunoprecipitation experiments were used to assess protein-protein interactions. We found that PAD2 was expressed in ECs at gene and protein levels. Treatment with Cl-amidine, a pan-PAD inhibitor, and AFM30a (a PAD2 inhibitor) as well as PAD2 genetic ablation decreased the levels of mesenchymal markers, N-cadherin and Snail, in ECs treated with TNF for 48h, indicating inhibition of EndMT. Overexpression of PAD2 in ECs increased the levels of the mesenchymal marker, N-cadherin, while decreasing the levels of endothelial markers, VE-cadherin and CD31, suggesting accelerated EndMT. We detected a citrullinated protein appearing in TNF-exposed ECs in total lysate and nuclear fraction. Co-Immunoprecipitation experiments demonstrated that recombinant PAD2 interacts with NFkB-p65 after TNF stimulation. Preliminarily, these results support that citrullination is involved during EndMT. Specifically, NFkB-p65 interacts with PAD2 during early stages of TNF signaling to promote mesenchymal polarization. Further experimentation will determine the role of citrullination during EndMT and its implications in endothelial dysfunction and end-organ damage in rheumatic diseases.

Vibha Dwivedi
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Cell Biology - General
Utilizing RNA devices to modulate PD-1 gene expression in mammalian cells for cancer immunotherapy
Immune checkpoint blockade, a major medical breakthrough in recent years, has become one of the most effective approaches in immunotherapy against a broad spectrum of cancers, including those in late metastatic stages. The agents against either the PD-1 receptor or the PD-L1 receptor-ligand have high efficacy and long durability. However, one of the problems associated with the current immunotherapy approach is the exhaustion of T lymphocyte cells. PD1 is an important gene to maintain peripheral tolerance and cellular homeostasis and hence, treatment procedures mediated by checkpoint inhibition are reported to impose deleterious effects. We propose a different approach. Instead of checkpoint blockade, we specifically control expression of genes key to checkpoint inhibition by using riboswitch devices to control the expression of PD-1 directly at the levels of transcription, splicing and translation through bypassing the host cell regulatory networks and pathway altogether. We have developed a system for rapid testing of rationally designed tetracycline regulated RNA devices to control PD1 gene expression by CRISPR knock-in (in EL4 cells). We have established the proof of concept that a ribozyme and a couple of functional RNA devices work efficiently in mammalian cells and demonstrated their reversibility and re-inducibility to regulate the gene expression. We have also made a lentiviral library of devices by randomizing the sequences in communication module with the help of our in-house script and we are in the process of performing high throughput screening to identify RNA devices with high efficiency and test the applicability of the RNA devices in animal models before possible clinical application. In summary, our system clearly indicates the robustness, efficiency, and reversibility of the

functional riboswitch devices to control PD1 gene expression for the development of a proper treatment regimen for cancer and viral infections with higher success rate relative to the currently available approaches.

Xinh-Xinh Nguyen
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Cell Biology - General
3D Biofabricated Immunocompetent Skin Tissue Model of Atopic Dermatitis for Testing the Efficacy of Immunomodulatory Mesenchymal Stromal Cell Therapies
Atopic dermatitis (AD) is a complex inflammatory skin disease that involves multiple cell types and immune system imbalance. During disease progression, T-helper type 2 (Th2) cells migrate to the skin and secrete pro-inflammatory cytokines such as IL-4 and IL-13, which induce type 2 inflammation underlie the skin barrier dysfunction, inflammation, and itch in AD. We have developed a protocol to fabricate normal and full thickness skin (FTS) and vascularized (VFTS) tissues. We have demonstrated that we can establish AD-like FTS and VFTS tissues by treatment with the cytokine IL-4. The IL-4 induced AD skin models recapitulate the hallmarks of AD including spongiosis-like intercellular spaces, epithelial hyperplasia, and the loss of barrier function, further highlighting the rescue of AD-like phenotypes by potent inhibitors of the JAK-STAT pathways. However, the lack of immune components in this model has significantly hindered immunological studies and testing immunomodulators. Therefore, in place of adding IL-4, we have increased the physiological complexity and relevance of the previous model by incorporating Th2 cells to induce the AD-phenotype. Human naive CD4+ T-cells isolated from peripheral blood mononuclear cells (PBMCs) are polarized in vitro to Th2 cells and incorporated within the fabricated skin tissues. AD skin tissue phenotypes are assessed by histological analysis, loss of barrier function, and secretion of AD relevant cytokines. Our data show that Th2-incorporated skin tissues exhibit AD-like phenotypes including the loss of barrier function, suggesting the barrier function of the skin is impaired, and undifferentiated epidermal keratinocytes by histology. Our data also shows that Th2-incorporated skin tissues produce a broad range of cytokines and chemokines such as TNF-α, IFN-γ, CXCL9, and CCL17. We also utilize our immunocompetent AD model test whether cell-based immunological therapies, such as mesenchymal stromal cells (MSC), can be used to monitor the suppressive T-cells response. Additional MSC treatment helps to restore the vasculature phenotype in our AD model. Taken together, our 3D fabricated tissue models have potential to improve understanding of immune cell interactions and mechanisms during the initiation and progression of such complex immune driven skin diseases, while also providing testing platforms for different therapeutic approaches, including small molecules and biologics.

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Cell Biology - Intracellular Trafficking and Cell Signaling
Distinct microglial populations and their NF-κB dynamics with age

Microglia are immune sentinels in the brain that are capable of orchestrating potent inflammatory responses in aging and neurodegenerative diseases like Alzheimer's and Parkinson's diseases. NF-κB signaling pathway is commonly recognized as a significant regulator of inflammation and aging. Therefore, it is essential to understand the roles of NF-κB signaling during aging and neuroinflammation. Mapping the spatiotemporal complexity of NF-κB signaling is crucial to understand its impact and function in vivo, but the lack of tools to directly monitor NF-κB protein components has hindered such efforts. Our lab has generated reporter mice with the endogenous RelA (p65) and c-Rel labeled with distinct fluorescent proteins and a double knock-in line with both subunits labeled. To understand how aging affects microglial function, we isolated primary microglia from young and old animals. We cultured them in vitro and verified their microglial identity by P2RY12+, CD11b+ and CD45int. Interestingly, we observed two different microglial populations, one motile, and the other forming clusters. Microglia from aged animals showed a higher prevalence of motile, free-roaming microglia. We also observed these distinct microglial populations in brain slices from the mice ex vivo. Live-cell imaging of NF-kB dynamics in primary microglia from young and old mice revealed a shift towards c-Rel in old brains. Further, we stained for Ki67 as a proliferation marker to functionally characterize these microglia populations. Interestingly, we observed only the free-roaming microglia to be proliferative in nature. In addition, we are exploring the differential cell surface markers, phagocytotic capability, senescence pattern, antigen presentation capacity, and finally NF-kB dynamics of these two distinct microglial populations. Lastly, we are decoding the biological significance of c-Rel shift in old brains. These findings will help us better understand the molecular mechanisms associated with age-dependent and inflammation-derived changes in young and old brains, which can ultimately shape the cell-targeted therapies for neurological disorders.

Kazuki Obashi
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Cell Biology - Intracellular Trafficking and Cell Signaling
Revealing conformational dynamics of endocytic proteins on the plasma membrane using FRET-CLEM
Clathrin-mediated endocytosis is the primary internalization pathway in eukaryotic cells. It is important for many cellular processes, including nutrient uptake, signaling, and the recycling of membrane components. Therefore, impairment of this process is associated with many human diseases. During clathrin-mediated endocytosis, endocytic proteins are assembled on the plasma membrane. Subsequently, the clathrin coat grows and curves. Finally, the invagination is separated from the plasma membrane by scission to generate cargo-loaded vesicles. In this process, the curvature of clathrin lattices is key step. However, the control of the lattice curvature remains unclear. To investigate conformational changes in endocytic proteins that regulate lattice curvature, we used fluorescence resonance energy transfer (FRET). FRET efficiency is strongly dependent on the distance between the donor and acceptor, allowing the detection of nanometer-scale molecular events. The diffraction-limited spatial resolution of FRET imaging cannot discriminate morphological differences in the clathrin lattices. However, to understand how endocytosis works, FRET measurements must be correlated with the distinct stages of endocytosis. To overcome this gap, we developed a correlative FRET and platinum replica electron microscopy method, named FRET-CLEM. Here, FRET-based atomic distances can be mapped directly to individual clathrin lattices visualized by EM at the plasma membrane. We used this

method to measure conformational changes in the clathrin light chain (CLC), a component of the clathrin triskelion and assembled clathrin lattice. Structural changes in CLC have been shown to regulate triskelia assembly in solution; however, the nature of these changes and their effects on lattice growth, curvature, and endocytosis in cells are unknown. Using FRET-CLEM, we discovered that CLC undergoes a conformational switch as clathrin lattices curve. Preventing this conformational switch using acute chemical tools increases the lattice size and inhibits endocytosis. Therefore, a specific conformational switch in CLC, which we discovered by FRET-CLEM, regulates the lattice curvature and endocytosis in mammalian cells. These data will help develop a complete mechanistic model of endocytosis. Generally, FRET-CLEM can map molecular interactions and conformational changes at targeted membrane-associated proteins in identified cellular compartments, including exocytic sites and neuronal synapses.

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Cell Biology - Intracellular Trafficking and Cell Signaling

Characterization of a ciliopathy-related disorder caused by WDR44 variants

Primary cilia are microtubule based antenna-like structures required for developmental signaling pathways such as Hedgehog (Hh). These organelles are derived from the mother centriole (MC) through a complex process called ciliogenesis which is initiated by preciliary vesicle (PCV) trafficking and docking to the MC. Notably, ciliopathy disorders have been linked to defects in PCV-MC docking, but not PCV trafficking. RAB11 is required for PCV transport and interaction with WDR44 through its NH2-terminal RAB11 binding domain (RBD) prevents ciliogenesis initiation by a poorly understood mechanism. Here we describe a new X-linked ciliopathy-related disorder caused by mutations in the RAB11 effector WDR44. We identified seven missense and one nonsense WDR44 variants in the COOH-terminal WD40 repeat domain from male patients that display ciliopathy-related features including musculoskeletal abnormalities, craniofacial dysmorphism, and kidney disease. Fibroblasts isolated from severely affected patients have aberrant ciliogenesis initiation and reduced Hh signaling implicating dysfunctional cilia as causative in disease. Zebrafish embryos expressing human WDR44 variants show ciliopathy-related phenotypes that correlate with the severity of the disease spectrum observed in these patients. Moreover, the expression of WDR44 variants in human cells and zebrafish embryos reduces ciliation. Remarkably, missense variants associated with more severe disease show strongly reduced expression, which was unexpected based on the WDR44 function in blocking PCV trafficking. We discovered that the gain-of-function for these proteasome-sensitive WDR44 variants is associated with enhanced vesicular localization resulting from higher affinity binding to RAB11. Molecular structure predictions and coimmunoprecipitation studies support a direct interaction between the WDR44 RBD and the WD40 repeat domains, and strikingly the WDR44 patient missense variants disrupt these interdomain interactions. Together, our work demonstrates that WDR44 interdomain associations are important for regulating RAB11 effector binding, and the mutations observed in our patients alter these interactions resulting in impaired ciliogenesis initiation causing a ciliopathy-related disorder.

Suneet Kaur
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Cell Biology - Intracellular Trafficking and Cell Signaling
Unraveling the AKAP79-Orai1 Interaction: Implications for Immune Response Regulation
Ca2+ release-activated Ca2+ (CRAC) channels are composed of STIM1 and Orai1 proteins and are responsible for extracellular Ca2+ entry after depletion of internal stores. CRAC channels regulate Ca2+ signaling in immune cells and activate transcription factors such as NFAT1 that regulate cytokines and chemokines secretion for an effective immune response. This is made possible by a scaffolding protein AKAP79 which associates with Orai1 on plasma membrane and brings NFAT1 and its activator calcineurin to a proximity of CRAC channels. However, the molecular details of this critical AKAP79-Orai1 interaction that facilitates NFAT1 activation following Ca2+ entry remain unclear. Our aim is to elucidate the molecular basis of AKAP79-Orai1 interaction and how it shapes the immunomodulatory responses. For the ease of biochemical characterization of AKAP79-Orai1 interaction we chose HEK293 cells. To pinpoint the AKAP79 site which binds to Orai1 we generated and validated isogenic AKAP79 knockout (KO) cells which would allow expression of truncation mutants of AKAP79 in a clean background. Using genomic DNA and cDNA sequencing we found that CRISPR/Cas9 resulted in truncation of AKAP79 leaving only a 73 amino acids long putative protein lacking calcineurin and NFAT1 binding motifs. Surprisingly, the KO of AKAP79 was without an effect on NFAT1 activation which is in contrast with our findings that silencing AKAP79 diminishes the activation of NFAT1. We assessed CRAC channel activity in KO cells to address if overactive Ca2+ influx compensated for normal NFAT1 activation but found no changes. Furthermore, overexpression of the truncated AKAP79 did not show any association with calcineurin. We thus hypothesized that a complete loss of AKAP79 protein may alter the expression of other AKAP protein(s) which in turn compensate for the loss of AKAP79 and assume its role in NFAT1 activation. To investigate this, we are performing RNASeq with the KO and WT cells. For a detailed characterization of AKAP79-Orai1 interaction, we are using a combination of different biochemical and imaging techniques such as co-immunoprecipitation, FRET, and airy scan microscopy. Revealing molecular details of AKAP79-Orai1 interaction will facilitate the development of an AKAP79 peptide that specifically blocks its interaction with Orai1 and abrogates immune responses without disrupting other crucial physiological functions driven by Ca2+ ions.

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Activation of PTH1 receptor using nanobody-facilitated in situ click chemistry assembly of ligand
Bioactive compounds sometimes possess unfavorable properties for drug development due to on-target, off-tissue mediated side effects or poor pharmacological properties. One alternative is to use fragments that can react to form a bioactive product upon exposure to a certain stimulus or chemical. We sought to apply this approach for activation of the parathyroid hormone receptor-1 (PTHR1), which regulates skeletal development, bone turnover, and mineral ion homeostasis. Two fragments (PTH1-11 and PTH12-34) together comprise the prototypical peptide agonist of PTHR1 (PTH1 34). Here we describe methodology for the in situ synthesis of conjugates that resemble PTH1 34 through peptide template-induced dimerization and click (azide-alkyne) reactions between the fragment peptides PTH1-11 and PTH12-34. Dimerization is facilitated through the binding of nanobodies (Nbs) to peptide tags as described below. Nbs are single domain fragments of heavy-chain only antibodies from camelids, which exhibit specificity and affinity for biological macromolecules comparable to conventional antibodies. Templated dimerization was achieved fusing PTH fragments containing click chemistry handles to one of two Nbs that bind to short peptide epitopes. A heterodimeric peptide (DP), comprised of the two distinct Nb-binding peptide epitopes, was used to bring fusions comprised of Nbs and PTH fragments with complementary click handles, into proximity. This platform is hypothesized to allow DP to act as a template for the in situ synthesis of a PTHR1 agonist. Gel electrophoresis analysis of the kinetics of the reaction between azide- and alkyne-functionalized Nbs showed that DP accelerated product formation. We also used a fluorescence resonance energy transfer assay and fluorophore-labeled Nbs to corroborate the finding that DP induces proximity between Nbs. Finally, we sought to test whether DP could facilitate click chemistry-induced dimerization in the context of a cell-based assay. Whereas PTH fragment-click handle-Nb conjugates were weakly active alone or when added in tandem, the addition of DP to the tandem mixture resulted in strong activation of the PTHR1. Altogether, this approach entails a new strategy for in situ synthesis of a bioactive agonist from its inactive fragment peptides. These results offer a path towards in situ assembly of bioactive molecules based on targeted delivery of DP template, with opportunities for minimization of on-target, off-tissue side effects.

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Chromosomes, Chromatin, and Nuclear Architecture
Synthetic analysis of chromatin tracing and live-cell imaging indicates pervasive spatial coupling between genes
The role of the spatial organization of chromosomes in directing transcription remains an outstanding question in gene regulation. Here, we analyze two recent single-cell imaging methodologies applied across hundreds of genes to systematically analyze the contribution of chromosome conformation to transcriptional regulation. Those methodologies are: 1) single-cell chromatin tracing with super-resolution imaging in fixed cells; 2) high throughput labeling and imaging of nascent RNA in living cells. Specifically, we determine the contribution of physical distance to the coordination of transcriptional bursts. We find that individual genes adopt a constrained conformation and reposition toward the centroid of the surrounding chromatin upon activation. Leveraging the variability in distance inherent in

single-cell imaging, we show that physical distance – but not genomic distance – between genes on individual chromosomes is the major factor driving co-bursting. By combining this analysis with live-cell imaging, we arrive at a corrected transcriptional correlation of approx. 0.3 for genes separated by less than 400 nm. We propose that this surprisingly large correlation represents a physical property of human chromosomes and establishes a benchmark for future experimental studies.

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Chromosomes, Chromatin, and Nuclear Architecture

MLL3/MLL4 methyltransferase activities control early embryonic development and embryonic stem cell differentiation in a lineage-selective manner

Enhancers are cis-regulatory DNA elements controlling cell-type-specific gene transcription and are marked by histone modification H3K4me1. In mammals, our lab previously identified MLL3 and MLL4 as the major methyltransferases responsible for enhancer H3K4me1. They are critical for enhancer activation during cell fate transition and are broadly required for organism development and cell differentiation. In addition, MLL3 and MLL4 are frequently mutated in human developmental diseases and cancers. However, roles of MLL3/4-mediated enhancer H3K4me1 and MLL3/4 enzymatic activities in general in these processes remain unclear. To address above issues, we first generated MLL3/4 enzyme-dead single and double knock-in mice by the CRISPR/Cas9 method. Removing enzymatic activity of MLL3 or MLL4 does not affect mouse development until the perinatal stage. Strikingly, simultaneous elimination of both prevents gastrulation and leads to embryonic lethality around day 6.5, suggesting that MLL3/4 enzymatic activities are redundant and together are essential for early embryonic development. To uncover the underlying mechanism, we eliminated MLL3/4 enzymatic activities in embryonic stem cells (ESCs) and evaluated the differentiation ability of these cells. Interestingly, ESCs lacking MLL3/4 enzymatic activities can develop towards the three embryonic germ layers in embryoid body and teratoma formation assays, but show deficiencies during lineage-specific differentiation to extraembryonic cells. Using ChIP-seq analysis, we attribute the defect in extraembryonic lineage differentiation to impaired enhancer-binding of lineage-determining transcription factor GATA6. Consistent with ESC data, Sox2-Cre-mediated removal of MLL3/4 enzymatic activities in embryonic, but not extraembryonic, tissues does not affect mouse gastrulation. These conditional MLL3/4 enzyme-dead mice can develop through the early embryonic stage without noticeable abnormalities. Finally, we demonstrated that MLL3/4-catalyzed H3K4me1 is dispensable for enhancer activation during early embryoid body differentiation and neural differentiation by epigenomic and transcriptomic analyses. Together, these findings suggest a lineage-selective role of MLL3/4 enzymatic activities in early embryonic development and ESC differentiation. Our data support a model that MLL3/4 enzymatic activities function through selectively modulating genomic binding of transcription factors rather than directly activating enhancers.

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Chromosomes, Chromatin, and Nuclear Architecture
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Vinutha Balachandra
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Chromosomes, Chromatin, and Nuclear Architecture
Genome-wide screening identifies histone H3 chaperones as regulators of CENP-A mislocalization and chromosomal instability
Centromeric Protein A (CENP-A) is an evolutionarily conserved histone H3 variant that defines the centromere, a specialized genomic region essential for chromosome segregation. Stringent regulation of CENP-A levels and deposition ensure the specific loading of CENP-A at the centromere. However, CENP-A is frequently overexpressed in several types of cancers, and often correlates with poor prognosis. We have previously shown that overexpressed CENP-A mislocalizes to non-centromeric regions and drives chromosomal instability (CIN), a major hallmark of cancer. Interestingly, suppressing the mislocalization of overexpressed CENP-A alone rescued CIN. Despite these studies, pathways that prevent CENP-A mislocalization are poorly defined. Here, we devised an image-based RNAi screen to identify gene depletions that regulate CENP-A mislocalization at the genome-wide level. Among the top candidates, we identified two hits which are required for histone 3 deposition, namely CHAF1B and DNAJC9. We observed that knockdown (KD) of CHAF1B or DNAJC9 led to CENP-A mislocalization to non-centromeric regions based on analysis of metaphase chromosome spreads, and higher enrichment of CENP-A in chromatin. In addition, DNAJC9 KD cells exhibited CIN phenotypes marked by a higher frequency of defective chromosome segregation. To identify the factors that contribute to CENP-A mislocalization in DNAJC9 KD cells, we analysed CENP-A interactome by mass spectrometry. One of the lead candidates which showed increased interaction with CENP-A was MCM2, which along with DNAJC9 is known to form an H3 chaperone complex. Depletion of MCM2 in DNAJC9 KD cells suppressed the mislocalization of CENP-A suggesting that MCM2 contributes to the mislocalization of CENP-A in DNAJC9 KD cells. Together, our studies define novel regulators of CENP-A localization and highlight the multifaceted roles of H3 chaperones in restricting CENP-A mislocalization and CIN.

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Clinical and Translational Research - Animal Models
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Julia Port
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Clinical and Translational Research - Animal Models
Exposure of genital or anal mucosae with monkeypox increases virus dissemination and shedding, thus facilitating increased risk of transmission
Monkeypox virus (MPXV), the etiological agent of monkeypox disease, belongs to the Poxviridae family. Historically, spillovers from posited rodent reservoirs to humans were self-contained. Yet, the 2022 outbreak totaled over 85,900 cases, most of which reported in counties not considered endemic, and uniquely supported human-to-human transmission. The virus spread through sexual networks of men who have sex with men. Whether this new increase in transmissibility resulted from epidemiological differences alone or because of an intrinsic advantage of MPXV to spread via contact with genital and anal mucosae during sex remains unknown. Experimental animal infection has, so far, been limited to artificial routes not suited to addressing the observed epidemiological patterns of the 2022 outbreak: No study has compared exposure through sexual contact-associated mucosae (vagina and rectum) with transdermal exposure. As no small animal model of disease and transmission is readily available, we first demonstrated that the African multimammate rat Mastomys natalensis, a posited intermediate host due to its peridomestic lifestyle, was susceptible to infection with a contemporary strain (Clade IIb). Intraperitoneal infection resulted in asymptomatic infection, wide tissue dissemination on day 8, seroconversion, and intermittent oral, rectal, and urogenital shedding. Strikingly, tissue dissemination and shedding magnitude and duration increased significantly after inoculation via the vagina or rectum, but not through the skin. We found that mucosal inoculation also increased T-cell, but not B-cell, activation and proliferation. In accordance with mild-case human pathology, self-clearing localized skin (transdermal inoculation), reproductive (vaginal inoculation), or rectal lesions (rectal inoculation) were observed on day 7/8. We then addressed if these findings translated to a measurable difference in transmissibility for the two routes most crucial to the human outbreak: Importantly, contact transmissibility was increased after rectal inoculation compared to transdermal inoculation. This data demonstrates for the first time, that MPXV is uniquely suited to replication in the urogenital mucosae, which facilitates increased shedding and suggests increased risk of transmission during sexual contact. Transmission mitigation strategies in non-endemic countries may, therefore, prioritize preventing mucosal exposure during close contact over skin or fomite contact.

Stacey Piotrowski
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Clinical and Translational Research - Animal Models
Characterization of the common marmoset as a preclinical model of gammaherpesvirus infection and associated lymphoma
Epstein-Barr virus (EBV) is a ubiquitous gammaherpesvirus that persistently infects humans. EBV is involved in the pathogenesis of multiple malignancies, and it is currently being investigated as a potential contributor in neurodegenerative diseases, such as multiple sclerosis and Alzheimer's disease. The limited susceptibility of animal models to experimental infection makes studying this virus

challenging. Callitrichine herpesvirus 3 (CalHV-3) is a gammaherpesvirus that naturally infects the common marmoset (Callithrix jacchus), a nonhuman primate of growing importance in biomedical research. While CalHV-3 was first identified in the early 2000s, characterization of the virus in marmoset research colonies and its related pathology is thus far limited. To further determine the utility of CalHV-3 as a model of EBV infection and associated disease, we performed the first reported large-scale investigation of the prevalence, demographics, and pathology of CalHV-3 infection in the common marmoset. We screened peripheral blood mononuclear cells (PBMCs) from over 140 animals in the NINDS colony via a CalHV-3 droplet digital PCR (ddPCR) assay we developed in our laboratory. Longitudinal saliva and PBMC samples were similarly screened in a subset of animals. Archived marmoset tissues were searched for neoplasms, and we extracted DNA from tissue blocks for CalHV-3 ddPCR. We performed immunohistochemistry (IHC) on these tissues to confirm neoplastic origin. 19% of colony was positive for CalHV-3, with fluctuations in PBMC and saliva viral loads over time. Aged marmosets over the age of 8 had a significantly higher prevalence of CalHV-3 infection compared to other age groups. Lymphoma was diagnosed postmortem in an animal with a high PBMC viral load of over a million copies of CalHV-3 per million cells. All archived B-cell lymphomas (8/8, 100%) were positive for CalHV-3, with millions of copies of virus per million cells in neoplastic tissue. Characteristics of CalHV-3 infection in the common marmoset, including persistent infection, variations in viral loads, and an increased prevalence with age, are comparable to human EBV infection. All analyzed cases of B-cell lymphoma are associated with CalHV-3 infection, similar to the relationship of EBV infection with subsets of lymphoma in humans. CalHV-3 in the common marmoset is a potentially useful model of EBV-like gammaherpesvirus infection and its role in disease, including virally induced hematopoietic neoplasia.

Teruhiko Yoshida
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Clinical and Translational Research - Animal Models

Preeclamptic phenotype in a transgenic mouse model with fetuses carrying an APOL1 risk variant

African-Americans experience more pregnancy complications, including preeclampsia, compared to other groups. Coding variants in apolipoprotein L1 (APOL1), present only in individuals with sub-Saharan ancestry. Recent clinical studies showed that fetuses with two APOL1 kidney risk variants predispose to preeclampsia. The mechanisms of APOL1-asociated preeclampsia remain poorly understood. Here we made use of a transgenic mouse model. We in-vitro fertilized CD-1 female mice using sperm from human APOL1 gene locus transgenic mice carrying a bacterial artificial chromosome bearing either the APOL1-G0 allele (common variant) or the APOL1-G1 allele (risk variant). Systolic blood pressure was measured with a tail cuff. Mice were euthanized at embryonic day (E)18.5 and fetuses, placenta and maternal plasma were collected. Phenotypes were assessed using plasma chemistry (dam), fetuses (weight) and placenta (weight). Placental transcriptomic profiles were assessed by single-nuclear RNA-seq. Compared with dams carrying APOL1-G0 fetuses, dams with APOL1-G1 fetuses had higher systolic blood pressure, 158 [141-163] vs 116 [115-131] mmHg; median [IQR], P=0.01). These mice had lower plasma placental growth factor-2 levels (32 [20-82] vs 126 [98-260] pg/mL; P=0.047). APOL1-G1 fetuses had lower relative weights, (0.95 [0.90-0.98] vs 1.0 [0.96-1.04]; P=0.03) when compared with wild-type (WT) fetuses. By contrast, APOL1-G0 fetuses had weights similar to WT fetuses (0.99 [0.95-1.08]; P=1.0).

Single-nucleus RNA-seq and in situ hybridization demonstrated increased expression of inflammation-related genes, e.g. Spp1, encoding osteopontin and Fn1, encoding fibronectin1, in APOL1-expressing endothelial cells and trophoblasts in placenta of APOL1-G1 fetuses compared with placenta of APOL1-G0 and WT fetuses. This preeclampsia model involving expression of APOL1 suggests that the APOL1-G1 allele promotes endothelial and trophoblastic inflammation and thus contributes to placental dysfunction. This mouse model manifests an accelerated preeclamptic phenotype with APOL1-G1 fetuses but not APOL1-G0 fetuses, corresponding to human disease. This model may help to identify mechanisms by which APOL1 variants perturb placental biology, promoting preeclampsia. Future studies might offer insight into clinical pharmacologic management.

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Clinical and Translational Research - Clinical Trials
Brain effects of 5-2 calorie restriction in a randomized clinical trial
Insulin Resistance (IR) is involved in Alzheimer's disease (AD) pathogenesis; older adults with IR are at increased risk for developing AD. An extensive animal literature suggests pro-cognitive and beneficial systemic and brain effects of intermittent calorie restriction (CR) that may mitigate AD risk. We conducted a Randomized Controlled Trial to examine whether 8 weeks of intermittent CR can lower IR, improve cognitive performance, brain metabolism and function, and induce beneficial changes in AD-related biomarkers. Forty cognitively intact individuals > 55 years old with body mass index (BMI) > 27 and Homeostatic Model Assessment for Insulin Resistance (HOMA2-IR) > 1.8, were randomized (1:1) to 2 consecutive days of 500 Kcal/day consumption plus non-restricted eating for 5 days (5-2 CR) or low-intensity "healthy living" (HL) diet with education on portion control and calorie reduction. After 8 weeks, both diets decreased BMI and HOMA2-IR equally suggesting high compliance, and increased beta-hydroxybutyrate and acetoacetate in blood equally suggesting ketone metabolism upregulation. Additionally, 5-2 CR improved performance on two executive function tasks (that involve strategizing and planning and the ability to switch between two tasks) over HL diet; however, no differences between the two diets were observed in composite scores and memory tasks. Moreover, 5-2 CR decreased brain glucose concentration by magnetic resonance spectroscopy (MRS) indicating more efficient glucose metabolism. Both diets decreased equally regional BrainAGE (brain-age-gap estimate assessed by structural magnetic resonance imaging (MRI)) in an anterior cingulate cluster. In cerebrospinal fluid (CSF), 5-2 CR increased light chain neurofilaments (Nf-L) and (at trend) neurogranin. However, no change was observed for Abeta42, Abeta40, total Tau and P181-Tau with either diet. Finally, neuron-derived extracellular vesicle biomarkers indicated that both diets decreased neuronal IR, as assessed by P-S312-insulin receptor substrate (IRS)-1, and P-Tau load. Nevertheless, similarly to CSF, Abeta42 and Abeta40 did not change with either diet. In conclusion, 5-2 CR was non-superior compared to HL diet for most systemic and brain outcomes. Moreover, the increase in CSF Nf-L with 5-2 CR raises concerns for potential neuronal injury. Overall, our findings provide little support for the hypothesis that 5-2 CR in mid- to late-life can modulate the AD pathogenic cascade.

Melissa Abel
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Clinical and Translational Research - Clinical Trials
Targeting Replication Stress and Chemotherapy Resistance with a Combination of Sacituzumab Govitecan and Berzosertib: A Phase I Clinical Trial in Patients with Advanced Solid Tumors with DNA Repair Mutations and Endogenous Replication Stress
Despite provocative preclinical results, dose-limiting toxicities have precluded rational combinations of cytotoxic chemotherapies that increase DNA damage with DNA damage response (DDR) inhibitors. We hypothesized that tumor-targeted delivery of cytotoxic chemotherapy might enable tolerable and active combinations with DDR inhibitors. We conducted a phase I clinical trial combining ataxia telangiectasia and Rad3-related (ATR) inhibitor berzosertib with sacituzumab govitecan, a trophoblast cell surface antigen 2 (Trop-2) directed antibody drug conjugate (ADC) that delivers high tumoral concentrations of topoisomerase 1 (TOP1) inhibitor SN-38. Depletion of ATR, the main transducer of replication stress is synthetically lethal with double-strand breaks (DSB) generated by TOP1 inhibitors. Primary end point was identification of the maximum tolerated dose of the combination. Efficacy and pharmacodynamics were secondary end points. The combination was well tolerated, with improved safety profile over conventional chemotherapy-based combinations, which allowed dose escalation to the highest planned dose level. There were no dose limiting toxicities. Common treatment-related adverse events (TRAE) were neutropenia (41.7%), diarrhea (50%), and fatigue (50%). Grade 3 TRAEs occurred in 58.3% of patients and included neutropenia (25%) and diarrhea (8.3%). There were no instances of febrile neutropenia or clinically significant grade 4 TRAEs. While no tumor responses were seen in three patients with DDR defects including BRCA1 and ATM mutations, two patients with neuroendocrine prostate cancer, a highly aggressive subtype of prostate cancer, showed partial or metabolic responses. A patient with EGFR-transformed small cell lung cancer (SCLC) also experienced partial response, together yielding objective responses in 3 of 12 evaluable patients (25%). Ongoing phase II expansion cohorts are evaluating efficacy of sacituzumab govitecan 10mg/m2 and berzosertib 210mg/m2 in patients with SCLC, extra-pulmonary small cell cancers, and DDR-mutated solid tumors. ADC-based delivery of cytotoxic payload represents a new therapeutic paradigm to extend the benefit of DDR inhibitors to target replication stress and chemotherapy resistance, with minimal added toxicities.

Abhishek Basu
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Clinical and Translational Research - Drug Discovery
Inhalation Delivery of MRI-1867 (zevaquenabant) As A Novel and Effective Therapeutic Modality in Pulmonary Fibrosis
Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease with a poor prognosis and necessitates prolonged therapy. Over the last decade, cannabinoid receptor 1 (CB1R) emerged as a potential target for the treatment of IPF. Following this line, we have developed MRI-1867 (zevaquenabant) as a peripherally restricted dual CB1R/iNOS inhibitor. Systemic administration of MRI-1867 is effective in mitigating experimental pulmonary fibrosis (PF) in mice, and it has completed a

Phase 1 clinical trial. We also found that alveolar macrophages (AMs) and epithelial cells expressing CB1R in the local fibrotic environment are likely responsible for the progression of PF. Since alveolar cells are accessible via the inhalational route, we hypothesized that pulmonary delivery of MRI-1867 may yield high exposure to critical target cells and thus could further guarantee safety by limiting circulatory distribution to other tissues. During trials with different oropharyngeal (O.P.) doses of MRI-1867, we found O.P. dose of 0.5 mg/kg b.w. in mice could achieve the same lungs concentration of 20 micromolar as the therapeutic I.P. dose of 10 mg/kg b.w. and did not cause significant accumulation in the blood, brain, and liver. In the next step, we investigated the antifibrotic efficacy of MRI-1867 at 0.5 mg/kg b.w. O.P. dose compared to the established dose of 10 mg/kg b.w. I.P. in bleomycin-induced murine PF model. Mice treated with MRI-1867 via both O.P. and I.P. dosage showed similar improvement in pulmonary functions and reduced fibrosis. Phenotypic characterization of lung macrophages revealed that MRI-1867 treatment significantly reduced monocyte-derived AMs, particularly profibrogenic M2 macrophages. Multiplex cytokine analysis from the BALF also showed a reduction in the levels of inflammatory cytokines (IL1b, IL6, TNFa, LIF) and chemotactic chemokines (CXCL10, CCL2, CCL5, CCL11) by MRI-1867. Transcriptomics analyses revealed that MRI-1867 treatment restored 34 pathways out of 51 pathways that are involved in fibrosis initiation and modification, fibroblast proliferation, as well as inflammation. In summary, pulmonary delivery of MRI-1867 is as effective as systemic delivery in mitigating PF, and it offers a more targeted therapeutic modality that could reinforce safety during prolonged therapy. Based on these findings, the inhalational formulation for humans is under development and clinical trials with inhalational formulation are in progress.

Shivani Dixit
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Clinical and Translational Research - Drug Discovery

Development of a discovery pipeline to validate miRNA-based mesothelioma therapy by nanoparticle-hydrogel delivery: Role of miR-497

MicroRNA (miR)-mRNA interactions are associated with critical biologic processes in Malignant pleural mesothelioma (MPM). Slow-release, locoregional miR delivery by nanoparticle hydrogel system is a new and efficacious method for in-vivo MPM treatment. Thus, the most efficient miR(s) inhibiting MPM need to be identified for optimizing this therapy. We hypothesized these clinically relevant miR can be identified by assembling a discovery pipeline. This integrated discovery pipeline contains 3 parts. In Part A, Genome-wide unbiased high-throughput screening for loss of cell viability was performed using 2 human libraries representing >3000 miRs. miRs exerting >2-fold loss of cell viability when overexpressed in MPM cells (vs normal) were rank ordered. From top screening hits, MPM specific expression of prognostic miRs were analyzed in 50 MPM and 26 unmatched normal human pleura tissues. Of the top 5% of inhibitory miRs, miR-497 expression had favorable prognosis for MPM. Mean expression of miR-497 in MPM was 4.2-fold lower vs normal pleura tissues. These criteria suggest that miR-497 is a clinically relevant anti-MPM agent, so it continued in the discovery pipeline. In Part B functional assays were performed to identify mechanisms regulated by miR-497 using a panel of patient derived MPM and normal mesothelial cell lines. Re-expression of miR-497 severely retarded MPM cell viability without exerting toxicity in non-MPM cells. miR-497 caused G1-phase arrest of cell cycle which ultimately led to apoptotic cell death in MPM cells. Gene targets of miR-497 were systematically identified by

biotinylated-miR pulldown affinity purification coupled with RNA-seq. This assay revealed 186 directly bound and differentially expressed novel gene targets of miR-497. Among them, 18 genes were negatively prognostic (Kaplan-Meier analysis of TCGA-Meso dataset) for MPM. Bioinformatics analysis revealed that miR-497 regulates PI3K/Akt, FAK/Src, ERK1/2, and Wnt pathways in MPM. In Part C, in-vivo anticancer efficacy of top-scoring miR-497 was evaluated in orthotopic and subcutaneous MPM xenografts in mice. miR-497-nanoparticle hydrogel complex caused potent tumor growth suppression and increased overall survival in in-vivo models. Conclusively, this novel discovery pipeline efficiently identifies biologically active miRs and elucidates their molecular mechanisms. In preclinical studies, miR-497 has a potential pathophysiologic role in MPM and could be a relevant therapeutic.

Hiroshi Ichise
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Clinical and Translational Research - General

Ce3D-IBEX: Achieving multiplex 3-dimensional imaging for deep phenotyping of cells in tissues

2D multiplexed antibody-based imaging provides a framework to study cell-cell interactions in thin tissue sections but lacks the ability to interrogate spatial relationships in larger 3D anatomical structures. The structures of nerves and lymphatics as well as cell interaction with those structures are largely under-sampled in a 2D imaging modality. Several methods for 3D imaging of optically cleared tissue exist, but current approaches do not provide the marker depth to delineate cell types and structures afforded by high-content 2D imaging because of the lack of a cyclic staining method that allows overcoming the color barrier in fluorescence microscopy and a computational algorithm to precisely register 3D images with a high density of cells acquired by cyclic imaging. To overcome this constraint, we combined a fast hydrophilic tissue clearing technique, clearing-enhanced 3D (Ce3D), with the Iterative Bleaching Extends Multiplexity (IBEX) technique, and created Ce3D-IBEX. Ce3D-IBEX offers a quantifiable, subcellular resolution multiplex 3D imaging method incorporated with a machine learning-based 3D image registration algorithm. It has been used to analyze murine lungs, lymph nodes, small intestine, and tumors as well as human tonsil, liver, and spleen yielding seamless views of large-scale tissue structures while obtaining single-cell resolution of multiple markers, permitting the identification of discrete cell subsets and structures. To date, up to 25 markers have been visualized in a single sample using 5 iterative staining and imaging cycles. This method can be applied not only to fixed frozen samples but also formalin-fixed paraffin-embedded (FFPE) samples which are major resources for archived human materials. In parallel, multiplex cyclic imaging methods require a computational algorithm to properly register images acquired separately where tissue undergoes both global and local deformation because of the repetitive antibody staining and tissue clearing. We developed a robust computational algorithm and workflow to compensate for those tissue deformation to achieve precise registration and downstream analysis. Ce3D-IBEX will open new avenues for characterization of rare cell subsets missed in thin tissue sections, visualization of structures like nerves and vessels whose trajectories in a tissue are disrupted by sectioning, and delineation of mesoscale tissue domains that contribute to homeostasis and are often the substrates for pathology.

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Clinical and Translational Research - General

WASF3 disrupts mitochondrial respiration and may mediate exercise intolerance in myalgic encephalomyelitis/ chronic fatigue syndrome

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is characterized by various disabling symptoms including exercise intolerance and is diagnosed in the absence of a specific cause, making its clinical management extremely challenging. A better understanding of the molecular mechanism underlying this apparent bioenergetic deficiency state may reveal new insights for developing targeted treatment strategies. We report that overexpression of Wiskott-Aldrich Syndrome Protein Family Member 3 (WASF3), identified in a 38-year-old woman suffering from long-standing fatigue and exercise intolerance, can disrupt mitochondrial respiratory supercomplex formation and is associated with endoplasmic reticulum (ER) stress. Increased expression of WASF3 in transgenic mice markedly decreased their treadmill running capacity with concomitantly impaired respiratory supercomplex assembly and reduced complex IV levels in skeletal muscle mitochondria. WASF3 induction by ER stress using endotoxin, well-known to be associated with fatigue in humans, also decreased skeletal muscle complex IV levels in mice, while decreasing WASF3 levels by pharmacologic inhibition of ER stress improved mitochondrial function in the cells of the patient with chronic fatigue. Expanding on our findings, skeletal muscle biopsy samples obtained from a cohort of patients with ME/CFS showed increased WASF3 protein levels and aberrant ER stress activation. In addition to revealing a potential mechanism for the bioenergetic deficiency in ME/CFS, our study may also provide insights into other disorders associated with fatigue such as autoimmune diseases and long COVID.

Yong Kim
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Clinical and Translational Research - General

Endogenous HiBiT tagging of PAX3-FOXO1 in rhabdomyosarcoma cell lines identifies a critical vulnerability to CDK9 inhibition for maintenance of the fusion oncogene protein levels and cancer cell viability.

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children and despite aggressive cytotoxic chemotherapy, 5-year overall survival for high-risk and recurrent disease is only 30% and 17%, respectively. PAX3-FOXO1 fusion oncogene is the major driver in fusion-positive rhabdomyosarcoma (FP-RMS) and represents a unique target. To identify repressors of PAX3-FOXO1 protein, a direct measure of PAX3-FOXO1 protein amenable to high throughput screen was required. We used the HiBiT system where a small 11 amino acid fragment of luciferase is tagged to PAX3-FOXO1 and when HiBiT combines with its partner Large BiT they form a functional enzyme. We used CRISPR to tag the endogenous PAX3-FOXO1 with HiBiT in 2 FP-RMS cell lines RH4 and SCMC. Single cell clones were validated for the tag using Large BiT Western analysis. Sanger sequence verified the integration of HiBiT at the carboxy terminus of PAX3-FOXO1. By RNA-seq analysis we demonstrate a similar transcriptome profile of the HiBiT clones to their parental cell lines RH4 and SCMC. By ChIP-seq analysis we showed

that HiBiT-tagged PAX3-FOXO1 binds to the same genomic regions as endogenous PAX3-FOXO1. Validated clones were used in a drug screen using the Mechanism Interrogation PlatE 5.0 library of compounds which are 1,912 compounds with known targets, the majority of which are FDA approved (42%) or in clinical trials (22%). We selected compounds which down regulated PAX3-FOXO1 protein as detected by HiBiT without significant cell death at the early time point of 24 hours. We narrowed the compounds to those active in both RH4 and SCMC which resulted in 183 hits. Focusing on targets covered by at least 3 drugs identified 14 drug classes. Drug classes included those already known to downregulate PAX3-FOXO1, such as HDACi and BRDi, as well as new classes such as cyclin-dependent kinase (CDK) inhibitors. We further characterized TG02, a multi-kinase inhibitor with high specificity for CDK9, which is in clinical trial for glioblastoma. We showed by Western analysis that TG02 treatment decreased PAX3-FOXO1 and RNA-seq analysis showed down regulation of PAX3-FOXO1 target transcripts. TG02 delayed tumor progression and prolonged survival in xenograft mouse model of FP-RMS. Thus, PAX3-FOXO1 HiBiT platform is an innovative methodology amenable to high throughput screening utilized here to discover novel vulnerabilities of FP-RMS to CDK9 inhibition which is a promising candidate for clinical translation in FP-RMS.

Ayse Gokce Keskus
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Computational Biology/Systems Biology
Long-read, assembly-based characterization of rearranged cancer karyotypes
Recent pan-cancer whole-genome sequencing studies revealed the rich landscape of structural variants (SV), from simpler indels to complex events involving multiple breakpoints and sequence gain/loss. SVs may contribute to tumorigenesis through direct modification of coding sequence or deregulation from copy number alterations, enhancer hijacking, or topological domain modification. A substantial part of the variation in the human genome is not accessible to short reads due to mapping ambiguities. Recent benchmarking studies reported that the best short-read methods only have 30-70% SV sensitivity. Long-read sequencing can overcome the limitations of short reads, however the current methods were not designed for the analysis of rearranged cancer genomes with complex copy number profiles. Current long-read analysis methods fail to capture complex events shown to be involved in tumorigenesis and cancer prognosis like chromothripsis, chromoplexy, or bridge-fusion-bridge(BFB). We developed a method that combines the ideas from long-read assembly and breakpoint graph frameworks. Our method detects abnormally mapped reads and builds a breakpoint graph that characterizes the structure of derived cancer karyotypes. Complex events with multiple breakpoints form connectivity clusters and are classified based on the subgraph properties. It also takes advantage of phased haplotypes and can incorporate multiple related datasets (such as in tumor-normal comparison or multi-site tumor sampling) allowing to identify somatic and germline variations. We first analyzed three cancer cell lines and corresponding matching normals (HCC1954, H2009, and COLO829). We identified a somatic chromoplexy event involving chr13 and chr1 in H2009 and translocation and inversion events between chr3, chr10, and chr12 within RARB, BICC1, and TRHDE genes in COLO829. Homologous recombination deficient HCC1954 has the highest number of complex events including the chr17q arm which hosts ERBB2, chromoplexy between chr8, chr5, and chromothripsis in chr21. In addition, we analyzed three other HPV-infected cell lines (CaSki, SCC152, SNU1000). In each of them, we observed

complex clusters of HPV-HPV and HPV-human breakpoints that formed cycles, suggesting extrachromosomal amplification. We also observed karyotype-scale changes that did not involve HPV sequence, such as the simultaneous exchange of six chromosome arms of chr2, chr7, and chr17 in CaSki and BFB in all three cell lines.

Piyush Agrawal
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Computational Biology/Systems Biology
Network-based approach elucidates critical genes in Breast cancer subtypes and chemotherapy response in Triple Negative Breast Cancer
Breast cancers (BRCA) exhibit substantial transcriptional heterogeneity, thus posing a major clinical challenge. Transcriptional heterogeneity, say, across BRCA subtypes, is conventionally represented by differentially expressed genes (DEGs). However, DEGs can be very noisy due to inherent stochastic variability and do not capture critical but lowly expressed gene classes such as transcription factors and kinases. Ultimately, DEGs do not necessarily point to the causal upstream genes underlying the global transcriptomic differences. Here, we propose a novel network-based approach (NNBA), which integrates transcriptomic data with protein-protein interaction networks to detect potentially functional genes underlying global transcriptome of a sample. I applied NNBA to TCGA BRCA cohort, with 1059 tumors and 112 healthy control samples across 4 subtypes (Basal, Her2, Luminal-A & Luminal-B) to identify central genes in each sample and then most frequent central genes in each subtype. Analogously I obtained most frequent DEGs as a control. Compared to DEGs, NNBA-identified genes (i) exhibit greater commonality across tumors and subtypes and reveal both pan-subtype as well as subtype-specific biological processes not identified by DEGs, (ii) better recapitulate BRCA associated driver genes in multiple benchmarks, (iii) exhibit greater copy number variation, (iv) exhibit greater dependency scores in CRISPR screens of subtype-specific cancer cell lines. NNBA identified central genes in each subtype supported by prior literature, i.e., MYC, MAX, and E2F2 in Basal; JAK2, ERBB2, and MAPK9 in Her2, and MMP2, FGFR3, ADCY1 in Luminal. Single cell RNA-seq analysis reveals expression of NNBA genes in malignant and immune cell types. NNBA application to Triple Negative Breast Cancer (TNBC) clinical data of responders and non-responders upon Doxorubicin treatment followed by Paclitaxel revealed key genes potentially mediating resistance, including FOXA1, EP300, CTNNB1 and TNF. In two independent clinical cohorts, these genes were able to predict responders with high accuracy (AUC of 0.76) whereas DEGs could not. Lastly, molecular docking was done to identify drugs targeting top novel genes underlying BRCA subtypes. Overall, our approach refines previous view of inter-tumor heterogeneity and identifies potential functional mediators of BRCA subtypes and therapy response.

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NIMH
Computational Biology/Systems Biology
Attractor dynamics reflect decision confidence in macaque prefrontal cortex

Decisions are made with varying levels of confidence. When we are confident about a choice, we are more likely to make the same choice consistently. Understanding the network computational mechanisms underlying decision confidence and choice consistency is a longstanding question in the neuroscience of decision making. Theoretical work suggests that attractor dynamics in networks can account for choice consistency. The attractor models operationalize the intuitive idea that choice related neural activity can be modeled as a ball rolling on an energy landscape characterized by two attractor basins separated by a hill. The ball starts on top of the hill and is pushed into one of the basins by evidence in favor of one of the choices. Decisions are made when network activity settles in the basin. Artificial network simulation suggests that choice consistency can be accounted for by the shape of the energy landscape around attractor basins. When energy landscapes are flat and attractor basins are shallow, decisions will be made less consistently, because it is easier to drive activity between basins. Despite advances in theory, these models have not been tested in the biological brain. In this work, we investigated population dynamics of prefrontal neurons using high channel-count Utah-array recordings (768 channels). We trained two monkeys to choose between rejecting and accepting offers of different reward size and delay. Monkeys consistently accepted good and rejected bad offers but made inconsistent choices for intermediate offers. To estimate the energy landscape in the biological network of prefrontal neurons, we analyzed neural activity in the “state space” spanned by the firing rates of neurons, with each dimension defined by the firing rate of one recorded neuron. Guided by the dynamical systems theory, we computed the flow field of neural activity by taking temporal derivatives of neural trajectories in the state space, and then empirically reconstructed the neural energy landscape by taking spatial integrals of flow vectors. Empirically, we observed the emergence of two attractor basins around the time of choice. Crucially, we found that the energy landscape is shallower for intermediate offers. Our finding was further validated by fitting a dynamical system model directly to the neural data. To our knowledge,we provide the first neural evidence that attractor dynamics in prefrontal cortex predicts choice consistency and reflects decision confidence.

Sofya Garushyants
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Computational Biology/Systems Biology
Interaction between anti-phage defense systems in bacteria
While such anti-phage defense systems as CRISPR and restriction-modification are well known and widely used in basic research and biotechnology, they represent only a small portion of all known defense systems in prokaryotes. Recently, mining of bacterial and archaeal genomes for antivirus defense systems has revealed a remarkable diversity of defense mechanisms. So far three main categories of molecular mechanisms of action for the defense systems were described - degradation of phage genome material, inhibition of DNA or RNA synthesis and abortive infection, but molecular mechanisms for many are still unknown. Each bacterial genome carries multiple defense systems, in many cases they are located in close proximity to each other forming so-called “defense islands”. For example, Escherichia coli isolates carry, on average, 5-7 defense systems per genome that are effective again various phages and act on different stages of infection. Surprisingly, the composition of defense systems even within closely related bacterial strains belonging to the same bacterial species can vary significantly, but why different systems are retained in different bacteria is not well understood.

Understanding the evolutionary interactions between defense systems is essential to unravel the bigger picture of anti-phage defense and understand limitations of individual systems. In our project we investigate the patterns of co-occurrence of 110 diverse defense systems in 26,362 complete E. coli genomes and show that positive and negative correlations are common among pairs of defense systems. Moreover, individual defense systems can have both positive and negative interactions with other systems. The observed effects can be clade specific, thus we show that E. coli phylogroup E have strong preference for retaining such systems as Zorya II and Druantia III that are rarely present in other phylogroups. We show that interactions between systems are not solely described by location in the genome, and that systems located on the same chromosome are not always positively correlated. Furthermore, experiments performed by our collaborators show that negative correlations do not result from antagonistic effects of the respective defense systems, but instead, even if partially redundant, negatively correlated systems can act in synergy and provide better defense when present together in the cell. Collectively, our work shows complex interaction between defense systems in bacteria.

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COVID-19 Methodologies and data analysis
Modification of a conventional deep learning model to classify simulated breathing patterns: A step toward real-time monitoring of patients with infectious diseases
The emergence of the global coronavirus pandemic in 2019 (COVID-19 disease) raised a need for remote methods to detect and continuously monitor patients with infectious respiratory diseases. Many different devices, including thermometers, pulse oximeters, smartwatches, and rings, were proposed to monitor the symptoms of infected individuals at home. However, these consumer-grade devices are typically not capable of automated monitoring during both day and night. This study aims to develop a method to classify and monitor breathing patterns in real-time using tissue hemodynamic responses and a deep convolutional neural network (CNN) based classification algorithm. Tissue hemodynamic responses at the sternal manubrium were collected in 21 healthy volunteers using a wearable near-infrared spectroscopy (NIRS) device during three different breathing conditions. In our previous study, we demonstrated that by using machine learning techniques, we were able to classify three different breathing patterns with 87% accuracy. Using the same data, we developed a deep CNN based classification algorithm to classify and monitor breathing patterns in real-time. The classification method was designed by improving and modifying the pre-activation residual network (Pre-ResNet) previously developed to classify 2-dimensional (2D) images.Three different 1-dimensional CNN (1D-CNN) classification models based on Pre-ResNet were developed. By using these models, we were able to obtain an average classification accuracy of 88.79% (without Stage 1 (data size reducing convolutional layer)), 90.58% (with 1×3 Stage 1), 91.77% (with 1×5 Stage 1). Among three models, the CNN model with 1×5 Stage 1 was best to classify the three breathing patterns. This study demonstrated the capability of a deep CNN based algorithm in classifying different breathing patterns with a high accuracy. The proposed classification method can be used to continuously analyze and monitor breathing patterns in real-time.

Adam Hage
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COVID-19 Virology/Mechanistic aspect
The Nuclear Transcription Factor HNF4a is a Potential Switch Between Proinflammatory and Antiviral Responses Following Infection with Highly Pathogenic Coronaviruses
Proper regulation of the innate immune response is critical for promoting pathogen clearance while avoiding host pathology. Dysregulation of innate immunity following viral infection is associated with poor patient outcomes due to failure to control virus replication and chronic proinflammatory cytokine production. Underlying medical conditions including diabetes have been associated with a greater risk for developing severe disease and a higher case fatality rate following severe acute respiratory syndrome coronavirus (SARS-CoV, MERS-CoV, and SARS-CoV-2) infection, although the molecular mechanisms leading to this are not well understood. To identify host factors that contribute to severe disease, we utilized CRISPR Activation (CRISPRa) to activate host genes at the transcriptional level for endogenous overexpression. Using this approach, we conducted a genome-wide screening in MERS-CoV infected cells and identified a host transcription factor, Hepatocyte Nuclear Factor 4 Alpha (HNF4a), as a potential proviral factor. HNF4a is broadly required for hepatic development and function, and mutations in HNF4a lead to the development of diabetes including the disease Maturity-onset diabetes of the young 1 (MODY1). CRISPRa cells expressing HNF4a revealed a preferential killing effect during coronavirus infection. Silencing of the HNF4a gene with siRNA significantly impaired the replication of SARS-CoV-2 in alveolar and colorectal epithelial cell cultures confirming our initial screen. Additionally, knockdown of HNF4a significantly reduced transcription of the proinflammatory cytokine IL-6 while enhancing expression of type-I and III interferons (IFN-I/III) and IFN-stimulated genes (ISGs). Consistent with this, overexpression of HNF4a promoted IL-6 transcription. Importantly, HNF4a alters expression of proinflammatory and innate immune genes specifically at the transcriptional level as loss of HNF4a did not affect upstream signaling events. Taken together, our study identifies a role for HNF4a as a potential switch co-opted by pathogenic coronaviruses to drive replication while also inducing proinflammatory cytokines and suppressing antiviral responses thereby exacerbating clinical outcomes for patients with comorbidities. Understanding the role of HNF4a in viral pathogenesis may inform the development of therapeutics that mitigate risks for patients with pre-existing conditions.

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COVID-19 Virology/Mechanistic aspect
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Brennan Streck
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Cultural, Social and Behavioral Sciences
Associations between Psychosocial and Lifestyle Factors and Biological Age Acceleration Among Long-Term Survivors of Childhood Cancer: A Prospective Study from the St. Jude Lifetime Cohort
Survivors of childhood cancers are at risk for premature, or accelerated, biological aging, which is previously associated with cancer treatment. However, life course theory suggests that psychosocial and lifestyle factors may also contribute. Our objective was to explore associations between psychosocial and lifestyle factors and biological age acceleration in the St. Jude Lifetime Cohort. In this cross-sectional study, we applied physiology-based methods (Klemera-Doubal method and Phenotypic Age) and DNA methylation-based epigenetic clocks (Horvath's, Hannum's, Levine's PhenoAge, GrimAge, and DunedinPACE clocks) to compare biological age acceleration among survivors (n=4,117) and age- and sex-frequency-matched non-cancer community controls (n=606). We compared psychosocial and lifestyle factors between survivors and controls using t-tests/Wilcoxan rank sums, and chi-square. Multivariable linear regression assessed associations of self-reported smoking, alcohol use, physical activity, overweight/obesity, and depressive symptoms with biological age acceleration in survivors, adjusted for age, race/ethnicity, sex, education, and cancer treatment. Survivors at a mean chronological age of 35.1 years (SD 10.2) were biologically older than controls according to KDM-BA, Phenotypic Age, Hannum's clock, Levine's PhenoAge clock, and DunedinPACE, with biological age acceleration ranging from 1.7-4.7 years (p<0.05.) A greater proportion of survivors reported elevated depressive symptoms (10.3% v. 8.7%), current smoking (17.8% v. 12.3%), <150 minutes of physical activity (47.3% v. 38.2%), and were overweight/obese (66.0% v. 59.9%) compared to controls (p's<0.001). In survivors, <150 minutes of physical activity (B (difference in biological age acceleration [years])=1.10-2.68), current (B=1.18-5.96) and former smoking (B=2.53-4.29) were consistently associated with higher biological age acceleration across measures (p's<0.05). Risky drinking and higher depressive symptoms were associated with higher biological age acceleration, but only for KDM (B=1.26, p<0.021) and DunedinPACE measures (B=0.15, p<0.001), respectively. Thus, biological aging in childhood cancer survivors is associated with psychosocial and lifestyle factors. Interventions designed to optimize healthy behaviors and mitigate poor emotional health may potentially prevent or remediate this phenotype in childhood cancer survivors.

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Examining the Bidirectional Associations between Screen Time, Sleep Duration, and Internalizing Symptoms in the Adolescent Brain Cognitive Development Study
Purpose: The likelihood of meeting sleep duration and screen time guidelines decreases as children develop towards adolescence. Simultaneously, the prevalence of internalizing symptoms (e.g., depression, anxiety, somatic symptoms) increases, with girls being disproportionately affected. Separate studies have reported associations between internalizing symptoms and screen time or sleep duration. Despite their co-occurrence, few longitudinal studies have considered both behaviors simultaneously in relation to internalizing symptoms, and little is known on possible bidirectionality of associations. Here, I examined bidirectional associations between sleep duration and screen time with internalizing

symptoms in a longitudinal study starting in late childhood. Methods: Participants were 10,828 youth (47.8% girls) enrolled in the Adolescent Brain Cognitive Development Study, a national multi-site cohort study. At baseline (aged 9.9 years) and follow-up (aged 10.9 years), youth reported screen time for weekdays and weekend days. Responses were separately dichotomized as >2 vs. ≤2 hours/day (meeting behavioral guidelines). Caregiver-reported youth sleep duration was dichotomized as less than 9 vs. 9-11 hours/night (meeting behavioral guidelines). Caregivers also reported youth internalizing symptoms via the child behavior checklist (CBCL). The withdrawn/depressed, anxious/depressed, and somatic symptom CBCL subscale t-scores were separately dichotomized as ≥65 (borderline clinical levels of symptoms and above) vs. less than 65. Gender-stratified multilevel logistic regressions estimated the longitudinal/bidirectional associations between screen time, sleep duration, and each internalizing symptom subscale. Results: In girls, longer baseline sleep duration was protective against withdrawn/depressed symptoms (OR 0.6, 95% CI 0.4-0.8) and somatic symptoms (OR 0.8, 95% CI 0.6-0.97) one year later. In girls, greater baseline weekend screen time was associated with increased risk of withdrawn/depressed symptoms (OR 1.6, 95% CI 1.1-2.2) one year later. No other significant associations were observed. Conclusions: In late childhood, sleep duration and screen time precede depressive and somatic symptoms, specifically among girls. Modifying these behaviors could be an important intervention strategy for supporting the healthy emotional development of girls entering adolescence, a population that is disproportionately affected by internalizing symptoms.

Rupsha Singh
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Racial/Ethnic Disparities in the Trajectories of Insomnia Symptoms from Childhood through Young Adulthood

Background: Insomnia, with an estimated burden of $100 billion in healthcare costs, contributes to common adverse health outcomes such as cardiovascular disease. Insomnia affects both children and adults, and the prevalence is likely higher and symptoms more severe among minoritized racial/ethnic groups than their non-Hispanic (NH) White counterparts. Further, insomnia symptoms in childhood tend to persist into young adulthood. However, no studies have considered racial/ethnic disparities in the trajectories of insomnia symptoms from childhood through young adulthood. Therefore, we examined differences in the temporal trajectory of childhood insomnia symptoms among Black/African American (BAA), Hispanic/Latinx (HL), and NH White groups. Methods: In the Penn State Child Cohort starting in 2000, 516 children were followed up 8 years later as adolescents and 15 years later as young adults. Approximately 77% (n=395) identified as NH White, 13% (n=66) as BAA, 7% (n=37) as HL, and 3% (n=18) “other”. Insomnia symptoms were based on parent-report in childhood or self-report in adolescence and young adulthood and defined as moderate-to-severe difficulty initiating or maintaining sleep. Longitudinal trajectories of insomnia symptoms were identified across three time-points and multivariable-adjusted odds ratios (OR) and 95% Confidence Intervals (CI) were estimated using binary logistic regression models, while adjusting for age, sex, body mass index, psychiatric disorders, psychotropic medication use, sleep apnea, and periodic limb movements. Results: Overall, insomnia symptoms persisted from childhood or adolescence into young adulthood in 23.1% of participants, 16.9% developed insomnia symptoms in adulthood, and 39.3% never developed insomnia symptoms.

BAAs (39.4%) and HLs (35.1%) had the highest proportion of insomnia symptoms that persisted into adulthood, while their NH White (18.7%) counterparts had the lowest. A smaller proportion of BAAs (6.1%) and HLs (5.4%) than NH Whites (9.6%) experienced insomnia symptom remission before transitioning into adulthood. BAAs compared to NH Whites with childhood insomnia symptoms were at significantly increased odds to persist into young adulthood (OR = 2.52, 95% CI [1.26, 5.06]) with a non-significant increased odds (OR = 1.66, 95% CI [0.71, 3.90]) among HLs. Conclusions: Our study is the first to reveal that racial/ethnic disparities in insomnia symptoms start early in childhood and persist into young adulthood.

Subhadeep Dutta Gupta
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No man is an island: Toward a neurobiology of social aging in a preclinical rat model

The worldwide population of individuals aged 65 years and above is estimated to reach 1.6 billion by 2050. It is unclear whether age-dependent deficits in social cognition mainly reflect the disruption of social network activity or are simply secondary to a more general impairment of cognition in older individuals. We propose that this fundamental issue can be better understood from a socio-neurocognitive perspective by investigating how cognition operates in a social situation using a rat model of aging. To address this, we used Long Evans rats and classified aged animals as either aged-unimpaired (AU; n = 15) or aged-impaired (AI; n = 16) based on their spatial memory performance relative to young (Y; n = 16). We assessed sociability and social novelty using a three-chambered social interaction test. In the sociability trial, experimental rats had a choice of exploring a trapped conspecific (a young rat) on one side of the apparatus or an empty chamber on the other. Rats from all groups (Y, AU, and AI) spent more time with the young conspecific, demonstrating an equivalent social preference. In the social novelty trial, rats were given a choice between a trapped familiar conspecific and a novel conspecific. Y and AU rats spent more time with the novel rat, whereas the AI rats exhibited no preference, displaying a deficit in social novelty (large effect size: Cohen's d of 0.88). To explore the basis of the individual differences observed among the aged rats, next, we quantified the number of oxytocin (OXY) immunoreactive neurons in the hypothalamic paraventricular nucleus (PVN), i.e., a neuropeptide implicated in social cognition. Preliminary results suggest a decrease in PVN OXY+ neuronal number in AI relative to Y and AU and we are extending this work using fiber photometry to interrogate real-time neural dynamics during social interaction. Overall, our findings encourage a novel perspective that an ecologically valid animal model can be established to illuminate the social neuroscience of cognitive aging.

Bechara Saykali
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Developmental Biology - Early Development/Embryology

Mapping CDK activity during early mammalian development

The short duration of the G1 phase in pluripotent stem cells (PSCs) limits the window of differentiation competency by restricting the time of exposure to morphogens and hence safeguards pluripotency. Importantly, constitutive cyclin dependent kinase (CDK) activity in PSCs has been closely linked to the fast-cycling G1. As these cells commit to an embryonic germ layer, their G1 phase extends and CDK activity becomes cyclic resulting in increased cell division time. These observations led to postulate the existence of an intertwined relation between cell cycle regulation and cell fate commitment. However, the relationship between CDK activity and cell identity in vivo has yet to be explored. To address whether differential CDK activity is associated with cell fate, we generated mouse embryonic stem cell (mESC) lines containing a CDK-Kinase translocation reporter (KTR) that allows for live imaging quantification of CDK activity according to its subcellular localization. As CDK activity oscillates, the fluorescent-based reporter translocates between the nucleus and the cytoplasm in a CDK-dependent manner, providing a quantifiable readout of CDK activity at a single cell level. Time-lapse imaging of the biosensor in proliferating mESCs and mouse embryonic fibroblasts (MEFs) confirmed the validity of our cell model. In addition, we generated a mouse model harboring the CDK reporter which allowed us to capture the CDK activity landscape in pre- and post-implantation mouse embryos. Interestingly, we observed drastic reduction of CDK activity in CDX2 positive cells in blastocysts. As a complementary approach, we also generated human ESC lines containing the KTR in wild-type as well as in CDK2 and/or CDK4-deficient hESC. We then set up a 2D differentiation system that recapitulates human gastrulation in vitro where we confirmed the decrease of CDK activity in CDX2-positive cells. These results suggest a link between CDK activity and the onset of CDX2 expression. We are currently generating hESCs lines harboring both the KTR and specific germ layer markers fluorescently tagged, including CDX2, to track precisely cell commitment and CDK activity in vitro. In summary, we developed different tools to examine CDK activity during mammalian development to assess interdependencies between cell fate and CDK activity.

Rashmi Patel
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Developmental Biology - Early Development/Embryology
Bmp relay signaling between Shh/ZPA and Fgf/AER restrains posterior digit number
Limb development is orchestrated by signaling centers, including Apical Ectodermal Ridge (AER), which secretes Fgfs to promote outgrowth, and posterior mesenchymal Zone of Polarizing Activity (ZPA), which secretes Sonic hedgehog (Shh) to specify digit number and pattern. Bmps cause AER regression. AER-Fgfs maintain Shh expression and Shh in turn maintains the AER by inducing Grem1 (Bmp antagonist) in a positive feedback loop. The extent of AER-ZPA overlap in posterior limb bud delimits posterior digit number. It is known that ZPA grafts cause immediately overlying AER to regress. The basis for this short-range AER inhibition is poorly understood, but direct Shh signaling to AER has been proposed. To explore this negative ZPA-AER interaction, we selectively deleted conditional alleles of Shh pathway components from either ZPA (ShhCre knock-in), or AER (Msx2Cre transgenic). The unbound Ptch1 Shh-receptor inhibits Smo, the membrane Shh-transducer that maintains the nuclear Shh effectors (Gli2/Gli3) in a target-activator state. Shh binding to Ptch1 relieves Smo inhibition. Selective Shh-response removal in ZPA, by deleting Smo (34/34) or Gli2/Gli3 (38/38), resulted in posterior AER extension and polydactyly, but removal in AER produced no abnormal phenotype. Conversely, enforcing

Shh response in ZPA, by Ptch1 deletion (22/22) or by Smo-constitutive activation (16/16), caused posterior AER attenuation and digit (d4/5) loss, whereas enforced response in AER had no effect. This suggests that Shh signals indirectly (via ZPA) to attenuate overlying AER, perhaps by secreted Shh-targets, such as Bmp 2/4. Using confocal imaging of fluorescent in situ hybridization chain reaction (HCR), we found that ZPA removal of Shh-response reduced Bmp2/4 RNA in the ZPA, and reduced Bmp-response (Msx2 RNA) in both ZPA and AER. We therefore checked effects of Bmp receptor (Bmpr1a) removal. Selective ZPA-Bmpr1a removal increased both Shh and Bmp expression, implying that Bmps act as Shh-negative feedback regulators in ZPA, and also cause AER attenuation and d4/5 loss (34/34). To test if AER attenuation/digit loss result from excess free Bmp ligands acting on AER, we removed Bmpr1a from both ZPA and AER (ShhCre+;Msx2Cre+), which completely restored normal d4/5 (32/32). In summary, we show that ZPA-AER negative interaction restrains posterior digit number indirectly (via Bmp relay), and that ZPA-produced Bmps are also direct negative feedback attenuators of Shh expression.

Shaun Abrams
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Developmental Biology - Early Development/Embryology
Tissue-specific requirements for centrioles in coordinating facial development
Centrioles make up the core of centrosomes which function as microtubule-organizing centers of the cell. Centrioles perform two distinct cellular functions: (i) they form core components required to build the centrosome and (ii) they form the basal body that templates formation of the cilium, a microtubule-based specialized signaling organelle. CENPJ is a central component of centrioles required for centriole duplication. Human mutations in CENPJ result in Seckel syndrome and primary microcephaly, both of which present with facial defects including hypoplasia of the lower jaw (micrognathia), expansion or narrowing of the midface, premature closure of cranial sutures (craniosynostosis), prominent nasal spine, and tooth abnormalities. While much progress has been made in defining how loss of centrioles leads to impaired brain development, the molecular basis underlying the development of facial defects is less clear. We set out to elucidate how loss of centrioles results in facial defects to identify actionable targets in the pathway that could be exploited for therapeutic intervention. Due to the lethal phenotype arising from global deletion of CenpJ in mouse embryos shortly after gastrulation, we performed conditional deletion of CenpJ (CenpJ cKO) in craniofacial tissues in mouse embryos using the Sox9-cre driver to investigate the role of CenpJ in craniofacial development. Our preliminary analyses show these mice display under-developed jaw (micrognathia), midfacial defects, and skull defects, recapitulating Seckel patient phenotypes and rendering these mice an excellent model to investigate the role of centrioles in facial development. We found that the defects first arise at embryonic day 10.5 (E10.5) in CenpJ cKO with a smaller lower jaw primordia and widening of the midface that increases in severity from E11.5-12.5 resulting in midfacial clefting when compared to littermate, stage-matched control embryos. This phenotype was associated with increased cell death in the mandibular arch and p53 activation. Interestingly, p53 deletion fully rescued the craniofacial phenotypes of CenpJ cKO mutants highlighting the critical role p53 activation plays in the development of craniofacial defects upon centriole loss or disruption. Our work identified the onset of facial defects and identified p53 as a critical

mediator driving facial dysmorphology providing novel insight into the pathways through which centrioles direct facial development.

Gustavo Ulises Martinez Ruiz
Other
NCI-CCR
Developmental Biology - Organogenesis
Expression of the transcription factor Klf6 by thymic epithelial cells is required for thymus development.
Thymic epithelial cells (TEC) are distributed in the cortical and medullary regions in the thymus where they control T cell development. A diversity of medullary thymic epithelial cells (mTEC) was recently uncovered by applying single cell-based approaches, that are referred to as mTEC I to mTEC IV. Reduction in mTEC subsets compromises the establishment of central tolerance, and it is therefore of importance to identify transcriptional controllers required for mTEC development. In particular, although mTEC I are widely recognized for their role in T cell development, cell-intrinsic transcriptional regulators are unknown. Here, by using Foxn1-Cre driven ablation of the Klf6 gene specifically in TEC, coupled with single-cell transcriptional and epigenomic profiling, we identify Klf6 as a critical factor in TEC development. Klf6 deficiency resulted in a hypoplastic thymus, evident from fetal stages into adulthood, with a dramatic increase in the frequency of apoptotic TEC. Ablation of Klf6 did not equally affect all adult mTEC subpopulations. We discovered a dramatic reduction of mTEC I and, in less degree, mTEC IV. To better assess these mTEC alterations, we evaluated chromatin accessibility changes at the single-cell level, and found that Klf6 deficiency greatly increased chromatin accessibility in mTEC I. Among cortical TEC (cTEC), a previously unreported cTEC population expressing the transcription Sox10 emerged from Klf6 ablation. Further, Klf6 deficiency compromised the function of the thymus. We determined that Klf6KO reduced the naïve αβ T and iNKT cell pools in the periphery of young adult mice, and we identified T cell infiltration in salivary and lacrimal glands. Thus, Klf6 has a pro-survival role in TEC, and impacts the development of specific TEC subsets contributing to thymic function.

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Developmental Biology - Organogenesis
Multi-level 3D Genome Reorganization during Human Retinal Organoid Differentiation
Genetic information is encoded in a linear fashion on nuclear DNA that is highly condensed in the form of chromatin, which undergoes extensive reorganization during development. Chromatin remodeling is considered as an important regulatory layer for gene expression and lineage specification. We wonder how changes in 3D chromatin architecture are involved in specifying and maintaining functionally distinct cell populations during human retinal development. To achieve this goal, we generated high-resolution Hi-C maps of developing human retinal organoids that recapitulate many developmental features of human retina. By integrating hierarchical genomic structure from Hi-C with RNA-seq-derived transcriptome data during differentiation, we have delineated the multi-level 3D genome structures in association with gene expression during retinal differentiation. At the compartment level, we

demonstrate progressive changes in chromatin structures that can correlate to specific stages of retinal development. The transition from early- to late-born retinal cell types coincides with dramatic shifts in genome architecture. Further, we show that genomic regions shifting towards active A compartment are enriched for upregulated gene expression whereas regions shifting towards inactive B compartment are enriched for non-coding genes and exhibit downregulated expression. At the topologically associated domain (TAD) level, we observed that differentially expressed genes associated with retinal development are enriched near lost boundaries of TADs. Notably, higher-order assemblages of TADs (termed as "TAD cliques") tend to localize in the active chromatin regions and are enriched for expressed transcription factors. We also analyzed chromatin loop communities formed by physical interactions or overlaps and found that these loop communities are decreasing in number with expressed genes increasing in them during differentiation. Interestingly, we found eye and central nervous system developmental genes are enriched in dense loop anchors regions. Finally, comparative analyses showed that the chromatin structure of developing retinal organoids increasingly resembles that of the adult retina. Our studies implicate that stage-specific hierarchical chromatin conformations are related to gene regulation and highlight the paradigm of using organoids as a model for investigating human retinal development.

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DNA Replication, Damage and Repair

END-seq at telomeres uncovers the terminal chromosomal sequences and unique features of ALT-positive cells

Like the little plastic cap at the end of a shoelace, telomeres, repetitive DNA found at both ends of eukaryotic chromosomes, protect chromosomes from degradation and loss of vital sequence. They also block any unwanted DNA damage repair that might result in end-to-end fusions and allow cells to distinguish between natural ends and any breaks that should be repaired to maintain genome stability. Human telomeres comprise 5–15 kilobases of tandem TTAGGG repeats. With each round of DNA replication, telomeres shorten progressively. In most eukaryotes, this problem is solved by the activity of telomerase, a reverse transcriptase; however, in human somatic cells, telomerase is not active. As a result, continuous proliferation causes telomere shortening and eventually triggers cellular senescence, a tumorigenesis barrier. Hence, cancer cells must overcome telomere shortening to acquire replicative immortality. While most cancers accomplish this by reactivating telomerase, a significant fraction uses a telomerase-independent recombination-based pathway, alternative lengthening of telomeres (ALT), to extend and maintain telomeres. These distinct pathways are incompletely understood for activation, propagation, and their associations with clinical outcomes. Interestingly, the structure or the precise sequence of telomeres is still the subject of debate, given their length and repetitive nature. In my project, I focus on unravelling the precise sequence of natural chromosome ends. For this, we employed an unbiased and high-resolution approach using the well-established END-seq method developed to investigate double-strand breaks. The analyses on various cell lines showed that independent of the telomere maintenance mechanism (TMM), the last nucleotide of the 5' end of human and mouse chromosomes is not random and is always a cytosine. Moreover, using a modified version of the END-seq to detect secondary structures, we found that telomeres of ALT cells can be readily distinguished

from telomeres of telomerase-positive cells or cells lacking any TMM. Also, we revealed that ALT-positive telomeres have structures driven by single-stranded DNA gaps. Surprisingly, these structures carry a distinctive motif indicating the existence of a mechanism that generates and preserves unique sequences. Our work provides insights into the source of those structures found in ALT telomeres, and it sets the grounds to explain the origin of single-stranded telomeric DNA in ALT cells.

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DNA Replication, Damage and Repair
Human DNA polymerase eta is a new substrate of the NEDD8 ubiquitin-like conjugating pathway
Skin cancer is a malignancy that arises due to mutations caused by DNA damage induced by solar ultraviolet radiation. In normal cells, the formation of DNA mutations is suppressed by DNA repair and replication mechanisms that allow damaged DNA to be corrected. This ensures that cells can survive, while preventing cancer-causing mutations. DNA polymerase eta (Pol eta) is an enzyme that enables efficient replication past ultraviolet light induced-DNA damage. Its biological significance is evident in patients with Xeroderma Pigmentosum Variant (XP-V), a disease caused by mutations in the Pol eta gene, and which is characterized by a high incidence of skin cancer in sunlight-exposed areas. Posttranslational modifications are important for regulating Pol eta function. This includes Pol eta mono-ubiquitination, which occurs at one of four lysine residues on the Pol eta C-terminus and regulates its interaction with replication sites. To identify other regulators of Pol eta, we used mass spectrometry to detect binding partners that co-immunoprecipiate with Pol eta. We found that Pol eta associates with NEDD8, a ubiquitin-like protein that shares ~60% sequence identity with ubiquitin and can also be covalently attached to lysine residues of a substrate protein. NEDDylation has been found to regulate DNA damage pathways including TLS and DNA repair. We therefore hypothesized that Pol eta might be regulated by NEDDylation. Indeed, using NEDD8 antibodies we detected mono-NEDDylation of Pol eta immunoprecipitated from HEK293T cells. Furthermore, we found by mutational analysis that NEDDylation occurs at the same four lysine residues in the Pol eta C-terminus that are also ubiquitinated. This suggests that Pol eta is regulated by competition between these two posttranslational modifications. In addition to being ubiquitinated, Pol eta can also interact with ubiquitin noncovalently via its ubiquitin-binding zinc-finger (UBZ) domain. HADDOCK modelling showed that NEDD8 might also interact with the Pol eta UBZ domain. This suggests that Pol eta might make similar interactions with NEDD8 and ubiquitin. In future experiments, we will focus on characterizing this interaction and explore the biological function of Pol eta NEDDylation. Our findings suggest a novel mechanism of regulation for Pol eta and sheds light on the complex interplay of posttranslational modifications in the DDR and TLS pathways, potentially leading to new avenues for skin cancer prevention and treatment.

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DNA Replication, Damage and Repair

Repair of nick-induced collapsed replication forks monitored at high resolution

DNA Replication is a highly regulated and accurate process that guarantees faithful DNA duplication in every cell cycle. Many obstacles and DNA lesions can interfere with replication fork progression and have the potential to stall or collapse replication forks. Collapsed forks are defined as replication forks that have lost the ability to perform DNA synthesis due to replisome loss and/or fork breakage. Unlike two-ended double-strand breaks (DSB) that can occur throughout the cell cycle, collapsed fork breaks are thought to be mainly one-ended. In yeast, one-ended breaks can be repaired through Break Induced Replication (BIR); however, it is still unclear if and how this process occurs in mammalian cells. To investigate collapsed fork structure and repair in human cells, we generated an inducible Cas9 nickase system and designed guides to target early replicating regions where the replication direction is unambiguous. Using this strategy, we produced nicks at the leading or lagging strands that collapse replication forks when the cells are released from G1 arrest into S-phase. By using END-seq, we were able to detect breaks at collapsed forks at high resolution. Both leading and lagging breaks are immediately resected (4-10kb), and the break signal eventually decreases, indicative of the repair of collapsed forks. We investigate the mechanism of nick-induced repair including the role of RAD51, BRCA1 and BRCA2-dependent homologous recombination. Surprisingly, we find that while leading strand collapsed replication forks generate mainly one-ended breaks, lagging strand collapsed forks show a more symmetric, two-ended break pattern. We propose a model that explains the difference between collapsed forks at leading vs. lagging strand nicks and the distinct consequences for repair.

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Receptor-mediated activation of G12/13 signaling in the arcuate nucleus regulates key metabolic functions

Obesity has emerged as a major threat to human health worldwide. The development of new anti-obesity therapies requires a detailed understanding of the underlying cellular and molecular mechanisms. The arcuate nucleus of the hypothalamus (ARC) exerts a multi-tier control over energy homeostasis, which involves appetite regulation and autonomic control of endocrine glands and metabolic tissues. Central to its role are the orexigenic AGRP and anorexigenic POMC neurons. These neurons express many G-protein coupled receptors which detect changes in hormone and metabolite levels. Among other G protein families, several of these receptors activate G proteins of the G12/13 family. The potential metabolic relevance of this signaling pathway remains unknown at present. To address this question, we generated mice expressing a G12/13-coupled designer receptor (G12/13 DREADD; G12/13D) specifically in AGRP neurons (AGRP-G12/13D mice) or POMC neurons (POMC-G12/13D mice), respectively. Deschloroclozapine (DCZ), a highly selective DREADD agonist which is otherwise pharmacologically inert, was administered via the drinking water for up to 8 weeks. G12/13 activation in AGRP-G12/13D mice resulted in a robust increase in body weight, increased adiposity, reduced insulin sensitivity, impaired glucose tolerance, and resulted in hyperglycemia. These effects were recapitulated in mice expressing a constitutively active version of Galpha12 selectively in AGRP neurons. Following chronic G12/13 activation, AGRP-G12/13D mice displayed a significant reduction in

total energy expenditure and fat oxidation. In contrast, G12/13 activation in POMC-G12/13D mice led to significant glycemic and metabolic improvements, characterized by reduced weight gain and improved glucose tolerance. In line with these metabolic improvements, mechanistic studies revealed enhanced insulin signaling in the ARC, elevated levels of plasma adiponectin, and increased expression of PPAR-gamma and adiponectin genes in white fat of POMC-G12/13D mice. Collectively, our data indicate that receptor-activated G12/13 signaling in AGRP and POMC neurons plays a critical role in the maintenance of glucose and energy homeostasis. Targeting endogenous G12/13-coupled receptors expressed in the ARC may prove useful to improve metabolic deficits present in various pathophysiological conditions.

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Thyroid Hormone Receptor α1: A Novel Regulator of Cancer Cell Differentiation

Thyroid hormone receptor α1 (TRα1) mediates the genomic actions of thyroid hormone (T3) in growth, differentiation, and development. TRα1 is known to function as a transcription factor to initiate and drive cellular programs to promote differentiation in different cell types under normal physiological conditions. However, how TRα1 could act as a transcription factor to regulate the differentiation of human cancers is largely unknown. My study aims to understand the role TRα1 in the differentiation of cancer cells using anaplastic thyroid cancer cells (ATC) as a model. Analysis of human thyroid cancer database of The Cancer Genome Atlas (TCGA) showed that THRA gene expression is lost in ATC cells. Accordingly, we explored the effects of TRα1 on the re-differentiation of ATC. We used human ATC cell lines 11T and 16T for our studies. These ATC cells exhibit different genetic lesions in that 11T cells harbor a KRAS mutation and 16T cells possess TP53, RB and PI3KCA gene mutations. In these cell lines, I did not detect expression of the THRA gene. We therefore stably expressed TRα1 in three clones each in 11T (11T-TRα1#2, #7 and #8) and 16T (16T-TRα1#3, #4 and #8) cells. I found that TRα1 expressing cells inhibited ATC cell proliferation and induced apoptosis. Using thyroid differentiation scores, we found the THRA gene expression was highly positively correlated with the paired box gene 8 (PAX8). I found that the PAX8 expression was barely detectable in parental 11T and 16T cells. However, the PAX8 gene expression was significantly elevated in 11T- and 16T-TRα1-expressing cells at the mRNA and protein levels. Using ChIP assays, we found that TRα1 bound to a thyroid hormone response element (TRE) on the upstream promoter of the PAX8 gene. Using luciferase reporters driven by the PAX8 upstream promoter encompassing the TRE, we demonstrated that TRα1 directly activated the expression of the PAX8 gene. By using single cell transcriptomic analysis (scRNA-seq), we demonstrated that TRα1 functioned as a transcription factor through multiple signaling pathways to suppress tumor growth. Importantly, scRNA-seq analysis showed that TRα1-induced PAX8, via its transcription programs, shifted the cell landscape of ATC toward a more differentiated state. Our studies indicate that TRα1 is a newly identified regulator of thyroid cancer cell differentiation and could be considered as a potential therapeutic target to re-differentiated ATC to improve the outcome of ATC patients.

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Protein arginine methyltransferase 1 regulates adult intestinal proliferation and enteroendocrine cells differentiation.
The adult intestinal epithelium is a complex, self-renewing tissue composed of specialized cell types with diverse functions. Intestinal stem cells (ISCs) at the base of crypts divide and differentiate into absorptive and secretory cells. Enteroendocrine cells (EEC), one type of secretory cells, are the most abundant hormone-producing cells in mammals and involved in the control of energy homeostasis. Many studies investigated the mechanisms that control cell fate determination; however, regulation of EEC development and function are still unclear or controversial. Here, we found that Protein arginine methyltransferase 1(PRMT1), a major arginine methyltransferase, is highly expressed in the proliferating transit-amplifying (TA) cells and ISCs of adult mouse intestinal crypts. By using tamoxifen-induced intestinal epithelial cell-specific deletion of PRMT1 in adult mice, we observed the number of EEC dramatically increased. Transcription analyses showed the expression levels of Enteroendocrine-specific hormone and transcription factors were upregulated in PRMT1-deficient small intestine. In addition, the top enriched upregulated pathways were all associated with EEC functions. Concomitantly, Neurogenin 3-expressing progenitor cells accumulated in the mutant small intestine. Also, the downstream target genes of Neurogenin 3 (such as Neuod1, PAX4, PAX6, and Insm1) were upregulated in the mutant crypts. Furthermore, intestinal epithelium of the mutant mice showed elongated crypts in the small intestine, while increased cell proliferation in TA cells. Additionally, inducible PRMT1 deletion led to increased cell death, which compensated for increased cell proliferation in the crypts to maintain overall intestinal morphology and intestinal homeostasis. Together, our results revealed that the loss of PRMT1 in the adult intestinal epithelium altered TA cell proliferation and EEC differentiation, which probably via enhancement of Neurogenin 3-mediated commitment to the EEC lineage. Thus, our results provide potential roles of PRMT1 as an essential transcriptional regulator of EEC determination.

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Dose-response of accelerometer-measured physical activity, step count, and cancer risk in the United Kingdom Biobank
Background: Walking is a highly accessible and intuitive measure of activity, making it one of the most widespread types of physical activity. Currently, little is known about the step count – incident cancer risk relationship. In this study, we describe the dose-response association between accelerometer-measured total activity expenditure, daily step count, and cancer risk using data from a population-based prospective cohort study. Methods: We examined data from United Kingdom Biobank participants who provided 7-days of valid accelerometer data and did not have a prior history of cancer (N=86,408, ages 43-78 at accelerometer wear). Total physical activity was estimated using average metabolic equivalents (METs) per day, and a hybrid step detection model was applied to ascertain

median daily step count. Overall and site-specific cancer incidence outcomes were obtained through linkages to national registries. Cox regression models were used to obtain hazard ratios and 95% confidence intervals after adjustment for age, sex, race, and other major cancer risk factors. Results: Over a mean of 5.37 years of follow up, 4,893 incident malignant cancers were reported. Compared to individuals in the lowest quintiles of average METs per day, individuals with greater activity levels had lower risk for incident cancer. Similar patterns were observed for breast, colorectal, and lung cancer, but not for prostate cancer. Associations for daily median step count and cancer incidence were weaker across for all cancers and site-specific cancers. Individuals in the lowest quintile of daily step count had the greatest cancer risk, but the association was not linear across quintiles. Conclusion: Our findings align with prior studies that reported greater physical activity was associated with decreased risk of breast, colorectal, and lung cancer, but not prostate cancer. Despite the highly translatable natures of step count, we did not observe a strong positive association between daily step count and cancer risk. Future analyses will quantify the dose-response relationship between accelerometer-measured physical activity and cancer risk and assess associations for other site-specific cancers. Ultimately, findings from this study could inform the precision of physical activity guidelines for cancer prevention.

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Epidemiology/Biostatistics - Etiology
Ingested nitrate and nitrite and end-stage renal disease risk among licensed pesticide applicators and spouses in the Agricultural Health Study
Background and Aim There are few established environmental risk factors for end-stage renal disease (ESRD). Nitrate and nitrite ingestion has been linked to renal cell carcinoma, possibly via the endogenous formation of carcinogenic N-nitroso compounds. These exposures might also play a role in ESRD, but there have been no epidemiologic studies on the associations. We investigated the associations of drinking water nitrate and dietary nitrate and nitrite intakes with incident ESRD among participants of the Agricultural Health Study. We explored modification of dietary associations by vitamin C and heme iron intakes, which may differentially mediate endogenous nitrosation. Method The primary drinking water source for participants was determined at enrollment. We estimated duration-specific average nitrate concentrations for public water supply users (N=14,769) from historical measurements data. For private well users (N=44,863), we developed random forest model estimates of nitrate concentrations based on well location, depth, nitrogen inputs, and other predictors. Dietary nitrate and nitrite were assessed among 30,177 participants who completed the NCI Dietary History Questionnaire at the first follow-up. Incident ESRD between study enrollment (1993-1997) or first follow-up (1999-2003) and December 2018 was ascertained through linkage with the U.S. Renal Data System. We estimated adjusted hazard ratios (HRs) and 95%CI for associations of tertiles (T) of exposure with incident ESRD overall and stratified by median intake of vitamin C and heme iron. Results We identified 469 ESRD cases, including 206 cases for the dietary analysis. Water and dietary nitrate exposures were not associated with ESRD risk overall or in strata of vitamin C and heme iron. Higher dietary nitrite was associated with a modest increase in ESRD risk (T3 vs. T1 HR: 1.36, 95%CI: 0.83, 2.21), with stronger associations observed among participants with vitamin C <median (T3 vs. T1 HR: 2.26, 95%CI: 1.05, 4.86) and those with heme iron ≥median (T3 vs. T1 HR: 1.73, 95%CI: 0.89, 3.39). No apparent

associations were observed among participants with vitamin C ≥median or with heme iron <median. Conclusions ESRD incidence was associated with dietary nitrite intake among participants with lower vitamin C or higher heme iron intake. Research using biomarkers of earlier renal dysfunction and other nutrient profiles may clarify the potential risks associated with nitrite exposures.

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Causes of Death among Adult Solid Organ Transplant Recipients in the United States, 1999–2019
Solid organ transplant recipients (SOTRs) are at high risk of mortality from underlying medical conditions, graft failure, and immunosuppression brought on by the medications used to prevent rejection. Yet, no published study has systematically evaluated causes of death (CODs) among the entire SOTR population in the US. To help inform strategies that can improve survival among SOTRs, we report the distribution of underlying CODs among US adult SOTRs and compare mortality to the general US population. The US SOTR registry collects information on CODs, but it is missing COD data for ~50% of deaths. For this reason, US SOTR registry data were linked to the National Death Index (NDI) to obtain CODs. Sampling fractions varied by year of transplant: 1999–2004 (40%), 2005–2009 (75%), and 2010–2019 (100%). The data were weighted to represent all deaths among adult (≥18 years) SOTRs during 1999–2019. Since graft failure is not an NDI-listed COD, deaths within 90 days of registry-documented graft failure were attributed to graft failure. To compare SOTR mortality to the general population, we calculated standardized mortality ratios (SMRs). Among 496,367 adults receiving a first transplant in 1999–2019, there were 153,491 deaths, where 99,373 had NDI-linked CODs. With an average follow-up of 7.3 years per person, the overall mortality rate was 4,258 per 100,000 person-years (range: 3,505 for kidney recipients, to 5,293 for heart recipients). Approximately one-fifth (19.2%) of deaths occurred within one year of transplant and half (49.6%) occurred 5+ years after transplant. Overall, heart disease, graft failure, and cancer were the top three CODs, together responsible for 46.2% of deaths. Graft failure was the leading COD for SOTRs aged 18–34, but it was heart disease for all older age groups. SOTRs had a 4-fold higher (SMR=4.0) risk of death compared to the general US population, and SMRs were elevated for 15 out of 16 COD analyzed (all CODs except Alzheimer's disease). The SMRs were 3.3 for heart disease (8.8 for heart recipients), 2.1 for cancer, 8.3 for diabetes, 11.0 for infections, 19.8 for kidney diseases (24.3 for kidney recipients), and 388.5 for cystic fibrosis. In summary, SOTRs had elevated mortality for a range of CODs, reflecting issues related to the transplanted organ, comorbidities, and immunosuppressive treatment. This information can be used to identify which CODs, such as heart disease, to prioritize for interventions and further research.

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Absolute lung cancer risk increases among individuals with >15 quit-years: analyses to inform the update

of the American Cancer Society lung cancer screening guidelines

Background: The United States Preventive Services Task Force (USPSTF) recommend lung cancer screening for individuals aged 50-80 years with ≥20 pack-years who currently smoke or quit within the last 15 years (<15 quit-years). However, lung cancer risk increases with increasing age, and age increases with increasing quit-years. We examined lung cancer risk beyond 15 quit-years, and project the impact of expanding eligibility to individuals with >15 quit-years. Methods: We fit Cox models to estimate 5-year lung cancer risk among Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial (PLCO) and National Lung Screening Trial (NLST) participants aged 55-74 years with ≥20 pack-years. Then, using the 2015-2018 National Health Interview Survey (NHIS), a US-representative sample, we calculated 5-year lung cancer death risk. The NHIS results were used to project the proportion of preventable lung cancer deaths prevented by screening those eligible (lung cancer death sensitivity), when expanding USPSTF eligibility criteria. Results: 5-year lung cancer risk initially decreases upon quitting smoking, but rebounds by 10-quit years (relative annual percentage change (RAPC) from 0 to 10 quit-years in PLCO: -0.3%, 95% CI: -1.3 to 0.6%, p=0.50). After the first 10 quit-years, the effect of aging outweighed the effect of increasing quit-years, so risk increased (RAPC beyond 10 quit-years: 3.8%, 95% CI: 2.6 to 5.0%, p<0.001). Results from the NLST were similar. Using the NHIS, we estimate that 14.3 million people aged 50-80 who ever smoked (32%) are eligible for screening under USPSTF recommendations. If the quit-year criterion were increased to 20, 25, or 30 quit-years, or removed, an additional 1.6 million, 2.5 million, 3.7 million and 4.9 million individuals who ever-smoked would be eligible, respectively. Increasing or removing the quit-year criterion could increase lung cancer death sensitivity from 63.7% to 67.4% (20 quit-years), 69.5% (25-quit-years), 71.9% (30 quit-years) or 74.2% (removing the quit-year criterion), corresponding to an additional 2,914, 4,597, 6,485 and 8,275 preventable lung cancer deaths over 5 years, respectively. Conclusion: Absolute lung cancer risk increases beyond 15 quit-years, which does not support restricting lung cancer screening to individuals who currently smoke or quit in the last 15 years. Removing the quit-year criterion could prevent an additional 10% of preventable lung cancer deaths.

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Epidemiology/Biostatistics - Etiology

Glyphosate use and mosaic loss of chromosome Y among male farmers

Background: Glyphosate, the most widely applied herbicide worldwide, has been implicated in the development of hematologic cancers and classified by the International Agency for Research on Cancer as a probable human carcinogen. While studies in human cells and animals support the genotoxic effects of glyphosate, evidence in human populations is scarce. Growing evidence suggests that certain occupational and environmental chemical exposures may contribute to mosaic loss of chromosome Y (mLOY), the most common chromosomal alteration among men and a marker of genotoxicity. However, no study has examined mLOY in relation to glyphosate exposure. We evaluated the association between lifetime occupational glyphosate use and mLOY among male farmers in the Agricultural Health Study. Methods: We analyzed blood-derived DNA from 1606 male farmers aged ≥50 years from Iowa and North Carolina. mLOY was detected using genotyping array intensity data in the pseudoautosomal region of the sex chromosomes. Cumulative lifetime use of glyphosate was assessed based on self-reported

pesticide exposure histories collected longitudinally across several decades prior to blood sampling. Using multivariable logistic regression, we estimated odds ratios (ORs) and 95% confidence intervals (CIs) for the associations between glyphosate use and any detectable mLOY (overall mLOY) or mLOY affecting ≥10% of cells (expanded mLOY). Results: Overall, mLOY was detected in 21% of farmers, and 10% of all farmers had expanded mLOY. Increasing total lifetime days of glyphosate use was associated with expanded mLOY (highest vs lowest quartile, OR=1.75, 95% CI=1.00-3.07, Ptrend=0.03) but not with overall mLOY; the associations with expanded mLOY were most apparent among older (age ≥70) men (2.30, 1.13-4.67, Ptrend=0.01), never smokers (2.32, 1.04-5.21, Ptrend=0.04), and non-obese men (2.04, 0.99-4.19, Ptrend=0.03). Similar patterns of associations were observed for intensity-weighted lifetime days of use, a metric accounting for factors known to influence pesticide exposure (e.g. personal protective equipment use). Conclusion: High lifetime glyphosate use may be associated with mLOY affecting a larger fraction of cells, suggesting glyphosate could confer genotoxic or selective effects relevant for clonal expansion. As the first study to investigate this association, our findings contribute novel evidence regarding the carcinogenic potential of glyphosate and require replication in future studies.

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Racial and ethnic disparities in hypertension medication efficacy using the All of Us Research Program
Hispanic ethnicities and non-White races continue to be underrepresented in clinical trials despite evidence of differences of drug efficacy in these groups. Essential hypertension affects 47% of Americans, and those of Hispanic ethnicity and non-White race typically have poorer rates of control. We developed a generalizable method to determine systemic race and ethnicity disparities in anti-hypertensive (anti-HTN) drug efficacy. The NIH All of Us Research Program (AoU) is a longitudinal cohort of 194,420 participants with electronic healthcare record (EHR) data, drug exposures, and demographics. We used a self-controlled case study design to determine drug efficacy by determining the reduction in systolic blood pressure (SBP) measurements after medication (delta SBP). We analyzed 16 anti-HTN medications to determine whether delta SBP for each medication was significantly different in each race and ethnicity, compared to non-Hispanic White participants using linear regressions. We were able to determine the most effective anti-HTN drugs within each demographic. Chlorthalidone was the most effective anti-HTN medication in Black participants (SBP mean reduction = 10.49, SE = 1.15mmHg), while labetalol was most effective in Hispanic participants (SBP mean reduction = 12.07, SE = 1.64mmHg), and HCTZ/Lisinopril was the most effective in White participants (SBP mean reduction = 10.06, SE = 1.42mmHg). We were also able to determine drugs which have the biggest variability of effectiveness based on race/ethnicity (e.g. labetolol delta SBP = -3.73±0.76 mmHg in White, -9.01±0.96 mmHg in Black, and -12.07±1.64 mmHg in Hispanic participants). 14 out of 16 anti-HTN drugs were less effective in Black and 8 out of 16 were less effective in Hispanic, compared to non-Hispanic White participants. Finally, we determined that Black participants were prescribed significantly more anti-HTN medications (mean = 2.94) than White (mean = 2.43) or Hispanic participants (mean = 2.53) and started on medications at a significantly higher SBP (Black mean = 139.07 +/- 0.42; Hispanic mean = 135.98 +/- 0.58; White mean = 133.2 +/- 0.3 mmHg). This work provides support for the further need to increase

racial- and ethnic-minority representation in clinical research and demonstrates the potential of real-world evidence to guide medication therapy selection. This method provides a scaffold for a systematic approach to determine drug efficacy in EHR data, which has many potential applications.

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Mosaic chromosomal alterations are independent predictors of chronic lymphocytic leukemia risk

Chronic lymphocytic leukemia (CLL) is strongly associated with mosaic chromosomal alterations (mCAs), a type of clonal hematopoiesis characterized by large structural chromosomal changes (e.g., gains, losses and copy neutral loss of heterozygosity). We aim to identify specific chromosomal regions that, when impacted by mCAs, are most predictive of CLL risk and evaluate their predictive utility in models that include established CLL risk factors. We utilized genotype array data from 436,784 participants in the UK Biobank (UKBB) and 35,382 participants in the Prostate, Lung, Colorectal, and Ovarian (PLCO) screening trial to identify mCAs. UKBB was split (90/10) into training and test sets, and PLCO served as an external validation set. We developed sequential regression models to predict risk of CLL, with a baseline model consisting of age, age-squared, sex, smoking status, and ancestry. Additional models evaluated mCAs, a CLL polygenic risk score (PRS), and blood cell counts. Area under the receiver operating characteristic curve (AUC) was used to evaluate the predictive utility for both 5-year and 10-year CLL risk. Any detectable mCA was associated with incident CLL (Hazards Ratio (HR)=26, 95% Confidence interval (CI)=21-31), and mCAs on each individual chromosome were associated with increased CLL risk. mCAs on segments of chromosomes 13, 12, 22, 14, and 11 had the largest effect size (e.g., chr 13q14 HR=199, 95%CI=155-257). The CLL prediction model, including baseline factors, CLL PRS, and any autosomal mCA status, showed strong predictive utility in independent UKBB (AUC=0.88) and PLCO (AUC=0.88) samples. Restricting to a subset of mCAs strongly associated with CLL did not improve performance over including any detectable autosomal mCA. After the inclusion of blood cell traits, predictive utility further increased in UKBB (AUC=0.91; no count data available in PLCO). We observed the strongest CLL predictive utility in 5-year risk compared to 10-year risk across all models. mCAs genome-wide are associated with CLL risk, with variable effects across chromosomes and specific chromosomal regions. Autosomal mCAs are strong independent predictors of CLL risk and substantially add to predictive utility over standard clinical measures. The predictive utility of autosomal mCAs is strongest within the first 1-5 years of sample collection. Evaluation of mCAs could provide added utility for risk stratification in populations at high risk of CLL.

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Observational and genetic associations between cardiorespiratory fitness and cancer: a UK Biobank and international consortia investigation

Background: High fitness is associated with good cardiometabolic health and therefore may be an important strategy for cancer prevention. However, the associations of fitness with cancer risk are poorly characterized, particularly for women who have been underrepresented in fitness assessments. Therefore, we aimed to investigate the associations of fitness with the risk of common cancers using observational and Mendelian randomization (MR) methods. This is the first study to use the genetic associations of fitness to investigate relationships with cancer, and the integration of evidence from these different approaches strengthens the basis for causal inference. Methods: Maximal cardiorespiratory fitness (defined as maximal oxygen consumption, VO2max) was estimated in a subset of 72,572 UK Biobank participants who underwent in a submaximal fitness test. VO2max was scaled to total body mass (tbm) and fat free mass (ffm), which represent differing components of fitness. In observational analyses, we used Cox proportional hazards models to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) for risks of lung, colorectal, endometrial, breast, and prostate cancers. We also used a two-sample MR framework, with genetically predicted VO2maxffm as an instrumental variable derived from UK Biobank participants and genetic cancer associations from international consortia for these cancer sites. Odds ratios (ORs) were estimated using the inverse-variance weighted method. Relationships between fitness and cancer may be partially mediated by body composition, therefore all associations were estimated with and without adjustment for adiposity. Results: After a median of 11 years of follow-up, 4,290 cancers of interest were diagnosed. Higher fitness levels were associated with lower risks of endometrial (HR per 1 standard deviation (SD) VO2maxttm=0.66, 95% CI 0.53-0.79), colorectal (0.88, 0.81-0.98), and breast cancer (0.92, 0.85-0.98). In MR analyses, higher fitness levels were associated with a lower risk of breast cancer (per 1 SD genetically predicted VO2maxffm=0.85, 95% CI 0.74-0.96). After adjusting for adiposity, both the observational and genetic associations were attenuated. Conclusion: Higher fitness levels may reduce risks of endometrial, colorectal, and breast cancer, though relationships with adiposity are complex. Aiming to increase fitness, including via changes in body composition, may be an effective strategy to reduce cancer risk.

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Occupational Farm Exposures Associated with Indoor Home Microbiota
Humans contribute and are exposed to environmental microbes, especially indoors where they spend the most time. Home dust microbiota can affect inhabitants' microbiome and is associated with allergic, atopic, and respiratory conditions. The indoor microbiota is influenced by environmental factors such as farming, which confers unique microbial exposures. In previous work, we've shown altered home dust microbiota with living on a farm and by farm type. However, farm work includes different tasks and exposures, including agrochemicals such as pesticides. It is unknown how these different occupational exposures will shape the microbes in workers' homes, which is important for their health and the health of any cohabitants. Therefore, the aim of this project was to determine if farm work activities and self-reported pesticide use were associated with changes in the indoor dust microbiome in the homes of 468 male participants of the Agricultural Lung Health Study, a nested asthma case-control study of licensed pesticide applicators, typically farmers, in Iowa and North Carolina. Vacuumed dust was collected from participants' bedrooms and underwent whole-genome shotgun sequencing for microbiome assessment.

We evaluated 6 different farm work tasks (hay, silage, feed, grain, soy, or fertilizer use) and 19 pesticide ingredients used in the past year. We found all 6 work tasks were associated with increased within-sample diversity, with a positive dose-response for the total number of tasks (p=0.001), and altered microbial compositions (weighted UniFrac permutational multivariate analysis of variance p=0.001). Analysis of composition of microbiomes with bias correction showed increased abundance of specific microbes, including pathogenic and beneficial commensals. Mixed results were seen with pesticides. Glyphosate was associated with increased within-sample diversity (p=0.04), and 9 pesticides were associated with altered beta composition (p 0.001-0.04), while atrazine and pyrethroid were associated with decreased abundance of select microbes. This work demonstrates that occupational exposures impact the microbiome inside homes through altered diversity levels and abundance of specific microbes. This is meaningful for the health of the workers and their families, as it sheds light on potential underlying mechanistic pathways for how work exposures can influence health through the role of in indoor microbiome and offers possible future intervention targets.

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Epidemiology/Biostatistics - Prevention and Risk

Accounting for cancer subtypes heterogeneity identifies eight breast cancer risk loci

Background: Breast cancer is a heterogeneous disease including various molecular subtypes, each with unique etiologies, clinical presentations, and outcomes. Growing evidence suggests that common germline variants are associated with breast cancer subtypes in a heterogeneous manner. Previously, a two-stage polytomous model was proposed to identify breast cancer variants using subtype information, but it has limited power and is computationally intensive when there are interaction effects. We aimed to develop a more powerful and computationally efficient model that incorporates tumor subtype information to identify novel breast cancer loci. Methods: We developed TOPO, a novel model that efficiently detects variants exhibiting subtype heterogeneity by employing a comprehensive test procedure with three different model structures. Using 138,209 breast cancer cases and 121,663 controls of European ancestry from the Breast Cancer Association Consortium, we conducted a genome-wide association study (GWAS) and defined breast cancer subtypes by estrogen receptor (ER), progesterone receptor (PR), human epidermal growth receptor 2 (HER2), and tumor grade. Covariate adjustments were made for age and principal components 1-10. After filtering out variants within ±500kb or with linkage disequilibrium (LD>0.1) with any previously published loci, the identified variants were ensured to be novel. Results: After filtering out known risk loci, we identified eight novel variants (P-value < 5×10-8) using the proposed model. The scaled genomic inflation factor is 1.002, which indicates no inflation for the association analysis. The identified variants exhibited strong evidence of subtype-specific associations across different breast cancer subtypes. For example, rs6677545 had a case-control odds ratio (OR) of 0.97 (95% confidence interval (CI): 0.95-0.99) for luminal-A like subtype (defined as ER positive or PR positive with HER2 negative) versus an OR of 1.04 (95% CI: 1.01-1.07) for triple negative subtype (defined as ER, PR, HER2 all negative). Conclusion: This study identified eight new breast cancer loci through subtype-specific analysis using a novel method that incorporates tumor characteristics for testing genetic association with cancer risk. Our findings demonstrate that

considering tumor heterogeneity can identify new loci, enhance understanding of breast cancer's etiologic heterogeneity, and inform subtype-specific genetic scores for precision prevention.

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Epidemiology/Biostatistics - Prevention and Risk
Ambient ultraviolet radiation and ocular melanoma incidence in the United States
Ocular melanoma (OM) is associated with significant risk of both vision loss and death. While ultraviolet (UV) radiation is a known risk factor for cutaneous melanoma, studies of OM have been inconsistent. These studies have usually been restricted to non-Hispanic Whites (NHWs) and have not been powered to report findings by anatomic sites. UV may play different roles for different sites of OM because of the specific positions of the structures within the eye. External ocular sites such as the iris are directly exposed to UV, while only a very small amount of direct UV can reach internal sites such as the choroid. Our objective is to examine the associations between UV and incidence of three major anatomical sites of OM (ciliary body/iris, choroid, conjunctiva) by race and ethnicity using 21 US cancer registries for years 2000–2019 from NCI's SEER program. We linked satellite-based noontime UVB estimates to county of residence at diagnosis. Incidence rate ratios (IRRs) were calculated for UVR quartiles (Q4 [highest] versus Q1 [lowest]) using Poisson models. UVR was associated with increased incidence of ciliary body/iris in NHWs (IRR/10 mW/m2=1.16;95%CI=1.08,1.24) but not in other races or ethnicities. The iris may be more UV-exposed as it is the most anterior segment of the uveal tract. UV was associated with reduced incidence of the choroid in the total population (highest UV quartile IRR=0.86;0.82,0.91). This reduced incidence was observed in NHWs, Hispanic Whites and American Indians, but not in Blacks or Asians/Pacific Islanders. One possibility is that UV exposure at higher latitudes could result in higher UV exposure to internal ocular sites. Greater internal ocular exposure to diffuse UV occurs when the sun is near the horizon, and at higher latitudes, the sun is close to the horizon for a longer period each year than at lower latitudes. Since our UV measure reflects noontime UVB exposure, it is inversely related to latitude and may result in the observed inverse association between UV and choroid. UV was not associated with conjunctiva for any race or ethnicity. In the largest population-based study of UV and incidence of three major anatomical sites of OM, our results suggest that direct UVR exposure is a risk factor of OM of the ciliary body/iris in NHWs, but not other races/ethnicities. Future studies should incorporate measures of UV exposure during morning and evening hours to confirm our findings for choroid.

Jayshree Advani
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NEI
Epigenetics
Integration of human retinal DNA methylome with genetic variants and gene expression identifies regulatory epigenetic mechanisms in age-related macular degeneration
Age-related macular degeneration (AMD) is a multifactorial neurodegenerative disease and a major

cause of blindness in elderly across the globe. Genome-wide association studies (GWAS) have identified 52 genetic variants at 34 AMD susceptibility loci. Previous retinal eQTL and TWAS analyses have uncovered several candidate genes in AMD GWAS loci. Complex traits, such as AMD, are the product of interaction between environmental and genetic factors. Epigenetic mechanisms and their genetic regulation can help dissect the impact of aging and environment on disease onset and severity. In this study, we have defined inter-relationships among gene expression, genetic variability, and DNA methylation (DNAm) in the human retina and investigated their potential causal effect on AMD. To achieve this goal, we generated DNAm profiles using MethylationEPIC chip from 159 post-mortem human retina, in addition to obtaining their genotypes and transcriptome. We mapped the genetic effects in cis(+/-1Mb) on DNAm of CpG sites (37,453 methylation quantitative trait loci, meQTLs) and gene expression (12,505 expression quantitative trait loci, eQTLs). Gene ontology analysis of mGenes showed enrichment of processes such as regulation of GTPase activity and synaptic signaling. Applying colocalization analyses with eCAVIAR and Summary-data-based Mendelian Randomization (SMR) between meQTL and eQTL or AMD GWAS signals uncovered meQTLs that potentially impact 14 disease associated loci, including meQTLs mapped to CFH, ARMS2/HTRA1, or LINC01004. We also identified over 5,000 eQTL-meQTL and meQTL-eQTL associations that may mediate methylation or gene expression and confirmed their target genes by combining with the retina Hi-C data. Furthermore, integration of expression with methylation identified 13,747 expression quantitative trait methylation (eQTMs). eQTM target genes were enriched in oxidative phosphorylation and electron transport chain. Our results contribute to a better understanding of genetic regulation, DNAm in the retina and their relationship with both transcriptome and AMD. The high correlation of genetic effects between methylation and gene expression highlights the target genes that may mediate the effect of aging and/or environmental factors on AMD. Future incorporation of age, environment, or lifestyle factors, such as smoking, to meQTL and eQTM detection, can potentially augment understanding of complex interplays among divergent factors contributing to AMD pathology.

Meagan Jezek
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NCI-CCR
Epigenetics

Characterization of cell type-specific enhancers in the human pancreas

The pancreas is a vital organ composed of multiple cell types with distinct functions. Accurate control of cell identity is integral to maintaining healthy, functional systems, and deviations from this regulation can lead to developmental defects and diseases such as pancreas cancer and diabetes. Therefore, it is crucial to understand the regulatory mechanisms that drive cell type-specific functions. Enhancers are non-coding genetic elements that regulate transcription and are overwhelmingly responsible for cell type-specific gene expression. Additionally, over 80% of disease-associated genetic variants map to enhancer regions, emphasizing the need to understand their function. While hundreds of thousands of candidate enhancer regions have been identified based on enhancer-associated chromatin features, functional characterization of enhancers and linking enhancers to their gene targets has been a daunting challenge, particularly at genome-scale. In our prior work, we identified genomic enhancer elements unique to human pancreas cell types and computational analyses revealed that these regions are likely bound by cell type-specific transcription factors. This supports the hypothesis that these regions act as

cell type-specific enhancers to direct distinct cell fates and functions. To systematically characterize the drivers of cell identity among the unique pancreas cell types, we are using a novel massively parallel reporter assay to quantify enhancer activity. We have cloned thousands of candidate enhancers into lentiviral reporter plasmid libraries and are currently assessing the enhancer activity of these elements in primary pancreas cells obtained from healthy donors. To complement enhancer-reporter findings, we will be perturbing select active enhancers using a CRISPR-inhibition system to measure the impact of enhancer perturbations on target gene transcription at a cell type-specific resolution. Thus far, most enhancer assays have been performed in mouse models or human cell lines. While these models have been instructive, using primary human tissue will provide a more biologically relevant context. The results from this study will yield the first functional enhancer map of human pancreas cells and advance our understanding of how these enhancers orchestrate the remarkable cell type-specific gene expression programs in human pancreas cells.

Tara Doucet-O'Hare
Research Fellow
NCI-CCR
Epigenetics

Human Endogenous Retroviruses expression due to Defects in Chromatin Remodeling leads to Clear Cell Meningioma

Clear cell meningioma (CCM), a malignant tumor with a bimodal age distribution, is characterized by altered gene expression due to defects in SMARCE1, part of the chromatin remodeling complex. We recently discovered that a similar defect in SMARCB1 led to aberrant expression of HML-2 human endogenous retrovirus K (HERV-K) envelope protein (env), and maintenance of pluripotency critical for tumorigenesis in atypical teratoid rhabdoid tumor (AT/RT). Expression of HERVs, remnants of exogenous retroviruses which integrated into the germline of humans more than ten thousand years ago, is critical for early development of tissues which includes formation of syncytia or multinucleated cells; however, it is typically downregulated in mature differentiated tissues. Many HERVs have maintained open reading frames for viral proteins which are expressed early in placental and human embryo development. We hypothesized that either continued expression or reactivation of HERV expression due to defects in chromatin remodeling acquired early in development is critical to tumorigenesis in both CCM and AT/RT. We found increased expression of HERVs in multi-nucleated syncytial tumor initiating cells in CCM samples (n=8) and in neural stem cells with knock-down of SMARCE1. We observed expression of OCT4, a marker of cell stemness, significantly correlated with HML-2 env expression (p<0.033, Pearson’s R=0.7461, n=8). In addition, we found expression of CD63, a marker for exosomes, in the vesicles expressing HML-2 env, in all samples tested (n=8). Finally, we observed transcription and protein expression of both Syncytin-1 (n=8) and Syncytin-2 (n=8), genes of retroviral origin which, in addition to HML-2 env, facilitate formation of multinucleated cells. These findings suggest either the aberrant persistent expression or reactivation of HERVs due to SMARCE1 loss may be a critical step in oncogenic transformation in early developmental tumors with chromatin remodeling defects. Biallelic loss of SMARCB1, another chromatin remodeling protein, can lead to different tumors depending on the timing and cell lineage in which it occurs. Due to the bimodal distribution in age of CCM patients and our findings, we propose that both persistent activation or reactivation of HERVs may be critical for tumorigenesis in tumors with chromatin remodeling defects.

Arun Prasath Damodaran
Visiting Fellow
NCI-CCR
Gene Expression - Postranscriptional Regulation

Exon-resolution functional genomics uncovers critical protein regions for cell fitness

An integral step in mRNA maturation is splicing, during which non-coding intronic sequences are removed and exons are joined together. Through the process of alternative splicing certain exons can be either included or excluded from the mRNA in a context-dependent manner. Alternative splicing contributes to transcriptomic and proteomic complexity and diversifies cellular functions. Although CRISPR-Cas technology has revolutionized genetics, the role of individual exons in critical cellular phenotypes has been largely overlooked. This oversight hampers our complete understanding of genome regulation and limits the development of novel RNA-based therapeutics targeting individual exons. To enable exon-resolution functional genomics, we previously developed CHyMErA, a CRISPR-based screening platform for combinatorial genetic perturbations. CHyMErA employs co-expression of Cas9 and Cas12a nucleases and libraries of Cas9 and Cas12a guide RNA fusions. This technology is promising yet primitive due to limited Cas12a activity. After screening multiple Cas12a variants, we have now developed an enhanced CHyMErA screening tool with substantially increased exon deletion activity. Using this enhanced tool, we studied the impact of over 10,000 frame-preserving exons on cell fitness in human cells. Our screens revealed that ~20% of the interrogated exons affect cell fitness and are often present in highly expressed and essential genes. The essential exons frequently overlap protein domains and interaction interfaces and are rare in low-complexity regions. Among the identified hits, an alternative exon (exon8) in TAF5 overlaps a WD40 repeat and strongly affects cell fitness. TAF5 is a subunit of the general transcription initiation factor TFIID, which is critical for RNA polymerase II preinitiation complex formation and transcription initiation. Proteomic and biochemical analyses revealed that TAF5 exon8 functions as a switch to control the TFIID complex assembly and activity, the latter was confirmed by transcriptomic analyses. Future studies are directed at exploring the cell fitness properties of other exon hits from our screen. In summary, we have developed and applied an ultra-efficient exon deletion screening platform for interrogating phenotypically important exons at the genome-scale, revealing novel mechanisms that control gene expression and cell fitness.

Bruna Rodrigues Muys
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NCI-CCR
Gene Expression - Postranscriptional Regulation

RNA-binding protein RBM47 functions as a key regulator of alternative splicing in colorectal cancer

Although most RBPs are ubiquitously expressed, some are expressed in a tissue-specific manner indicating important context-dependent functions. We have found that human RBM47 (RNA-binding motif protein 47) is expressed in a tissue-specific manner, with robust expression in the colon. In human colorectal cancer (CRC), RBM47 is significantly downregulated and high RBM47 expression is associated with significantly improved prognosis suggesting tumor suppressor functions. We comprehensively identified the transcriptome-wide RNAs directly bound by RBM47 in crosslinked cells using PAR-CLIP (PhotoActivatable Ribonucleoside-enhanced CrossLinking and ImmunoPrecipitation) and the mRNAs up-

or downregulated upon RBM47 knockdown by performing RNA-seq from two CRC cells lines DLD1 and LS174T. PAR-CLIP analysis showed that RBM47 mostly binds to intronic (~50%) regions of pre-mRNAs. Integration of PAR-CLIP and RNA-seq data showed that RBM47-bound mRNAs tend to be upregulated after RBM47 knockdown, and this tendency is increased with the increment of binding sites on the targets. Due to the observed extensive RBM47 binding to intronic regions of RNAs, we hypothesized that RBM47 regulates alternative splicing. Indeed, we found that RBM47 is mostly involved in cassette exons events (~80%) and these events occur predominantly near RBM47 binding sites. Among the top RBM47 targets we focused on CTNND1 (Catenin delta 1), a gene that encodes a protein important for cell adhesion and signal transduction. The rationale for focusing on CTNND1 was that it showed one of the highest values of difference of Percentage of Splicing In (PSI) upon RBM47 knockdown and contains RBM47 binding sites around the alternatively spliced exon 21. We validated the exclusion of exon 21 in CTNND1 after RBM47 knockdown by semi-quantitative RT-PCR and immunoblotting. Analysis of TCGA data indicates that the CTNND1 isoform lacking exon 21 is highly expressed in CRC tumors compared to normal tissues and the contrary occurs for the exon-included isoform. In addition, the exon-included isoform expression correlates with RBM47, but the exon-skipped isoform negative correlates with RBM47 in CRC tumors. Collectively, these unpublished data reveal the molecular mechanism(s) by which RBM47 functions in CRC and uncover a key role of RBM47 as a regulator of alternative splicing in CRC.

Allison Mitchell
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NCI-CCR
Gene Expression - Transcriptional Regulation

Investigating the lineage-specific roles of the Forkhead box (FOX) family in cancer

The Forkhead box (FOX) superfamily is the largest transcription factor (TF) family in the human genome whose members perform critical functions in development and adult tissue homeostasis. Many FOX family members have been observed to drive oncogenesis and tumor progression and could serve as potential therapeutic targets. However, other FOX members are reported to perform tumor suppressor functions or display pro- or anti-tumorigenic roles dependent on the biological context. Further adding to this complexity, some FOX TFs have been found to act cooperatively or antagonistically with one another. Therefore, we sought to uncover the biological contexts that govern the role of individual FOX members in cancer through a comprehensive genomic analysis of cancer cell lines and patient tumor samples. We analyzed CRISPR-Cas9 fitness screening data from 558 human cancer cell lines spanning 19 lineages to determine the factors influencing cell dependency for each of the 50 FOX genes. In addition, we analyzed the gene expression profiles of cancer cell lines and patient tumor samples to elucidate the patterns of FOX gene co-expression and mutual exclusivity and relationships to patient outcomes. We observed that several cancer lineages were selectively dependent upon an individual member of the FOX family for cell fitness. Several of these findings were corroborated by patient data in which lineage dependency corresponded to an association between gene expression and overall survival in patients of the matching cancer type. We also observed several examples of potential FOX gene cooperativity. For example, cell lines derived from soft tissue displayed selective dependency on FOXO1 and FOXF1 for cell fitness. In sarcoma patients, this corresponded to an association between gene expression and survival with patients that displayed elevated expression of both FOXO1 and FOXF1 exhibiting the worst survival outcomes. These findings support the use of genomic and functional screening data to dissect the role

of FOX proteins across cancer types and the regulatory relationships within the family. In addition, this work will lay the foundation for prioritizing the contexts in which individual FOX family members may be most effectively studied and targeted to improve patient outcomes.

Kaustubh Wagh
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Gene Expression - Transcriptional Regulation
Chromatin and chromatin-bound transcriptional regulators switch between two low-mobility states at the single molecule level
Transcription is a tightly regulated process where multiple proteins act in concert to activate appropriate gene expression programs. Transcription factors (TFs) are key players in this process, with TF binding being the first step in the recruitment of the transcriptional machinery. TFs have two essential characteristics: the ability to (1) bind specific DNA sequences and (2) regulate transcription. TFs play a central role in numerous biological processes such as development, metabolism, and cellular homeostasis by activating appropriate gene expression programs. Conversely, dysregulation of TF function can lead to pathological conditions such as diabetes, cancer, autoimmune conditions, cardiovascular disease, and neurological disorders. Yet, how TFs navigate the complex nuclear microenvironment to rapidly and specifically assemble at appropriate genomic regions remains poorly understood. Recent technological advances have enabled us to follow single TF molecules within live cells. Most TFs have been shown to exhibit power law distributed dwell times on chromatin, arising from a broad distribution of binding affinities within the nucleus. This blurs the line between specific and non-specific binding, rendering it impossible to distinguish between different binding modes based on dwell times alone. In this study, we combine single molecule tracking with a machine learning-based algorithm to identify two distinct low-mobility states for chromatin (marked by histone H2B) and ten chromatin-bound transcriptional regulators within the nucleus. Ligand activation results in a dramatic increase in the propensity of steroid receptors to bind in the lowest mobility state. Mutational analysis revealed that only chromatin interactions in the lowest mobility state require an intact DNA-binding domain as well as oligomerization domains. Importantly, these states are not spatially separated as previously believed but in fact, individual H2B and chromatin-bound TF molecules can dynamically switch between them. Single molecules presenting different mobilities exhibit different dwell time distributions, suggesting that the mobility of TFs is intimately coupled with their temporal dynamics. This provides a way to identify different binding modes that cannot be detected by measuring dwell times alone. Together, these results identify two unique and distinct low-mobility states of chromatin that represent common pathways for transcription activation in mammalian cells.

Pelin Yasar
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NIEHS
Gene Expression - Transcriptional Regulation
Establishing Estrogen Receptor α Enriched Mouse Mammary Organoids to Investigate Transcriptional

Dynamics and Heterogeneity

Breast cancer is a heterogeneous disease with various subtypes, characterized by differences in gene expression, prognosis, and response to therapy. Approximately 80% of breast cancer patients have tumors that express estrogen receptor alpha (ERα) and rely on estrogen signaling for their growth and survival. However, the transcriptional response to estradiol is heterogeneous in the cell population. This expression heterogeneity is a hallmark of cancer and is considered a significant challenge in the treatment of breast cancer due to its crucial role in the development and progression of the disease. While the genetic aspects of transcriptional heterogeneity have been extensively studied, the mechanisms underlying non-genetic heterogeneity remain largely unknown. Recent studies focused on the transcriptional response to hormone in single cells have primarily used immortalized cell lines. However, cell lines can undergo genetic drift or accumulate genetic aberrations during prolonged culture. Additionally, cell lines are typically cultured in a simplified 2D environment, which does not fully capture the complexity of the tissue microenvironment. Here we developed ERα positive mouse mammary organoids as a complementary model system to study transcriptional heterogeneity. Using a transgenic strain which expresses ZsGreen reporter in ERα-expressing cells, I enriched for ERα positive progenitor cells and established organoids. These organoids form acinar-type structures with lumen, proliferate and express the ERα. Importantly, treatment with estradiol induces well known estrogen-responsive target genes after 5 days of stimulation. Moreover, chromatin profiling by CUT&Tag shows significant enrichment of active transcription mark, H3K27ac at well-known estrogen responsive promoters and nearby enhancers. I am working to integrate single cell fluorescence in situ hybridization and live cell MS2 imaging to characterize transcriptional dynamics of estrogen responsive genes in organoids. Our study aims to build a tractable system for investigating ERα-mediated transcriptional heterogeneity by enriching for ERα-expressing cells. This approach will facilitate mechanistic dissection of estrogen signaling dynamics, leading to the development of more precise therapeutic strategies for the treatment of the disease.

Shreeta Chakraborty
Visiting Fellow
NICHD

Gene Expression - Transcriptional Regulation

Robust enhancer-promoter interactions ensure faithful gene regulation during embryonic development independently of CTCF

CTCF-mediated chromatin loops play a crucial role in facilitating interactions between distal genomic regions. These loops have also been proposed to insulate enhancers from contacting with promoters in neighboring domains to prevent ectopic gene activation. However, the in vivo significance of this model has not been thoroughly tested. To explore the significance of CTCF as a boundary during early mammalian development we targeted a 5Kb region containing 2 CTCF motifs between Fgf3 and Fgf4 using CRISPR in mouse zygotes. These neighboring genes located in separate 3D domains have a critical function in early cell fate specification to determine how cells of the inner cell mass in blastocysts differentiate into the epiblast (Epi) and the extra-embryonic primitive endoderm (PrE). To visualize changes in chromatin structure we established ES cell lines representing Epi from control and mutant littermate embryos and differentiated them into PrE-like cells (XEN) using Retinoic acid and Activin A. Using capture Hi-C, we saw that loss of the CTCT motifs between Fgf3 and Fgf4 caused the fusion of their

domains. RNA sequencing in our ES and XEN cell lines confirmed high expression of Fgf4 and Fgf3, respectively, which confirms their Epi and PrE-state. Interestingly, mutant ES cell clones had a significant increase in Fgf3 expression suggesting an aberrant contact with epiblast specific enhancers, in absence of CTCF. Consequently, there was a decrease in negative regulators of FGF signaling such as Spry4, Lefty2 and Foxd1. Furthermore, in mutant XEN cells, there were alterations in a few cell proliferation markers and chloride channel Clc genes, but Fgf4 was not significantly affected by the boundary loss. Surprisingly, despite perturbations in domain structure, and some changes in gene expression our mutant mouse line lacks any implantation defects and fetal development was not affected. Our study indicates that some tissue-specific enhancer-promoter interactions are highly resilient to disruption of domain architecture to tightly regulate correct spatio-temporal gene expression during cellular differentiation and organogenesis. Altogether, our findings suggest that the role of CTCF in mediating insulation of domain boundaries is not universal and emphasize the presence of independent alternative mechanisms and factors to maintain higher-order chromatin organization.

Marika Oksanen

Other

NIA

Genetics - Diseases

The mechanisms of HNRNPU-mediated RNA regulation in neuronal development and HNRNPU-related neurodevelopmental syndrome

Heterogenous Nuclear Ribonucleoprotein U (HNRNPU) is a DNA/RNA-binding protein with many functions, including regulation of transcription, alternative splicing, and mRNA stability. Pathogenic variants in the HNRNPU locus have been connected to neurodevelopmental disorders including developmental delay, intellectual disability, and early-onset epilepsy. Our previous work revealed delayed neurogenesis in HNRNPU-deficiency cell models. However, the mechanism of HNRNPU-mediated regulation of human neuronal development has not been studied in detail. Here, we examined the function of HNRNPU by identifying associated proteins and RNA targets during early neuronal development in order to discover the mechanisms through which HNRNPU regulates RNA splicing. To study early neuronal development, we used induced pluripotent stem cell (iPSC)-derived neuroepithelial stem cell lines, including one from a patient with a heterozygous deletion spanning the HNRNPU locus (HNRNPUdel/+), one neurotypical control (CTRL), and isogenic cells in which siRNAs were used to silence HNRNPU (siHNRNPU). HNRNPU immunoprecipitation (IP) analysis in CTRL cells was followed by mass spectrometry (IP-MS) and ribonucleoprotein immunoprecipitation was followed by RNA sequencing (RIP-seq) to identify HNRNPU-associated proteins and RNAs at the neural stem cell state (D0) and after neural differentiation (D28) in culture. IP-MS analysis revealed 177 proteins at D0 and 46 proteins at D28 as protein partners of HNRNPU. Among them, 16 out of 177 and 4 out of 46, are human splicing factors and 4 proteins were consistent binding partners at both time points. By gene ontology enrichment analysis, the identified proteins were related to translation (FDR=4.11x10^-77 and FDR=2.91x10^-28, at d0 and d28, respectively), mRNA stability (FDR=8.945x10^-14 and FDR=1.76x10^-9) and mRNA splicing (FDR=1.52x10^-7 and FDR=6.20x10^-3) among other terms. The RIP-seq results were compared with our previous data on differential splicing (DS). Only 2.4% of the earlier DS transcripts in the HNRNPU-deficient cells were identified as direct RNA targets by RIP-seq analysis, suggesting that splicing aberrations were indirect events due to dysregulation of HNRNPU. We are

currently investigating whether HNRNPU-deficiency disturbs splicing factor complexes and leads to an incorrect pool of mRNAs and delayed neurogenesis.

Sounak Sahu
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NCI-CCR
Genetics - Diseases
Reducing uncertainty in BRCA2 genetic testing with CRISPR-Cas9 based saturation genome editing.
Large-scale genetic sequencing has not been widely used as a clinical paradigm due to our limited ability to determine the functional impact of genetic variants. This has resulted in the identification of many variants of unknown clinical significance (VUS) in ClinVar, a clinical variant database, particularly in BReast CAncer gene BRCA2. Several functional assays have been developed to ascertain the impact of VUSs on protein function that can be used to determine their pathogenicity. Our mouse Embryonic Stem cell (mESC)-based method includes the generation of individual BRCA2 variants in a bacterial artificial chromosome (BAC) by recombineering and assessing their expression in mESCs. These cells can be used to determine how the variants affect mESC viability and sensitivity to chemotherapeutic drugs. Over the last decade, we have functionally classified around 400 BRCA2 VUSs reported in ClinVar. While this approach has proven to be highly reliable, it is time-consuming and resource intensive with limited potential to multiplex these variants. To overcome this shortcoming, we have developed a high-throughput approach to rapidly generate and examine the functional impact of variants by Next Generation Sequencing (NGS). We have generated a mESC line expressing a single copy of human BRCA2 that can rescue the lethality of Brca2-deficient mESC. Specifically, we utilized CRISPR-Cas9-based saturation genome editing (SGE) to efficiently knock-in targeted mutations in mESCs using a library of single-stranded DNA donors. We change each nucleotide position to all possible non-wild type nucleotides along exons 15 to 26 of BRCA2 that code for the C-terminal DNA binding domain (CTD), known to harbor most of the pathogenic variants. Since the loss of BRCA2 function leads to cell lethality, we observed a significant drop in the frequency for pathogenic variants of BRCA2, whereas functional variants are enriched in the pool. Using this NGS-based approach, we have classified all possible variants in CTD leading to around 7000 missense variants, that not only support the ClinVar classification but also assign a clinical significance to several VUSs for which no functional data is currently available. We also demonstrate that cell viability coupled with drug sensitivity assays can enhance the accuracy of classifying BRCA2 VUSs. We anticipate that SGE coupled with drug sensitivity assays can be applied to other clinically actionable genes, thereby reducing VUSs in ClinVar.

Stella Hartono
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Genetics - Diseases
SMAD4 Mutations in Myhre Syndrome lead to recurrent infections, hypogammaglobulinemia, and low memory T and B cells
SMAD4 is a critical downstream signaling molecule in the transforming growth factor-β (TGF-β) pathway.

Upon ligand binding, TGF-βR phosphorylates and activates receptor-associated Smads, which subsequently associate with SMAD4 to control target gene expression. More recently, studies in murine models lacking SMAD4 have shown a role for SMAD4 in mediating CD8 T cell function and proliferation independent of TGF-β signaling, leading to decreased responses to viral infections. Germline mutations in human SMAD4 (most commonly p. I500V) cause Myhre syndrome (MIM#139210), a rare autosomal dominant disease with cognitive impairment, hearing loss, and musculoskeletal anomalies. The immunological phenotype has not been previously described, despite the critical role for TGF-β and SMAD4 in regulating immune responses. We performed extensive clinical and laboratory evaluations of ten patients with Myhre syndrome (age 4–51 years), including measurement of serum immunoglobulins and vaccine titers. Flow cytometry immunophenotyping was obtained on 7 patients and 14 age- and gender-matched healthy controls. Statistical significance was determined by non-parametric testing (Mann-Whitney). Eight of 10 (80%) patients evaluated reported a history of recurrent ear infections while 3 (30%) patients had recurrent sinus infections and pneumonia. All patients exhibited characteristic thickened skin, 2 patients endorsed delayed wound healing, and 1 had recurrent warts. Four of 8 (50%) patients had low total IgG (344 +/- 83 mg/dL) and IgA (35 +/- 19 mg/dL) with normal IgM. Three of 10 patients (30%) had nonprotective titers to tetanus and varicella. Flow studies revealed normal total T and B lymphocyte frequencies, but low central memory (p=0.012) and effector memory (p=0.006) CD4+ T cells, low CD8+ T effector memory cells (p=0.042), low switched memory B cells (p=0.016), and low NKT cells (p=0.024) compared to matched healthy volunteers. In conclusion, Myhre syndrome patients exhibit an increased frequency of ear and respiratory infections associated with hypogammaglobulinemia and potentially an impaired ability to form memory T and B cells. Further studies are ongoing to elucidate the underlying molecular mechanism of these defects.

Yu Ishimoto
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NIDDK
Genetics - Diseases

A novel kidney organoid system based on mouse nephron progenitor cells to study Autosomal Dominant Polycystic Kidney Disease (ADPKD) cystogenesis

ADPKD is a common genetic disease, affecting 13 million people worldwide, and is the 4th leading cause of end-stage kidney disease in the US. It is characterized by progressive initiation and growth of multiple renal cysts in all nephron segments. Most cases of ADPKD result from mutations in PKD1, which encodes polycystin-1 (PC1). It has been 30 years since PC1 was first discovered, yet its function remains poorly understood. One major obstacle to its study is that there are no good in vitro models that accurately recapitulate cystogenesis. A human iPS cell-derived nephron organoid system has been reported, but the process takes >30 days, differentiation efficiency is variable with high inter-experiment variability, and cysts are incorrectly oriented, with their primary cilia outwardly facing rather than into the lumen. Nephron progenitor cells (NPC) are renal progenitor cells that give rise to most renal epithelial cell lineages and can be induced to develop nephron organoids within a week. However, no one has successfully generated organoids from human NPC, and the only published approach for mouse requires that NPC express a fluorescent marker which allows their isolation using flow cytometry. My project is to establish a mouse-based nephron organoid system that recapitulates Pkd1-mutant cystogeneis using NPC isolated by antibodies that detect endogenous kidney lineage differentiation markers. I sorted

Robo2-high/Pdgfrb-neg/Podocalyxin-neg NPCs from E13.5 embryonic kidneys using flow cytometry and showed that they could be cultured for >40 passages and still maintain their nephrogenic potential. I established 15 NPC lines from mice with germline null and floxed Pkd1 alleles and showed that all could be induced to form nephron organoids within a week. All kidney organoids derived from NPC with Pkd1 mutations (either germline or conditionally induced) spontaneously formed appropriately polarized cysts with primary cilia facing the cyst lumen when grown in suspension culture. This is the first ADPKD organoid system that spontanteously makes cysts with correct orientation and the first using mouse NPCs. Mouse NPC form cysts more quickly than human iPS cells, and results from this system can be readily confirmed in vivo. My methods can also be used to generate NPC from any other mouse model. This method will be a powerful new tool to reveal mechanisms of cystogenesis in ADPKD, and more generally, in the study of other developmental disorders of the kidney.

Batel Blechter
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Genetics - General
Multi-ancestry polygenic risk score for lung adenocarcinoma in East Asian never-smokers
Lung cancer among never-smokers is a significant global health burden and the incidence in East Asian (EAS) women is among the highest in the world. Polygenic risk scores (PRSs), representing cumulative genetic susceptibility, can stratify women by their risk of lung cancer and may guide personalized preventative and screening strategies; however, most efforts to date have focused on developing PRSs in European (EUR) individuals who are active smokers. We therefore aimed to develop and validate single-and multi-ancestry PRSs for lung adenocarcinoma (LUAD) in EAS never-smokers, using the largest available genome-wide association study (GWAS) dataset. We developed PRSs for LUAD risk in EAS women using single-ancestry PRS methods that only use GWAS data from EAS women and multi-ancestry PRS methods that use data from both EAS and EUR individuals. We developed the PRSs using summary statistics from 3 studies with 3,564 never-smoking cases and 16,238 controls of EAS ancestry, as well as a study with 2,058 never-smoking cases and 5,575 controls of EUR ancestry. The performance of the PRSs was assessed in the Female Lung Cancer Consortium in Asia, an independent study of 4,438 never-smoking EAS cases and 4,544 controls, by estimating the area under receiver operating characteristics curve (AUC). Further, 10-year and lifetime absolute risk of LUAD was estimated based on age-specific lung cancer incidence and overall mortality rates in Taiwan. The multi-ancestry PRS derived using empirical-Bayes estimation and super-learning approach (CT-SLEB) on over 2 million single nucleotide polymorphisms had the best predictive performance with an AUC of 0.643 (95% confidence interval (CI): 0.633, 0.655) compared to the single-ancestry PRS (AUC= 0.508). Compared to women in the middle quintile, those in the lowest and highest 5% of the PRS had 4.40- and 0.42-fold risk of developing LUAD, respectively. Further, the lifetime risk of LUAD for never-smoking women in the lowest and highest 5% of the PRS were 0.73% and 7.20%, respectively. The multi-ancestry PRS had the best predictive performance of LUAD in EAS never-smokers, suggesting the importance of expanding GWAS in diverse populations. The observed level of risk discrimination may inform primary and secondary prevention efforts, such as targeted screening. Future studies incorporating lifestyle and environmental factors with the PRS may achieve further discrimination and risk stratification for LUAD.

Jakub Jankowski
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NIDDK
Genetics - General
Genetic programming of the female kidney: a baseline of its own
Background Published kidney pathology and physiology data is heavily skewed towards male sex. Both chronic diseases, like diabetes, and acute kidney injury lead to worse outcomes and are more common in men. This, combined with female mice being more resilient to renal injury models and subsequent reluctance to use them in research, leads to underrepresentation of female kidney datasets. Additionally, little to no attention is given to the effects of pregnancy and lactation on renal genetic landscape, despite the likelihood of significant changes to the kidney gene expression profile, as it adjusts to changes in filtration rate, blood pressure and plasma osmolality. Methods I harvested kidneys from 12-14 weeks old C57/Bl6 mice in the following groups: male (M), female non-breeding (N), day 19 of pregnancy (P19) and day 10 of lactation (L10). I performed RNA-seq to investigate the baseline differences in gene expression and used GSEA to indicate significantly divergent renal metabolic pathways. I also used ChIP-seq (H3K27ac, H3K4me3, PolII) to dissect sex-dependent chromatin activation and putative enhancer locations in male and female mice. Results RNA-seq analysis shows 400 up- and 456 downregulated genes in female kidneys compared to male. Impacted GSEA hallmark gene sets include epithelial-mesenchymal transition, xenobiotic and fatty-acid metabolism, and complement pathways. The expression data correlates with histone modification peaks visualized at gene promoters through ChIP-seq analysis, which also indicates presence of putative sex-specific enhancer elements located close to deregulated genes like Acsm3 and Cyp2j13. While 21 and 22 genes were differentially expressed between groups N and P19, and P19 and L10 respectively, 122 genes were deregulated between N and L10, suggesting long-term effects of pregnancy on the kidneys. Those effects involved cell proliferation, as mitotic pathway assembly and G2M checkpoint genes were among the most affected. Conclusions There is a need for increased awareness of the differences between male and female renal genetics in research. Recognition that sex and pregnancy have significant effect on transcriptional and epigenetic landscapes might lead towards an explanation for the increased female resistance to kidney injury. Planned incorporation of scRNA-seq and detailed ChIP-seq analysis will further bolster my findings, especially regarding the lasting changes caused by pregnancy.

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CC
Heart, Lung, and Vascular Disease and Biology
Endothelial Senescence in Acute and Late COVID-19 Vasculopathy
Manifestations of severe Coronavirus disease-2019 (COVID-19) include acute respiratory distress syndrome, cardiovascular instability, and thrombotic angiopathy involving multiple organ systems. The cardiovascular complications associated with COVID-19 indicate persistent endothelial injury with disruption of barrier and antithrombotic functions. The vascular manifestations of acute and long COVID-19 may be mediated by virus-induced endothelial cell (EC) senescence, an inflammatory, prothrombotic cellular phenotype with therapeutic implications. EC senescence may be due to direct

infection of endothelium or exposure to circulating viral proteins, including SARS-CoV2 spike protein (S1), which persists in the blood of patients with severe COVID-19 for weeks. We observed that human pulmonary arterial ECs (PAECs) exposed to recombinant S1 alone and SARS-CoV-2 nucleocapsid protein (NP) develop a dose-dependent senescent phenotype, as exhibited by senescence-associated β-galactosidase (SA-β-Gal) activity, reduced proliferation, and apoptosis resistance. Whereas influenza A (H1N1) hemagglutinin and nucleoproteins did not induce senescence in PAECs. S1 suppressed ACE2 and CD147 (SARS-CoV-2 entry receptors) expression in cultured PAECs. Similarly, siRNA silencing of either ACE2 and CD147 also induced senescence in these cells. Importantly, hospitalized COVID-19 patients had elevated circulating levels of senescence-associated secretory phenotype (SASP) markers, including GDF15, STC1, TIMP1, and SERPINE1 (PAI1). Finally, the senolytic agent, navitoclax (ABT263; BCL2 signaling inhibitor), or fostamatinib, a spleen tyrosine kinase inhibitor, reduced S1-induced senescence in PAECs. In conclusion, SARS-CoV-2 S1 spike protein reduced ACE2 and CD147 expression and induced a senescent phenotype in human endothelium. Endothelial senescence may underlie some of the acute and chronic vascular complications of COVID-19.

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Heart, Lung, and Vascular Disease and Biology

Novel mechanism to explain how the heartbeats are generated: Double autonomic receptor blockade of ex-vivo sinoatrial node impairs summation of heterogeneous local calcium signals to alter the sinoatrial node's rhythm

Introduction We recently discovered that mammalian sinoatrial (SAN) tissue cytoarchitecture bears a striking resemblance to that of the central nervous system and that spontaneous oscillatory calcium signals that are heterogeneous in size, amplitude, and frequency occur within and among the pacemaker cells - the process of HOW local calcium oscillations organize (summate) to initiate action potentials (APs) and the heartbeat within SAN tissue is not well understood. Aim As far as we are aware, this is the first study in the world in live mammal heart tissue where we tested the hypothesis that neurotransmitter release is involved in the summation of local oscillatory calcium releases within and among SAN cells. Methods We developed a novel high-resolution confocal imaging technique for live tissue and a novel analysis algorithm to detect the occurrence of LCRs and AP-induced Ca2+ transients (APCTs) in individual pixels across the entire mouse SAN images. Double autonomic blockade (DAB) was applied to the tissue. Results Prior to (DAB), very rhythmic low zoom AP-induced Ca2+ transients that emanated from within the SAN tissue occurred at a mean frequency of 6.7+/-0.4Hz (mean+/-sd). There is a time-dependent reduction noted as early as 3 to 4 minutes in the mean frequency of APCTs from 6.7+/-0.4Hz in control to 0.62+/-0.6 Hz at 3-4min and to 0.52+/-0.6 Hz at 20min of DAB. The variability between APCTs also became markedly increased from 5.6% in control to 45.2% and to 63.4% at 3-4min and 20min of DAB application. Small amplitude Ca2+ oscillations that were heterogeneous in shape emerged during pauses between APCTs. The frequency of these low amplitude oscillations was higher than the mean APCTs in control and changed minimally throughout the 20min (mean frequency 9.4-8.6Hz) over the time in which the frequency of APCTs became progressively slower and more arrhythmic. Conclusion Neurotransmitter release from nerve endings within the SAN is crucial to the generation of high frequency APs that emanate from the SAN to generate the heartbeat. Because

summation of local oscillatory Ca2+ releases are linked to the generation of APs, the results suggest a new paradigm - that autonomic neurotransmitter signaling is linked to summation of local oscillatory Ca2+ signals occurring within and among SAN cells. Further studies are required to determine which cell types express receptors that are impacted by autonomic neurotransmitters and DAB.

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Heart, Lung, and Vascular Disease and Biology

Perivascular expression of neuronal β-III Tubulin (Tuj1) leads to diminishing the severity of pulmonary fibrosis

RATIONALE: Idiopathic pulmonary fibrosis (IPF) is a devastating disease with a poor prognosis. Despite the advances in basic and clinical studies on IPF, effective treatment options for IPF patients are limited, and an improved understanding of the pathological mechanisms promoting IPF is crucial. We here discovered a unique expression of neuronal ß-III tubulin (Tuj1), a known pan-neuronal marker, as a potential target in pulmonary fibrosis. Our surprising discovery of Tuj1-expressing pericytes in the bleomycin-induced fibrotic lung tissues prompted us to pursue the functions of Tuj1 and Tuj1-expressing pericytes in pulmonary fibrosis. METHODS: Tuj1 expression in the fibrotic lung tissue was examined using 1) a series of spatial and temporal imaging experiments and 2) scRNA-seq analysis of the bleomycin-induced mouse lung fibrosis model. The origin of Tuj1-expressing cells was examined using a lineage-tracing experiment with cell-type specific Cre drivers. The role of Tuj1 in lung fibrogenesis was investigated by the phenotypic analysis of Tuj1 (Tubb3) knockout mice. RESULTS: Tuj1-expressing cells with highly branched morphology emerged in response to bleomycin injury. They were preferentially located in the fibrotic area where collagen type I was aberrantly deposited. Time course analysis revealed that Tuj1-expressing cells appear during the fibrotic phase (14 days after bleomycin treatment). scRNA-seq analysis and immunofluorescence imaging identified that the Tuj1-expressing cells belong to pericytes. Our lineage-tracing experiments using the pericyte-specific CreER mice (PDGFRß-CreER or NG2-CreER) and reporter mice (Rosa-LSL-YFP) confirmed that Tuj1-expressing cells are derived from pericytes. We also found Tuj1-expressing pericytes in human IPF tissues. Tubb3 knockout mice exhibited enhanced lung fibrosis without a significant neuronal abnormality after bleomycin-induced lung injury, suggesting that Tuj1-expressing pericytes function as a negative regulator to suppress lung fibrosis. CONCLUSIONS: Perivascular Tuj1 expression is a new biomarker in lung fibrosis in mice and humans. Moreover, Tuj1-expressing pericytes are directly or indirectly involved in suppressing the severity of lung fibrosis. This study will provide a potential clue for developing a novel therapeutic strategy targeting the Tuj1-expressing pericytes in the fibrotic lung vasculature.

Hana Veler
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HIV and AIDS Research

Antagonism of viral glycoproteins by guanylate binding protein 5

Guanylate binding protein (GBP) 5 is an interferon-inducible cellular factor with a broad antiviral activity, reducing the infectivity of progeny virions by interfering with processing and incorporation of viral glycoproteins. GBP5 is believed to inhibit the infectivity of viruses such as HIV-1, highly pathogenic influenza A, and dengue virus by reducing the proteolytic activity of furin. However, the exact mechanism by which GBP5 inhibits processing of viral glycoproteins and whether it only affects furin-dependent glycoproteins remains poorly understood. HIV-1 Δenv luciferase reporter viruses were pseudotyped with either HIV-1 envelope glycoprotein (Env), MLV Env, vesicular stomatitis virus G glycoprotein (VSV G), SARS-CoV spike (S) or SARS-CoV-2 S glycoprotein and the effect of producer-cell GBP5 expression on particle infectivity was determined. GBP5 was found to reduce the infectivity of particles bearing each of these viral glycoproteins in a concentration-dependent manner. Western blot analysis demonstrated that GBP5 causes a dose-dependent shift in the electrophoretic mobility of the viral glycoproteins in cellular lysates. Moreover, GBP5 strongly reduced glycoprotein incorporation into virions while increasing virion-associated levels of the immature glycoprotein precursors. To determine whether the change in electrophoretic mobility of the glycoproteins is due to altered glycosylation, cell-associated lysates were treated with peptide N-glycosidase F (PNGase F), which removes N-linked oligosaccharides from glycoproteins. PNGase F treatment abolished the GBP5-dependent shift in the electrophoretic mobility of the glycoproteins, indicating that GBP5 indeed affects N-linked protein glycosylation and glycan modification. These data confirm that GBP5 impairs viral infectivity by interfering with glycoprotein function. Moreover, our work provides evidence that GBP5 not only inhibits furin cleavage of viral glycoproteins but also affects protein glycosylation of both furin-dependent (e.g., HIV-1 and MLV Env and SARS-CoV-2 S) and furin-independent (e.g., SARS-CoV S and VSV G) viral glycoproteins. Furthermore, our data on VSV G indicate that GBP5 targets the glycosylation of proteins other than class I fusion proteins. These results provide novel insights into the broad antagonism of viral glycoprotein function by the cellular host innate immune response.

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HIV and AIDS Research

Molecular interactions between multiple FG motifs of NUP98 and HIV-1 capsid that facilitate nuclear import

We recently reported that intact HIV-1 cores are imported through nuclear pore complexes (NPCs) and uncoat in the nucleus near sites of integration. Interactions between the viral core and several NPC proteins, including NUP358, NUP153, and others are critical for HIV-1 nuclear import. Interactions between the HIV-1 core and single FG motifs in host cleavage and polyadenylation specificity factor 6 (CPSF6) and NUP153 are essential for facilitating viral core nuclear import. Despite these advances, how an intact HIV-1 core navigates through the NPC into the nucleus remains a poorly understood yet critical step during HIV-1 replication. To identify host factors that modulate HIV-1 core nuclear import, we performed a high-throughput imaging-based siRNA screen of nuclear envelope associated genes and nuclear epigenetic factors. After siRNA knockdown, HeLa cells were infected with HIV-1 labeled with APOBEC3F fused to mNeonGreen fluorescent protein, and viral core nuclear import events were imaged using high-throughput confocal microscopy. A custom MATLAB image analysis pipeline was utilized to determine the efficiency of nuclear import. Subsequent siRNA knockdown of candidate hits showed that

NUP98 depletion resulted in potent inhibition of HIV-1 nuclear import (>60%), indicating that it is a strong facilitator of HIV-1 nuclear import. Co-pelleting assays showed that NUP98 binds to HIV-1 capsid nanotubes. We sought to determine whether, similar to CPSF6 and NUP153, NUP98 interaction with HIV-1 cores is dependent on one or more of the 39 FG motifs in NUP98. To identify the NUP98 FG motifs that interact with HIV-1 cores, we established a TRIM5α fusion assay. First, three fragments of the N-terminal 500 amino acids of NUP98 encoding 17, 7, and 15 FG motifs were used to replace the SPRY motif of TRIM5α; all three fragments possessed FG motifs that bound to viral cores and inhibited virus infectivity. Further extensive mutational analyses identified 8, 3, and 9 FG motifs in the respective fragments that were essential for binding to viral cores. These studies show that, unlike the single FG motif-viral core interactions that facilitate CPSF6 and NUP153 binding, multiple FG motifs of NUP98 are required for capsid binding. These studies reveal new insights into how viral core interactions with NUP98 facilitate HIV-1 nuclear import; inhibition of HIV-1 core nuclear import could be potential new target for development of antiviral therapies.

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Immunology - Autoimmune
The ESCRT protein CHMP5 regulates tissue-intrinsic inflammation in skeletal muscle
Macrophages are a key immune cell population required for maintenance of tissue homeostasis through their ability to regulate tissue metabolism, immunity, and inflammation, and to promote repair after injury. In skeletal muscle, the coordinate activity of resident and monocyte-derived macrophages facilitates crucial crosstalk between satellite cells, fibroadipogenic precursors, and myofibers that drives regeneration of damaged muscles. Dysregulation of this crosstalk contributes to conditions such as myopathy, inefficient wound healing, and sarcopenia. Despite their central role in muscle homeostasis and regeneration, the tissue-intrinsic factors and mechanisms that coordinate the recruitment and activity of these macrophages is poorly understood. In this study, we investigated how skeletal muscle homeostasis is regulated by the endosomal-sorting complex required for transport (ESCRT) protein CHMP5, which has recently emerged as a regulator of mammalian tissue development. CHMP5 (charged multivesicular body protein 5) was initially characterized as a member of the ESCRT family of proteins that coordinate membrane scission events in eukaryotic cells. However, recent studies by us and others have revealed non-canonical roles for CHMP5 in development wherein CHMP5 promotes the stability of client proteins required for cellular differentiation and cell fate decisions in both hematopoietic and non-hematopoietic tissues. Using an in vitro model of myogenesis, we found that CHMP5 knockdown impaired the upregulation of key myogenic transcription factors including Myogenin and MyoD and impaired the differentiation of C2C12 myoblasts into myotubes. Furthermore, when wild-type bone marrow-derived macrophages were cocultured with CHMP5-knockdown C2C12 myoblasts, they failed to polarize into pro-regenerative macrophages. Transcriptomic analysis of CHMP5-knockdown myoblasts at various timepoints throughout differentiation revealed upregulation of interferon response-associated transcripts and downregulation of transcripts associated with muscle differentiation. In vivo, mice with muscle specific CHMP5 deletion (CHMP5-KO mice) showed increased pro-inflammatory macrophages in skeletal muscle. H&E staining of tissue sections from CHMP5-KO mice displayed pathologic changes

indicative of impaired regenerative capacity. These data together suggest a critical function for CHMP5 in dampening muscle-intrinsic inflammation and promoting muscle homeostasis.

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Immunology - Autoimmune
LRRK2 Kinase Function Regulates NLRC4 Inflammasome Activity and Thereby Modulates Intestinal Inflammation
The LRRK2 (Leucine-rich repeat kinase 2) gene bears multiple gain of function polymorphisms or mutations associated with increased risk of IBD and/or Parkinson's disease. It encodes a multidomain protein that interacts with and influences the function of several intra-cellular proteins, including those regulating immune responses. One such interaction, only recently described, involves its binding to and phosphorylation of NLRC4, the key component of the NLRC4 inflammasome. This LRRK2 function, however, has uncertain significance since it is not clear how NLRC4 phosphorylation affects NLRC4 inflammasome function. This study utilizes a specific, newly developed LRRK2 inhibitor, termed CS-82, to clarify this knowledge gap. In initial in vitro studies we found that inhibition of LRRK2 kinase activity by CS-82 inhibits both its binding to NLRC4 and the latter's phosphorylation upon activation of the NLRC4 inflammasome with needle protein, thus establishing that LRRK2 has an indispensable role as the kinase enabling NLRC4 phosphorylation. In further studies using THP-1 cells with ASC deletion or human PBMC-derived dendritic cells with shRNA ASC-knock-down we showed that ASC deficiency retards NLRC4 phosphorylation and inhibition of NLRC4 phosphorylation prevents ASC oligomerization; thus, while phosphorylation requires NLRC4 conformational change caused by association with ASC, lack of phosphorylation inhibits ASC function. Finally, we found that inhibition of NLRC4 phosphorylation by CS-82 has a major negative effect on NLRC4 inflammasome-mediated IL-1b cleavage and expression, but, surprisingly, is has only a minor effect on IL-18 cleavage and expression. This indicated that IL-1b expression requires ASC-caspase binding whereas IL-18 expression does not, the latter most likely due to ASC-independent caspase cleavage by NLRC4. In further studies, we found that systemic administration of an NLRC4 inflammasome activator (needle protein) caused increased intestinal permeability and that co-administration of inhibitor reversed the increased permeability. This correlated with the finding that LRRK2 inhibitor administration ameliorated the severity of DSS-colitis. These findings thus showed that inhibition of inflammasome production of IL-1b coupled with the preservation of production of IL-18 via LRRK2 inhibition blocks the potentially harmful effects of NLRC4 inflammasome activity on intestinal permeability during intestinal inflammation.

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Immunology - General
Adaptive immunity to ancient retroelements controls the skin tissue threshold of activation
The skin represents the body's largest and outermost barrier organ. As such, the skin is exposed to a

variety of environmental stimuli, including not only the microbiota, but also pollutants and detergents. A symbiotic dialogue between the microbiota and host is essential to promote protective immune responses in the skin, including wound healing. Integrated retroviral elements, or retrotransposons (RTNs), comprise up to 45% of the human genome. We found that encounter with commensal microbe reactivated RTNs, and that inhibition of RTN cDNA synthesis abrogated immune responses to the skin microbiota. However, the extent to which reactivation of RTNs contributes to protective immune responses in the context of environmental challenge remains unknown. Here, we hypothesize that RTNs may represent an abundant source of antigen, and that adaptive immune responses directed toward these antigens will confer barrier protection in response to environmental stress. We first established a model of sterile injury, where topical application of a mild detergent reactivated RTNs, and recruited CD8 T cells to the skin in an RTN-dependent manner. RNAseq analysis of RTN expression revealed Langerhans cells (LCs) have the most abundant RTN expression amongst skin antigen-presenting cells. Our work demonstrated that CD8 T cell responses to sterile injury were entirely dependent on LCs, supporting the idea that LCs could present RTN antigens to CD8 T cells. To identify RTN-specific T cells, we selected peptides that were uniquely expressed in LCs, but not in medullary thymic epithelial cells which mediate negative selection (the process of eliminating self-reactive T cells). In vitro recall identified several peptides which stimulated cytokine production from injury-elicited CD8 T cells, confirming the presence of RTN-reactive CD8 T cells in the skin. RNA-seq analysis revealed that injury-induced CD8 T cells express a strong immunoregulatory/ tissue repair signature, supporting the idea that adaptive immunity to RTNs may contribute to maintaining barrier integrity. Thus, tonic stress, such as that induced by mild detergent, is sufficient to promote adaptive immunity to defined antigens derived from RTNs. Our work also proposes that homeostatic immunity to ancient retroviruses promotes a class of adaptive response aimed at maintaining barrier integrity in response to environmental stressors.

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Immunology - General

Lineage-specific metabolic adaptation of ILC3 in the gut but not central lymphoid tissues requires transcription factor Tox2

Innate lymphoid cells (ILCs) are immune cells that lack specific antigen receptors but possess similar effector functions as T cells. Interestingly, ILCs and T cells express many of the same transcription factors. One such factor is Tox2, which is widely studied in T cells however its role in ILC lineages is unknown. Here, we report that Tox2 is highly expressed specifically in ILC3 in the gut. We generated germline Tox2-/- mice and found that they have reduced ILC3 numbers in gut; however, ILC3 in spleen, lymph node or thymus were intact. Depletion of Tox2 resulted in defective control of Citrobacter rodentium infection. We generated a conditional knock out allele of Tox2 (Tox2fl/fl) and crossed it with ERT2 Cre to ablate Tox2 from mature cells upon tamoxifen administration. Conditional ablation of Tox2 in ILC subsets of adult mice resulted in a significant reduction in gut ILC3, but mesenteric ILC3 numbers remained intact. This result suggested that Tox2 is important for persistence of mature gut ILC3. Next, we performed transcriptional profiling of adult ILC3 from gut and mesenteric lymph nodes in Tox2-/- mice. ILC3 from mesenteric lymph nodes of Tox2-/- mice were transcriptionally similar to their wild-type counterparts. However, gut ILC3 from Tox2-/- mice appeared transcriptionally distinct. Tox2-/- gut ILC3

showed reduced expression of Hexokinase 2 (Hk2), the first-rate limiting enzyme in glycolysis. Consistent with the requirement for hexokinases in glycolysis, Tox2-/- ILC3 displayed a decreased ability to utilize glycolysis for protein translation. Furthermore, supplementing Tox2-/- gut ILC3 with glucose did not rescue survival whereas the metabolite pyruvate and acetate, that is downstream of Hk2, partially rescued the survival of gut ILC3 from Tox2-/- mice both in vitro and in vivo. We assessed HIF-1α expression and observed higher HIF-1α expression in gut as compared to mesenteric lymph node. This suggested gut is more hypoxic than mesenteric lymph node. Similar to Tox2, Hk2 was upregulated in gut ILC3 as compared to mesenteric lymph node ILC3. Furthermore, we observed that hypoxia-mediated induction of Hk2 required Tox2 in ILC3, suggesting a mechanism by which ILC3 adjust to changing environments. Our results demonstrate distinct metabolic adaptations of ILC3 residing in different tissues, and they reveal the requirement for Tox2 in the enhanced dependence on glycolysis of gut ILC3

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Immunology - General
IL-10 promotes tissue-resident memory T cells in the respiratory tract during SARS-CoV-2 infection in rhesus macaques
T cells are thought to be important for control of SARS-CoV-2 infection, but little is known about the host factors that promote T cell responses in the respiratory tract. We used non-human primates to understand the regulation of host defense during mild SARS-CoV-2 infection. We first examined the kinetics of viral replication and host immune responses. Foci of inflammation detected with 8FDG-PET/CT imaging peaked within 2-3 days of infection. Single cell RNA sequencing revealed an early influx of IFN-activated inflammatory monocytes into the airways, which correlated with viral RNA loads. Antigen-specific T cell responses were first detected after inflammation and viral replication had resolved and were preferentially localized to the lower airways. We next examined the role of pro and anti-inflammatory cytokines, IFNg and IL-10, on immunity to SARS-CoV-2 infection by blocking these cytokines with monoclonal antibodies early during infection. IFNg blockade decreased lung inflammation but had no major impact on adaptive immune responses. In contrast, IL-10 blockade increased lung inflammation and enhanced accumulation of virus-specific T cells in the lower airways. IL-10 blockade also inhibited the differentiation of virus-specific T cells into airway CD69+CD103+ tissue resident memory cells (TRM). While virus-specific T cells were undetectable in the nasal mucosa, IL-10 blockade similarly reduced the frequency of bystander TRM cells in the nasal mucosa. Thus, in the setting of mild SARS-CoV-2 infection IL-10 has a key role in suppressing local lung inflammation and the accumulation of SARS-CoV-2-specific T cells in the lower airways, while also promoting TRM at respiratory mucosal surfaces.

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Immunology - General

Androgen signaling shapes the sexual differences of skin immunity by regulating ILC2 and dendritic cells
Sex dimorphism can lead to sex differences in disease susceptibility and is associated with vastly different clinical manifestations. For instance, numerous skin diseases have strong sex dimorphism with females more susceptible to autoimmune diseases and more resistant to infectious diseases and skin cancers. The determinants of such differences within the skin are still unclear. This study aims to investigate sex differences in the skin immune system and decipher the underlying regulatory factors. We found that female mice have a significantly higher baseline level of defined skin resident antigen-presenting cells (CD103+ dendritic cells (DCs)) and type 2 innate lymphoid cells (ILC2s) than males. Females have also enhanced responses to encounter with microbes and inflammatory triggers within the skin. Using complementary approaches, we found that sex differences within the skin were regulated by male sex hormones. ILC2s are the dominant population of ILCs in the skin, which rapidly respond to environmental stimuli to promote immunity or tissue homeostasis and play a critical role in coordinating innate and adaptive immune responses. Moreover, ILC2s are a major source of GM-CSF, a factor required to maintain CD103+ DC survival and homeostasis. ILC2s express a higher level of androgen receptor (AR) and female ILC2s have a more activating gene expression signature than ILC2s from males and express a higher level of GM-CSF. Further in mice deficient in ILC2 the DC network of the skin is profoundly dysregulated with the level of CD103 significantly reduced. Collectively, these results propose that androgen receptor signaling negatively regulates the level of skin resident ILC2s, thereby limiting the level of GM-CSF within the tissue and as such the level of DC. Our current results uncover a mechanism that explains sex differences within the skin and how a specialized subset of cells responses to sex hormones dictate the differential outcome for immunity and inflammatory responses in males and females.

Mohammad Mansoori
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Immunology - General
CD4+Foxp3+ Tregs suppress antigen-specific CD8+ T cells in vivo by generating a novel immune synapse.
Tregs suppress T cell responses in vivo and in vitro by using different mechanisms. Our studies have demonstrated that one mechanism utilized by antigen-specifc CD4+Foxp3+ Treg involves capture of their target peptide-MHC-II (pMHC-II) antigen from the dendritic cell (DC) cell surface thereby limiting antigen presentation. It remains unclear how this mechanism would result in suppression of CD8+ T cell responses. We have demonstrated that MHC-II restricted OT-II Tregs could suppress OT-I CD8+ T cells in vivo only when challenged with DC pulsed with both OVA323-339 and OVA257-264 Under these conditions,we found that OT-II Tregs could uptake pMHC-I complexes from DC, and could also form clusters with OT-I cells, thereby acting as antigen presenting cells (APC) for OT-I T cells. The amount of uptake of pMHC-I complexes is directly dependent on the strength of TCR-MHC-II interaction in vitro and OT-II Tregs could also uptake pMHC-I in vivo. When peptide-pulsed Treg were injected in vivo with OT-I T cells, they induced a partial response as manifest by induction of CD44, but lack of induction of CD25, and a deficient proliferative response. Taken together, MHC II-restricted Treg form a high avidity interaction with DC resulting in removal of both their target pMHC-II complex as well as pMHC-I complexes from DC expressing both peptides. Treg expressing pMHC-I complexes form a synapse with responder CD8+ T cells resulting in aberrant T cell activation.

Lanqi Gong
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Immunology - Immunotherapy

Integrated multiomics reveals CD70 as a metabolic switch for regulatory T cell-mediated immunotherapy resistance in head and neck cancer

Anti-PD-1 immunotherapy that unleashes anti-tumor immunity of CD8+ T cells has benefited numerous cancer patients. However, patients with solid tumors have strong in situ immunosuppressive signals that contribute to intrinsic and acquired resistance to PD-1 blockade, particularly in head and neck cancer (HNC). Recent clinical trials show that only 20-30% of HNC patients can benefit from anti-PD-1 treatments. Hence, there is an unmet clinical need to enhance the immunotherapy response by targeting immunosuppressive signals in tumors. Here, we establish a multi-center single-cell cohort, containing 357,206 cells from 50 patient samples, and find out that a high infiltration of regulatory T cells (Tregs) is the major source of immunosuppression. We integrate our single-cell data with Visium spatial transcriptomics data from additional 7 primary tumor tissues and confirm a strong spatial co-localization between HNC cells and Tregs in PD-1 resistant patients. Based on spatial cell-cell interaction analysis, we reveal that CD70+ HNC cells enhance the suppressive activity of proximal Tregs via interacting with surface CD27. At the tumor-immune interface, CD70-CD27 interaction is significantly enriched for immune evasion from CD8+ T cells. Functionally, CD70 blocking using cusatuzumab (a human anti-CD70 monoclonal antibody) reverts Treg suppressive activity on CD8+ T cells in antigen-specific co-culture assays. Lower Treg-secreted immunosuppressive factors, including IL-10, TGF-β, and cyclic adenosine monophosphate, revitalize T-cell proliferation and cytotoxicity against tumor cells. Anti-CD70 + anti-PD-1 combination therapy is systematically evaluated in patient-derived xenograft, organoids, and peripheral mononuclear cell-engrafted humanized mice, showing an enhanced pre-clinical efficacy and consistent safety profile compared to monotherapy. Mechanistically, mass spectrometry-based metabolomics and proteomics show that targeting CD70 inhibits the lipid signaling in Tregs involving mitochondrial oxidative phosphorylation, cholesterol and fatty acid metabolism, mediated by SREBF1/2. Overall, our findings identify CD70+ NHC cells as a metabolic switch that enforces the lipid-driven functional specialization and homeostasis of Tregs, leading to immune evasion and PD-1 resistance. Our study also prospectively demonstrates that CD70 blockade can act synergistically with PD-1 blockade to reinvigorate T-cell immunity against head and neck cancer.

MEIJIE Tian
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Immunology - Immunotherapy

FGFR4 and CD276 dual targeting CAR T cells demonstrate synergistic antitumor activity in childhood rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children. Patients with relapsed or refractory RMS have a dismal cure rate, and effective therapies are urgently needed. Despite their success for hematological malignancies, chimeric antigen receptor T cell (CAR T) therapies perform poorly in solid tumors partially due to heterogeneous expression of target antigens, limited T cell

persistence and exhaustion. We have generated a clinical grade CAR T targeting FGFR4 that is currently being developed for a clinical trial at the Pediatric Oncology Branch. This standard FGFR4-CAR with a CD8 hinge and transmembrane domain (HTM) and a 4-1BB co-stimulatory domain (CSD) demonstrated potency in low burden RMS disease but has a more modest effect for bulky disease in RMS intramuscular (I.M.) xenograft model. To improve the CAR efficacy, we replaced CD8 HTM of 4-1BB-based CAR with a CD28 HTM and found that the resulting CAR demonstrated enhanced activity while maintaining persistence. Moreover, substitution of 4-1BB CSD with that of CD28 outperformed 4-1BB CAR leading to rapid tumor eradication. Despite these optimization strategies, these modified FGFR4-CARs, even FGFR4.28HTM.28z CAR were unable to eradicate solid tumors in an aggressive RMS559 model due to its short persistence and increased exhaustion. Furthermore, we show that CAR T cells targeting another cell surface protein CD276, a direct target of the RMS core-regulatory transcription factor MYOD1, is potent and effective, but could not eradicate all tumors. Therefore, we engineered a bicistronic CAR (BiCisCAR) to target both FGFR4 and CD276, with CD28 and 4-1BB CSDs, respectively, which showed remarkable and rapid tumor eradication compared with either single CAR alone. The BiCisCAR also prevented tumor escape showing a persistent anti-tumor response in an RMS orthotropic model with heterogenous expression of antigens. Using cellular indexing of transcriptomes and epitopes by sequencing (CITE-Seq), the FGFR4-CD276 BiCisCAR using the two different CSDs demonstrated higher cytolytic activity and less exhaustion. Furthermore, they demonstrated more robust downstream signaling activation of AKT, ERK1/2 and p65, likely due to the synergistic effect of dual engagement of their cognate antigens and the utilization of two distinct CSDs. Thus, this BiCisCAR represents a promising and potent CAR that can be utilized against this aggressive RMS disease in future clinical trials.

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Immunology - Immunotherapy

Engineering “Fab”ulous CARs: Fab chimeric antigen receptors enhance T cell potency against solid tumors

Chimeric antigen receptor (CAR) T cell therapy has revolutionized the treatment of hematologic malignancies. However, its efficacy in solid tumors has been suboptimal, primarily due to T cell exhaustion and limited in vivo persistence. A potential cause of premature T cell exhaustion is tonic signaling (TS) caused by antigen-independent CAR aggregation on the cell surface that results from unnatural pairing of the heavy (VH) and light (VL) chains in the single chain variable fragment (scFv): the antigen binding motif of a CAR. This self-aggregation leads to chronic T cell activation in the absence of CAR antigen and is known to result in T cell exhaustion and poor in vivo efficacy. While reversing the VH–VL orientation, and mutations to scFv framework can sometimes reduce TS, these approaches are only partially effective. We hypothesized that replacing the scFv with a Fab for antigen recognition could alleviate TS by allowing stable pairing of VH–VL chains. To test this, we specifically selected CARs targeting disialoganglioside (GD2) and herceptin (HER2) which are known for their TS and poor efficacy. Using retroviral vectors to express these CARs in scFv or Fab format in human T cells, we compared their phenotype and function. While expansion and viability of scFv and Fab CAR T cells were similar for both GD2 and HER2 CARs, TS was observed only in scFv CAR T cells, as indicated by higher interferon gamma secretion without antigen stimulation. We also detected elevated expression of activation and

exhaustion markers CD25, CD69, PD-1, and TIM-3 in scFv CAR T cells. In co-culture experiments with human IMR5 (GD2+ neuroblastoma) and JIMT1 (HER2+ breast cancer) cells, GD2 and HER2 Fab CAR T cells showed superior tumor-killing in their respective models. Next, in preclinical xenograft studies, we compared the CARs for their activity against metastatic IMR5 or orthotopic JIMT1 tumors. In both models, scFv CARs only transiently slowed tumor growth, while Fab CARs rapidly regressed tumors. Live cell imaging in the JIMT1 model revealed more T cells in tumors of Fab CAR recipients, suggesting improved proliferation/persistence due to reduced TS. Remarkably, more recent data revealed that conversion to Fab format can even benefit CARs with no discernible TS by mechanisms yet to be understood. Taken together, our findings show that Fab CARs can drive potent anti-tumor responses against solid tumors with implications for widespread improvement of CAR T cell therapy.

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Immunology - Infectious Disease
PI3Kd: a key driver of effector differentiation under conditions of T cell exhaustion
In chronic infection and cancer, prolonged antigen exposure drives a state of T cell hyporesponsiveness, called T cell exhaustion. Exhausted T (Tex) cells are maintained by a small subset of TCF1+ stem-like progenitor Tex (pTex) cells, which are able to respond to checkpoint blockade. Understanding mechanisms regulating these populations may help identify approaches to counter exhaustion and maintain T cell function. Phosphatidylinositol 3-kinase-delta (PI3Kd) is a lipid kinase and an important component of T cell signaling and differentiation. PI3Kd activates the serine/threonine kinase AKT, which phosphorylates and inhibits the FoxO1 transcription factor. We have found that expression of TCF1, a FoxO1 target, is repressed in CD8+ T cells from patients with Activated PI3Kd Syndrome and a mouse model of activated PI3Kd (Pik3cdE1020K/+) in acute viral infections. However, how activated PI3Kd affects T cell exhaustion is unknown. To address this, I challenged mice with a chronic infection model, Lymphocytic Choriomeningitis Virus (LCMV) clone 13. Interestingly, half the Pik3cdE1020K/+ mice rapidly died, yet surviving mice recovered and cleared virus faster than wildtype (WT) mice. RNA sequencing revealed decreased expression of pTex associated genes, including the gene encoding TCF1, in antigen-specific CD8+ T cells; this was associated with loss of pTex cells in Pik3cdE1020K/+ mice. In contrast, Tex cells in Pik3cdE1020K/+ mice showed decreased levels of inhibitory receptors and increased cytokine production. Furthermore, I observed expansion of CX3CR1+ KLRG1+cells, with increased effector functions, suggesting these cells might induce early immunopathology yet increase viral clearance. Transfers of WT and Pik3cdE1020K/+ LCMV-specific P14 TCR transgenic CD8+ T cells

revealed that loss of TCF1+ pTex cells in Pik3cdE1020K/+ mice was cell intrinsic and could be recapitulated by CRISPR-mediated gene disruption of FoxO1. In contrast, the effector-like Tex cell expansion was partially cell extrinsic: transfer of WT P14 cells into a Pik3cdE1020K/+ host generated more effector-like Tex cells among transferred cells. This was prevented by use of depleting antibodies against CD4+ T cells and IL-21R. Together, these findings suggest that PI3Kd is a critical rheostat that balances effector versus exhausted CD8+ T cell differentiation, and may provide insight into therapeutic strategies for reinvigorating cells during exhaustion in cancer and chronic infection.

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Immunology - Infectious Disease
Sulfides mediate protection against gastrointestinal infection via alterations to local immunity and the microbiome
Sulfide is a gaseous molecule, which has toxic effects at high concentrations yet plays key roles in homeostasis throughout the body. Sulfides are produced endogenously by both host tissues and the bacterial cells of the gut microbiome, which results in the gut containing the highest concentrations of sulfide in the body. We sought to assess the role of the highly abundant sulfide molecule in gut homeostasis and its ability to promote resistance to enteric infections. Local sulfides can be depleted by the compound bismuth subsalicylate (BSS), a commonly used anti-diarrheal medication, which acts locally by sequestering sulfides in the gut. Depletion of gut sulfides resulted in significantly enhanced susceptibility to oral Salmonella infection, with a greater than 5 log increase in bacterial CFU 24 hours post infection. In addition to loss of pathogen resistance, treatment with BSS induced a significant impairment to local gut immunity. There was a 50% reduction in CD4+ T cells in the small intestine, which was especially pronounced in the terminal ileum, the main site of Salmonella invasion. Additionally, we observed significant alterations in the gut microbiome, with a loss of Lactobacillus species that are key competitors of Salmonella in the gut. Finally, we sought to take advantage of the protective capacity of sulfide by increasing gut sulfide concentration, supplementing mice with sodium hydrosulfide or dietary cysteine. These treatments were able to enhance protection against a lethal oral Salmonella infection. These data suggest that loss of gut sulfides reduces pathogen resistance via impairment of gut immunity and dysregulation of the beneficial microbiome. Mechanistically, our results support the idea that sulfide may be acting directly on both immune cells and local microbes by interfering with aerobic respiration. Notably, physiological sulfide concentration is associated with CD4 T cells reduced aerobic respiration capacity and mitochondrial membrane potential. Similarly, Salmonella

and other pathogens grew slower in microaerobic conditions in the presence of sulfide. Our results indicate that sulfides play a key role in homeostasis, and that management of gut sulfide levels may be a key component of gastrointestinal health.

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Immunology - Innate and Cell-mediated Host Defenses
T cell response in Aggregatibacter actinomycetemcomitans-associated periodontal disease
Periodontitis (PD) is a noncommunicable inflammatory condition, where severe disease affects approximately 9% of adults in the USA. The oral microbe Aggregatibacter actinomycetemcomitans (Aa) is associated with severe PD, however, the physiologic consequences of Aa recognition and subsequent immune response in PD is poorly understood. CD4+ helper T cells with active T cell receptor (TCR) engagement have been shown to be critical regulators of periodontitis pathogenesis in mouse models of PD. However, whether microbe specific CD4+ T cells mediate periodontal pathology requires further investigation. To understand the T cell response in Aa-associated periodontal disease, we utilized the mouse model of ligature-induced periodontitis and cultured ligatures with Aa (AaLIP). Using a combination of microcomputed tomography, flow cytometry/sorting, and in vitro T cell culture, we identify the importance of T cell response in AaLIP and generate T cell hybridomas (TcHyb) reactive to Aa. We found that AaLIP significantly increases alveolar bone loss compared with sterile ligature or no ligature, suggesting that Aa induces specific host responses that lead to pathogenic bone loss. We observed that mice deficient in recombinant activating gene 1, which lack mature T and B cells, are significantly protected from pathogenic bone loss associated with AaLIP compared to strain-matched wild-type animals, suggesting that T cells play an important role in AaLIP-induced bone loss. Additionally, AaLIP mice displayed lymphadenopathy in oral draining (cervical) lymph nodes (cLN) and an increase in the number of CCR6+ (a marker of mucosal tissue homing and proposed marker of antigen-specificity) CD4 T cells in the gingiva and cLN compared to controls, which indicates an antigen-specific response to bacterial pathogen. Sorted CCR6+ CD4 T cells isolated from the cLN of AaLIP (but not sterile ligature) treated mice expand only when exposed to Aa antigen, which occurs in antigen-specific T cells upon antigen recognition. TcHyb generated from T cells fused with TCRalpha/beta null BWZ.36 myeloma cells showed specific TCR activation in the context of Aa. These observations support the notion that Aa-specific T cell responses contribute to tissue destruction in AaLIP. Further characterization of Aa-specific TcHyb will enable the development of highly specific tools to study the role of Aa in periodontal disease.

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Immunology - Innate and Cell-mediated Host Defenses
Cell-Intrinsic Effects of TGF-b Signaling in Mast Cell Effector Function that Modulate Allergic Anaphylaxis
IgE mediated mast cell activation is a key feature of allergic disease, although the mechanisms governing mast cell homeostasis are not fully understood. The TGFb signaling pathway has been shown to regulate

mast cell development and effector function. Furthermore, variants in the TGFb signaling pathway are associated with allergic diseases; TGFb mediated mast cell regulation is likely involved in the allergy diathesis. Patients with Loeys Dietz Syndrome (LDS), a disorder caused by loss of function variants in TGFBR1 and TGFBR2, are highly predisposed to develop allergic diseases, thus provide an opportunity to study the role of mast cell TGFb signaling in allergic diseases. Mast cells are activated through the high affinity IgE receptor, FcERI, causing the release of granules containing mediators that are directly involved in anaphylaxis and other allergic symptoms. IgE mediated activation can be modulated by other co-stimulatory signals such as the type 2 alarmin IL-33. We examined murine mast cells carrying an LDS mutation, Tgfbr1mut, or with conditional deletion of Tgfbr1, and found that they degranulated less in response to IgE cross-linking, in vivo and in vitro. This phenotype was not tied to changes in the IgE and SCF receptor expression or mast cell tissue distribution in mice and was recapitulated in human LDS mast cells, in vitro. Furthermore, this phenotype was found to be cell intrinsic since TGFbRI WT mast cell deficient mice reconstituted with Tgfbr1mut mast cells also displayed diminished anaphylaxis compared to the WT recipients. Additionally, Tgfbr1mut mast cells responded more to IL-33 stimulation. Mechanistically, the Tgfbr1mut anaphylaxis phenotype was linked to IL-33, as the reduction of anaphylaxis in Tgfbr1mut mice was partially restored in IL-33RKO Tgfbr1mut mice. Thus, the TGFb-IL-33R axis likely plays a major role in controlling IgE mediated mast cell functions. Taken together, TGFb signaling upregulates mast cell effector function most likely by disrupting an IL-33/ST2 mediated regulatory pathway.

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Immunology - Innate and Cell-mediated Host Defenses
Activin A-induced SMAD3 activation restrains macrophage-mediated inflammation
SMAD3 (Mothers against decapentaplegic homolog 3) is a critical mediator of the signaling initiated by TGF-beta (Transforming growth factor beta), which has been reported to be an important regulator of adaptive immune responses, notably in regulating the differentiation of regulatory T cells as well as Th9 and Th17 cells. In innate immune cells like macrophages, the activation of SMAD3 by TGF-beta has been described to promote tissue repair and control inflammation. However, whether SMAD3 can be regulated in macrophages in TGF-beta-independent contexts remains unknown. Here, we report that SMAD3 is activated through the phosphorylation of its C-terminal residues (Ser 423/425) in human and mouse macrophages in response to the bacterial ligand Lipopolysaccharide (LPS). Surprisingly, this activation is independent of TGF-beta since the use of TGF-beta receptor I-deficient macrophages or the blockade of TGF-beta signaling with an anti-TGF-beta antibody in wild-type macrophages did not affect SMAD3 phosphorylation induced by LPS. Mechanistically, we discovered that SMAD3 activation was dependent on Activin A (a member of the TGF-β superfamily), as normal macrophages treated with the natural Activin A inhibitor Follistatin or macrophages with a deficiency in Activin A receptor 1b (Acvr1bf/f-Lyz2cre) showed no SMAD3 activation in response to LPS stimulation. We also showed that Acvr1bf/f-Lyz2cre macrophages exhibited a similar proinflammatory phenotype to SMAD3 KO macrophages. In the mouse models of LPS- or CLP-induced sepsis, the levels of Activin A increased dramatically 6h after onset of the septic disease, and the increased Activin A levels induced SMAD3 phosphorylation in vivo. Importantly, Acvr1bf/f-Lyz2cre mice succumbed more to sepsis due to

increased inflammation compared to control mice, which photocopied the SMAD3 KO mice phenotype. Furthermore, the Activin A-SMAD3 pathway in macrophages also protected against inflammation in a mouse model of imiquimod-induced psoriasis. Thus, we have revealed a previously unrecognized natural brake to inflammation in macrophages that occurs by the activation of SMAD3 in an Activin A-dependent but TGF-β-independent manner. Targeting this axis could have clinical implications in inflammatory contexts like sepsis or psoriasis.

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Immunology - Lymphocyte Development and Activation
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Immunology - Lymphocyte Development and Activation
Optimal CXCR5 expression controlled by the Bhlhe40-Pou2af1 axis is critical for GC-Tfh generation
Tfh cells, especially GC-Tfh cells locating in germinal center (GC) of B cell follicle, play a critical role during humoral immune response by helping B cells in antibody production. CXCR5, a typical cell surface marker of Tfh, its expression is critical for both Tfh cell development and their migration into GC. However, the regulation of CXCR5 expression during Tfh cell differentiation is still unclear. Therefore, finding the regulators of CXCR5 and revealing the mechanism behind it can not only answer the basic questions of Tfh cell differentiation, but also provide clues for modifying appropriate humoral immune response to help host defend diseases. In this study, we found that the transcription factor Pou2af1/Bob1, which was reported to be essential for GC formation and B cell development, was also required for Tfh cell differentiation in a T cell intrinsic manner. On the other hand, Bhlhe40 inhibited Tfh cell generation partially by suppressing the expression of Bob1. By generating Bob1-tdTomato reporter mice using CRISPR-Cas9 and crossing it with Bhlhe40 knockout mice, we further confirmed that Bob1 up-regulation after loss of Bhlhe40 both in vitro and in vivo. Bhlhe40 expression was analyzed in detail during Tfh development, showing that it gradually decreased from CXCR5-Bcl6-, to CXCR5-Bcl6+, to CXCR5+Bcl6+, which was accompanied by increased expression of Bob1. By using Bhlhe40 and Bob1 double knockout mice, we found that Tfh cell differentiation was blocked at the pre-Tfh (CXCR5int Bcl6int) stage indicating that Bob1 works at the downstream of Bhlhe40. In the mixed bone marrow model, we further verified, through confocal imaging, that Bob1-deficient CD4 T cells largely failed to migrate into GC in competition with WT counterparts. By contrast, Bhlhe40 deficient T cells were more efficient than wide type cells in becoming GC Tfh cells. Such migration behavior change can be explained by a corresponding alteration in the MFI of CXCR5. Genome-wide RNA-Seq analysis also confirmed an important role of Bhlhe40 in suppressing Bob1 and the requirement of Bob1 upregulation in optimal CXCR5 expression. Furthermore, Bob1 ChIP-Seq analysis uncovered a potential binding site of Bob1 on

Cxcr5 enhancer. Thus, our study revealed a novel and critical transcriptional regulatory circuit involving Bhlhe40/Pou2af1 in regulating optimal CXCR5 expression and Tfh cell migration during the transition from pre-Tfh to GC-Tfh cells.

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Immunology - Lymphocyte Development and Activation

Two developmental pathways generate functionally distinct populations of natural killer cells

Natural killer (NK) cells function by eliminating virus-infected cells or tumor cells during early defenses. However, the early development of NK cells and lineage relationships between NK cells and helper innate lymphoid cells (ILCs) remain elusive. Common precursors for ILCs (ILCPs) can differentiate into both helper ILCs and NK cells. Here, we identified a NK lineage-biased progenitor population, early NK progenitor (ENKP), which does not develop from ILCPs, thus ENKP may represent the ILCP-independent pathway of NK cell development. Competitive chimera experiment shows ENKPs generate NK cells more efficiently than ILCPs, suggesting that ENKP-dependent pathway is the major pathway for NK cell development. scRNA-seq shows ENKP-derived NK cells (ENKP_NK) express Ly49 receptors and higher levels of cytotoxic genes whereas ILCP-derived NK cells (ILCP_NK) have very low expression of Ly49 receptors and express higher levels of genes related with cell-cell adhesion and activation, which implicated tissue residency. Furthermore, Ly49H+ NK cells which response to MCMV infection mostly develop from ENKPs but not ILCPs. Consistently, ENKP_NK but not ILCP_NK expanded dramatically after MCMV infection. However, ILCP_NK express higher levels of IFNgamma than ENKP_NK upon stimulation. Interestingly, human CD56dim and CD56bright NK cells are both transcriptionally and functionally similar to ENKP_NK and ILCP_NK, respectively. Our findings establish the existence of two pathways of NK cell development that generate functionally distinct NK cell subsets.

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Immunology - Tumor Immunology

Mechanisms of tumor dormancy induction mediated by abrogation of myeloid TGF-β signaling

Acquisition of programmable proliferative arrest of disseminated tumor cells hinder the efficacy of cancer treatments, which eventually give rise to context dependent overt metastasis at distant organ sites. The mechanisms for dormancy induction or reactivation remain unclear. In this study, we report that the abrogation of myeloid specific TGF-βRII (TβRIImyeKO) induced tumor dormancy which was rescued upon TGF-βRII reintroduction in mouse models of breast cancer metastasis. Transcriptomics analysis of sorted p27+ CVC+ dormant vs p27- CVC- proliferative tumor cells identify 504 differential expressed genes, including those involving cell cycle arrest, IFNg response, MTOR signaling as well as MYC targeted genes. Spatial lung imaging showed close association of CD8+T cells and CD103+ DCs with dormant tumor cells in TβRIImyeKO mice. Moreover, TβRIImyeKO mice depicted increased frequency of CD103+ DCs in concomitant with elevated IFNg level in CD8 T cells. Importantly, depletion of CD103 DCs

in tumor-bearing TβRIImyeKO mice diminished the dormancy phenotype along with IFNg level in CD8 T cells. Mechanistically, secreted Ly6/PLAUR domain containing 1 (SLURP1) was enriched exclusively in dormant tumor cells from TβRIImyeKO mice. Overexpression of SLURP1 induce growth arrest of the tumor cell in vivo, which was also observed in 2D and 3D in vitro culture system. This induction of tumor dormancy by SLURP1 was particularly evident when cells were under stress conditions such as in low serum culture condition. Consistently, treatment with recombinant SLURP1 protein also induced dormancy phenotype in the 2D and 3D in vitro culture. Further dissecting SLURP1 downstream mediators by proteomics revealed a critical involvement of cellular communication network factor 1 (CCN1) that is known to inhibit the Caspase3 mediated cell death. Our studies uncover an important mechanism in immune microenvironment regulation of tumor dormancy mediated by myeloid specific TGF-β. These insights provide mechanistic understanding and potential treatment options to address cancer treatment resistance and relapse.

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Immunology - Tumor Immunology

Mesothelin-targeting, nanobody-based CAR T cells effectively target solid tumors in fully immunocompetent hosts

Nanobody-based chimeric antigen receptor (CAR) T cells are currently being tested in early phase clinical trials against blood and solid tumor antigens. Owing to the low molecular weight, increased stability, and low immunogenicity of nanobodies, nanobody-based CAR T cells are an attractive alternative to antibody-based CAR T cells for improving in vivo persistence and overcoming loss of antigen in solid tumors. However, no such agent has been described that can target mesothelin, a tumor antigen that is highly expressed in mesotheliomas, pancreatic, ovarian, and lung carcinomas. Here, a novel nanobody-based CAR T cell that targets both mouse and human mesothelin was created and evaluated in a pre-clinical model of lung adenocarcinoma that encompasses a fully functional murine immune system. The cell line used to establish the model was 344SQ which expresses high levels of mouse mesothelin. Murine CAR T cells demonstrated antigen specificity by selectively targeting the tumor cells expressing mesothelin in co-culture assays. When mice were treated with a single dose of 10 million CAR T cells, there was a threefold reduction in tumor size one week post-treatment (n = 6) with complete responses observed approximately two weeks post-treatment in 83% of the treated mice (n = 5/6). Complete responses were maintained for at least two months after treatment in those mice. Collectively, this demonstrates that the nanobody-based CAR T cells can effectively target established subcutaneous tumors even after a single dose and without the need to manipulate the immune system of the host to enhance persistence. This study constitutes a first attempt to establish an immunocompetent pre-clinical model for the study of mesothelin-targeting, nanobody-based CAR T cells in unprimed hosts. This model will facilitate the study of the dynamic changes occurring in the tumor microenvironment following nanobody-based CAR T cell therapy while also providing valuable insight into the tumor resistance mechanisms and methods to overcome them.

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Immunology - Tumor Immunology
Vagal-CD8+ T Cell Neuroimmune Axis Modulates Liver Cancer
Hepatocellular carcinoma (HCC) remains a leading cause of cancer-related death. In contrast to most solid tumor malignancies, HCC mortality is predicted to rise in part due to the immunosuppressive liver landscape. Beyond immune and stromal components, peripheral nerves infiltrate the tumor microenvironment. Nerve innervation alters tumor burden across various cancers, although the role of nerves in HCC has not been reported and precise mechanisms of nerve-cancer interactions remain largely unknown. The parasympathetic vagus nerve innervates visceral organs, regulating homeostatic and inflammatory pathways via acetylcholine (ACh) release. To assess putative neuroimmune HCC interactions, we utilized mice that underwent a surgical hepatic branch vagotomy (HV) or sham procedure (SV). Here we report that precise liver denervation reduces tumor growth in three models of primary (RIL-175) and metastatic (B16-F10, A20) liver cancer with luciferase-labeled cancer cells assessed by in vivo imaging. Importantly, HCC findings remained organ specific as HV mice exhibited reduced tumor burden of intrahepatic, but not subcutaneous, models. HV livers exhibited decreased ACh levels (ELISA) and expression of ACh receptors Chrm1/Chrm3 (RT-qPCR) compared to SV controls. Treatment with bethanechol (ACh receptor agonist, 400 ug/mL drinking water) increased tumor burden. Flow cytometry analyses revealed increased CD8+ T cells in HV tumor-bearing livers and increased expression of intracellular cytokines (IFNg, TNFa) within hepatic lymphocytes. We then examined whether these findings were a cause or consequence of smaller HV tumors. ACh exposure significantly reduced intracellular cytokine levels in ex vivo hepatic CD8+ T cells following anti-CD3/CD8 activation and altered Chrm levels. Bethanechol failed to promote tumor growth in Rag1KO mice lacking mature B and T cells, while targeted depletion of CD8+ T cells abrogated the effects of vagotomy. Intriguingly, immunofluorescence analyses revealed that CD8+ T cells colocalize with peripheral hepatic nerves (HCC tumor resection cohort, n = 12). These collective findings highlight a vagal-CD8+ T cell axis mediating HCC tumor burden via ACh signaling. Ongoing scRNA-Seq analyses will identify hepatic CD8+ subsets and immune pathways altered by parasympathetic denervation. This study furthers the emerging field of cancer neuroscience and identifies nerve-dependent targets to modulate immunosuppressive HCC features and outcomes.

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Microbiology and Antimicrobials
RoaM is a transcriptional regulator in Rickettsia rickettsii governing the transition to a cellular program of active spreading.
Rickettsia rickettsii is an obligate intracellular pathogen causing Rocky Mountain spotted fever, the highest morbidity tick-borne disease. In the natural environment, the bacterium must navigate the transition from its arthropod vector to a vertebrate host, and back. Once R. rickettsii has invaded a host cell, it can spread from cell to cell by polymerizing actin filaments to propel the bacterium into adjacent

cells. While a strategy of aggressive spreading might be necessary in the vertebrate to achieve horizontal transmission of R. rickettsii between co-feeding ticks, unchecked spread within the vector could ultimately cause death of the arthropod vector and become a dead end for the bacterium. We recently discovered that production of the rickettsial protein RoaM (Regulator of Actin-based Motility) negatively regulates the production of actin tails and its abrogation induces hyper-spreading behavior in many laboratory-adapted strains of R. rickettsii. Using R. rickettsii strains derived from the virulent Sheila Smith strain that express varying levels of roaM ranging from a genetic knockout to high overexpression, an RNA-seq experiment was performed. The experiment revealed that roaM-deficient strains upregulate at least seven hypothetical proteins which may link the regulatory effects of RoaM to the phenotypic effect on motility. Among the genes regulated is the effector RarP2 which disrupts host cell signaling by dispersing the trans-Golgi. RNA-seq hits were confirmed with RT-qPCR and disruption of the RarP2 disruption of the trans-Golgi in a RoaM-dependent manner was confirmed in infected Vero cells. Two of the hypothetical proteins were shown to be secreted via fusion to a glycogen synthase kinase beta tag, which when phosphorylated reveals exposure to the host-cell cytosol. Additionally, we performed growth assays in a tick explant model that showed hyper-spreading strains multiply to a higher number by four days post infection. Taken together, these data support the hypothesis that RoaM is a transcriptional regulator down-regulating rickettsial genes important in the mammalian host but detrimental in the tick vector.

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Microbiology and Antimicrobials
Co-cultivation mediated elicitation of Fungal Natural products against Mycobacterium tuberculosis
Tuberculosis caused by Mycobacterium tuberculosis is a leading cause of global mortality with about eight million new cases and 1.6 million deaths ascribed to it annually. To combat the threat of drug-resistant strains of Mtb, newer drugs with novel mode of actions are needed. Microbial natural products (NPs) are gaining renewed attention as their complex structural scaffolds have evolved to specifically inhibit essential cellular targets. Herein, we utilized co-cultivation approach to identify cryptic biosynthetic gene clusters (BGCs) from fungal genomes eliciting the expression of genes that are silent or poorly transcribed in axenic cultures. Fungi were isolated from sphagnum peat bog collected from different regions of North-eastern USA because aspects of this ecological niche reflect the critical microenvironment of the human tuberculosis granuloma. In addition, sphagnum peat bogs are a natural habitat for slow growing mycobacteria that compete for limited nutrients with other microbes. Bioactivity-guided assay against reporter strain mScarlet Mtb H37Rv led us to identify two fungal isolates that selectively produce growth inhibitory metabolites during co-cultivation with Mtb. Interestingly, counter-screening against ESKAPE pathogens and M.smegmatis did not cause any growth inhibition. Whole genome sequencing showed these isolates were a Penicillium and Talaromyces sp., denoted as F2 and Fun31 respectively in our study. Fungal mRNA sequencing from co-cultured isolates facilitated the identification of elicited Type I Polyketide Synthase BGCs that were silent in axenic cultures. These highly induced BGCs upon co-cultivation were bioinformatically linked to their products including patulin from F2 and unique emodin/chrysophanol derivatives from Fun31. These induced filtrates led to a highly responsive redox-stress homeostasis within Mtb. Our study illustrates a co-

cultivation mediated elicitation of unique fungal NP resulting in a thiol-reactive oxidative stress mediated killing of Mtb. Subsequent chemical experiments will be helpful to confirm the molecular structure of the unknown metabolite as well as target identification would benefit us to validate the mode of Mtb killing. These results illustrate that two different fungi have convergently selected different molecules to target the same vulnerability of slow-growing mycobacterial species in nature and suggest that this target may be uniquely vulnerable in the context of human caseous lesions.

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Microbiology and Antimicrobials
Manipulation of the human ubiquitination by a novel virulence factor in Legionella pneumophila
Ubiquitination represents one of the most important post-translational modifications in eukaryotes. The ubiquitination cascade involves several enzymes, most notably E3 ubiquitin ligases, to covalently attach the modifier ubiquitin to substrate proteins. Intracellular bacterial pathogens often encode molecular mimics of host E3 ligases in order to hijack these processes during intracellular replication. In my project, we discovered a novel virulence factor of the bacterium Legionella pneumophila (Lp), the causative agent of Legionnaires' pneumonia, that manipulates the host ubiquitination pathway. This effector protein interacts with the cullin-4 E3 ubiquitin ligase (CRL4) complex that is known to play a critical role in cell cycle regulation and DNA damage responses. We named this novel Lp virulence factor LiuC for Legionella effector interfering with ubiquitination by CRL4. LiuC shows no homology to known proteins in databases, yet its high conservation within the genus Legionella suggests an important role for virulence. Upon gene silencing by CRISPR interference, we found that liuC is important for intracellular growth of Lp in its native amoebal host Acanthamoeba castellanii. Mass-spectrometry-based proteomic analyses together with co-precipitation assays and yeast 2-hybrid assays validated that LiuC directly binds the scaffolding E3 ligase CUL4A and the substrate receptor DCAF8, the two components of the CRL4 complex. To our surprise, our preliminary data suggest that LiuC interferes with rather than promotes substrate ubiquitination by the CRL4-DCAF8 complex, suggesting that it is not just another mimic of a host E3 ligase. I am currently investigating the LiuC-CRL4 complex formation at a structural level using negative stain electron microscope. For that, I have already optimized the purification protocols for all the CRL4 complex components as well as LiuC and will soon begin to acquire high-resolution data by cryo-electron microscopy (in collaboration with Dr. Doreen Matthies, NICHD). I am also in the process of determining how LiuC alters CRL4-mediated signaling within mammalian macrophages by performing proteome analyses in collaboration with Dr. Aleksandra Nita-Lazar (NIAID). My project will shed light on a potentially novel role of CRL4 in microbial pathogenesis and offer new avenues for combatting infections with Lp and related pathogens.

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Microbiota

Cutaneous commensal induces humoral immunity via formation of dermal tertiary lymphoid organs
The skin is one of the largest barrier sites which harbors a plethora of microorganisms. Whether skin microbiota promotes humoral responses remains unknown. Here we show that colonization with common skin commensal Staphylococcus epidermidis profoundly modulates cutaneous immunity that results in serum responsiveness with antigen-specific IgG antibodies. Such response is uncoupled from inflammatory signals and is surprisingly associated with the induction of dermal tertiary lymphoid organs that closely mimic classical germinal centers. Of note, such responses occur in a tissue autonomous manner as evidenced by the preserved antibody response in mice deficient in professional lymphoid organs. Our data also uncover a non-redundant role for Langerhan's cells, the largest population of skin resident dendritic cells, for the generation of commensal specific antibodies. Further, our work proposes a model by which commensal specific antigens are collected in the hair follicle leading to the generation of T follicular helper cells that further expand the local B cell pool in an IL-21-dependent manner. This mechanism is central for host's ability to control local cutaneous bacterial burden. Antibody responses to the skin microbiota also provides protection against systemic infection with the same microbe demonstrating the fundamental importance of this line of defense in maintaining barrier protection and allowing for symbiotic coexistence of the skin microbiota with its host. Collectively, our observations highlight the autonomous potential of skin organizational flexibility and for the first time demonstrate that B cells represent an indispensable local cutaneous compartment that actively maintains skin immunity.

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Microbiota
Intratumoral bacteria are associated with patient outcome in primary liver cancer
Humans are metaorganisms that evolve from symbiosis between host cells and microorganisms (the microbiota), yet imbalances in this interaction may lead to disease. Recent work showed tumor type-specific intracellular microbiota in the tumor microenvironment (TME), but the consequence for patients is rarely addressed. Considering the physiological connection within the gut-liver axis and the unique ability of microbes to modulate host-microbes' interactions, it is crucial to characterize the microbial profile in primary liver cancer (PLC) and its influence on patient outcome. However, current technologies limit the ability to comprehensively characterize this low ratio of microbes per host cell in the TME. We hypothesized that outcomes in PLC patients are influenced by intratumoral microorganisms, particularly bacteria. We unbiasedly characterized the bacterial profiles of 740 surgically paired human samples (tumor and adjacent non-tumor) of two histologically distinct subtypes of Thai PLC patients, i.e., hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA), as part of the TIGER-LC consortium. For this, we used a newly developed technique that starts with RNA before 16S rRNA sequencing, followed by bacterial read mapping and bioinformatics analysis. We observed distinct bacterial profiles among tumors and adjacent non-tumors, as well as between HCC and iCCA, providing evidence that despite all tumors developing within the liver, unique microbial ecosystems are maintained depending on the tumor subtype. Second, we observed a higher bacterial load in the tumor compared to the adjacent non-tumor, implying a potential role for specific bacteria within the TME. Third, patients with a high bacterial load showed a decreased overall survival,

demonstrating that bacteria influence patient outcome. Interestingly, specific gut-bacteria were enriched in iCCA patients and associated with survival, pointing to a potential mechanism where an imbalance in the gut may increase bacteria's translocation to the liver and influence patient outcome. Finally, by integrating metabolomics and transcriptomics generated within the same cohort, we were able to identify host-microbe interactions influencing patient outcome. Collectively, we characterized the distinct intratumoral microbial profile in HCC and iCCA and observed that specific bacteria influence patient outcome by modulating microbe-host interactions.

Stefan Katharios-Lanwermeyer
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Microbiota
Genetic determinants underlying Pseudomonas aeruginosa-mediated aggregation of Stenotrophomonas maltophilia
Polymicrobial infections are frequently observed in patients with cystic fibrosis (CF) and can lead to worse disease outcomes. Despite the urgent clinical interest, how interspecies dynamics affect disease progression and outcomes is only beginning to be understood. Stenotrophomonas maltophilia (Sm) and Pseudomonas aeruginosa (Pa) are two opportunistic pathogens often co-isolated from the lungs of CF patients, where they likely exist in close physical proximity to one another and interact via their respective secreted products. We examined the interactions between these co-infecting species and found that Sm rapidly aggregates in response to Pa cell-free supernatant. These Pa supernatant-induced aggregates of Sm were ~100-fold less susceptible to the antimicrobial cetrimonium bromide compared to non-aggregated bacteria, suggesting that aggregation can protect Sm against environmental insults. To ascertain the genetic determinants of Sm aggregation, we experimentally evolved Sm via repeated exposure to Pa supernatant, selecting for non-aggregating cells. We identified mutations in the smf-1 gene that encodes a fimbrial adhesin in the evolved populations. A Δsmf-1 strain was aggregation-deficient when challenged with Pa supernatant, suggesting that Smf-1 is necessary for Pa-induced aggregation of Sm. To identify the Pa genetic determinants involved in Sm aggregation, we used a high-throughput microscopy-based screen of a Pa transposon mutant library. Preliminary data from mutants identified from this screen indicate that Pa supernatant-mediated aggregation of Sm Requires the Type VI Secretion System, a molecular secretory machine that can impart Pa a fitness advantage via iron acquisition and contact-dependent killing of competing bacteria. Furthermore, Pa mutants involved in a shared glycine betaine and choline catabolic pathway were also aggregation-deficient, suggesting that Pa utilizes this metabolic pathway to produce or secrete factors which induce Sm aggregation. This work thus identifies a novel interspecies interaction between the co-infecting pathogens P. aeruginosa and S. maltophilia, delineates underlying genetic determinants in each species that are required for this phenotype, and illuminates survival benefits afforded by P. aeruginosa-induced aggregation of S. maltophilia. Future work will focus on identifying the specific P. aeruginosa effector molecule(s) as well as the signaling pathways in S. maltophilia that lead to aggregation.

Gamze Ayaz Sen
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Molecular Biology - General

Mutant p53 condensation induced by transcription inhibition is required for breast cancer metastasis

Breast cancer is the second leading cause of cancer-related deaths in US women, with over 90% of deaths caused by metastasis. This understanding of breast cancer metastasis is crucial for developing effective treatments. As the most frequently mutated gene in human cancers, TP53 plays a crucial role in breast cancer metastasis. Previous studies have shown that mutated p53 (mtp53) not only loses tumor suppressive function but also gains new oncogenic functions (called GOFs) that are independent of wild type (WT) p53. Specifically, GOFs of mtp53 include promoting cancer cell mobility, invasion, and metastasis of breast cancer cells. However, the molecular mechanisms underlying mtp53-mediated metastasis remains unclear. To elucidate the molecular mechanisms, I utilized an unbiased proteomics approach, proximity-dependent biotinylation (BioID) assay, to identify new binding partners of mtp53 in human metastatic breast cancer cells, MDA-MB-231. BioID identified dozens of previously unreported binding partners of mtp53. Among verified binding partners, I focused on NONO, an RNA-binding protein critical for forming membraneless condensates in cells, which play important functions in the stress response. Interestingly, mtp53 but not WT p53 formed condensates with NONO in several metastatic breast cancer cell lines based on results from super-resolution microscopy. In the xenograft model, depletion of NONO or mutp53 inhibited lung metastasis, suggesting that these two proteins regulate the same pathway to promote metastasis. RNAseq and CHIPseq data revealed that the genes involved in oxidative phosphorylation and fatty acid metabolisms are regulated by mtp53 and NONO. Mechanistically, stresses such as transcription inhibition via DRB, an inhibitor of transcription elongation by RNA polymerase II, induce mtp53 and NONO condensation in the nucleolus, and this formation of NONO-mediated condensation is dependent on mtp53. Transcription of key metabolic enzymes, MDH1, POR, and FASN (fatty acid synthesis), was diminished in the absence of NONO or mtp53 when cells were treated with DRB compared to parental cells. Importantly, an inhibitor of FASN reduced breast cancer metastasis. In summary, mtp53 may promote breast cancer cell metastasis by forming condensates to overcome stress caused by transcription inhibition. My results suggest a new way to inhibit metastasis in breast cancer patients with mtp53 by targeting the fatty acid synthesis pathway.

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Evidence of High-altitude windborne migrating mosquitoes infected with arboviruses, plasmodia, and filariae: a new epidemiological frontier

Windborne mosquitoes have been recently collected at high-altitudes (40-290 m above ground) over the Sahel using sticky nets attached to tethered helium-filled balloons. The high diversity of mosquito species at altitude, the predominance of gravid females indicating that they took at least one blood meal prior to their migration, and their abundance during the rainy season suggest that windborne mosquitoes at altitude are a key driver in the spread of mosquito borne pathogens. Indeed,

epidemiologists inferred that windborne vectors spread arboviruses, plasmodia, and even filariae over hundreds of kilometers. However, until now there was no empirical evidence demonstrating that mosquitoes at altitude were infected with pathogens. Here, we analyzed the infection status of mosquitoes collected at altitude over different ecozones from the Sahel of Mali to the forest of Ghana by targeted assays on pathogen genera using qPCR followed by sequencing as well as by metagenomics. Our results reveal high infection rates with plasmodium: 6.9% and 2.8% abdominal and thoracic infection, respectively. Because thoracic infection indicates development sporozoites, such infection allows to incriminate natural vectors. These parasites consisted of 14 avian Plasmodium species, including P. relictum, P. vaughani and P. matutinum and additional two haemosporida: Paraheamoproteus vireonis and Haemoproteus pastoris, which are vectored by biting midges. The pan-filaria screen revealed infection of avian and mammalian (caprine-antelope) filariids. RT-qPCR revealed infection with flaviruses, including the zoonotic West Nile virus and an alphavirus. A metagenomic analysis of 48 individual mosquitoes enriched in qPCR positive samples revealed the presence a second zoonotic human pathogen—M'Poko virus among nearly 50 insect-specific viruses (not transmitted to vertebrates). Additionally, this metagenomics data detected other non-mosquito borne pathogens (e.g., Trypanosoma spp.). This study provides the first direct evidence of the spread of mosquito borne pathogens by windborne mosquitoes and suggests that aerial sampling would be valuable component of broad disease surveillance, informing about the sources of pathogens and vectors on the move and the likely locations of disease outbreaks.

Srinivas Pittala
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Molecular Biology - General
Activation of receptor-mediated G12 signaling has pronounced effects on hepatic glucose fluxes
G protein coupled receptors (GPCRs) play a key role in regulating whole body glucose homeostasis. In the presence of agonist ligands, GPCRs can activate four distinct classes of heterotrimeric G proteins: Gs, Gi/o, Gq, and G12. At present, little is known about the metabolic consequences of GPCR-mediated activation of G proteins of the G12 family. The hepatocytes of the liver play a central role in maintaining euglycemia. To elucidate the vivo metabolic roles of hepatocyte G12 signaling, we generated a new mouse line that expresses a CNO-sensitive, G12-coupled designer GPCR (G12 DREADD; DREADD = designer receptor exclusively activated by a designer drug) selectively in hepatocytes (Hep-G12D mice). CNO is a synthetic small molecule that can selectively activate the G12 DREADD but is otherwise pharmacologically inert. In addition, we generated a mouse model that lacks functional Ga12 selectively in hepatocytes (Hep-G12 KO mice). These mutant mice were subjected to a series of metabolic tests to examine how activation of hepatic G12 signaling or hepatocyte G12 deficiency affect glucose tolerance, insulin sensitivity, in vivo glucose uptake, and various other metabolic parameters. We found that selective activation of hepatocyte G12 signaling led to greatly enhanced blood glucose levels under both fed and fasting conditions. In vivo studies of hepatic glucose fluxes showed that these effects were due to an increased rate of glycogenolysis and gluconeogenesis. The G12D-mediated hyperglycemic effects were absent in mice lacking functional G12 in hepatocytes, indicating that the G12D designer receptor selectively activates G12. Studies with isolated hepatocytes prepared from Hep-G12D mice demonstrated that G12D-mediated glucose production required signaling via RhoA and ROCK1. We also

showed that activation of endogenous sphingosine-1-phosphate receptors (subtype 1) stimulates hepatic glucose production via a similar pathway. In conclusion, our results show, for the first time, that hepatic G12 signaling plays a key role in regulating whole body glucose homeostasis.

Melissa Arroyo-Mendoza
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A unique sigma 70 variant of an Adherent Invasive Escherichia coli, LF82, a pathobiont associated with Crohn's disease, results in specific gene expression changes
Crohn's disease (CD), an inflammatory bowel disease, arises from an immune attack of the GI tract, affecting roughly 1.6 million Americans (0.5% of the pop). Although the etiology of CD is unknown, Adherent Invasive Escherichia coli, such as the pathobiont strain LF82, is frequently found associated with CD, suggesting that it may play a role in CD pathogenesis. Interestingly, LF82 contains no virulence factors or toxigenic genes, but it can adhere to and invade the intestinal epithelium, form strong biofilms, and survive and replicate within macrophages. A whole genome analysis of LF82 by the lab of our collaborator Dr. Greg Phillips (Iowa State University) revealed multiple nucleotide substitutions (SNPs) that distinguish it from commensal and toxigenic Enterobacteriaceae. The SNPs include an extremely rare mutation, D445V (.01% out of > 20,000 Enterobacteriales sequences), within the primary specificity subunit (sigma 70) of RNA polymerase (RNAP). From the structure of E. coli RNAP, we predict that D445 might affect interactions with the spacer region between the conserved -10 and -35 promoter elements, positing that it might affect transcription in general. However, our in vitro transcription results indicate that the mutation does not simply render RNAP less active at a general, strong promoter. Westerns demonstrated that the mutation also does not affect the level of sigma 70 within the cell. Consequently, we hypothesized that this variant may affect transcription from specific promoters. Using RNA-seq and isogenic strains of E. coli MG1655 with either the wild type RpoD D445 or variant V445, we have found that the D445V SNP does result in specific changes in gene expression. Furthermore, observed transcriptome and phenotypic changes are consistent with multiple phenotypes observed for LF82, including increased antibiotic resistance and biofilm formation, modulation of motility, and increased capacity for methionine biosynthesis. Our work demonstrates that a single residue change within the bacterial primary sigma factor can lead to multiple alterations in gene expression and phenotypic changes, suggesting an underrecognized mechanism by which pathobionts and other strain variants with new phenotypes can emerge.

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Neuroscience - Cellular, Molecular, and Glia
Single Nucleus RNA Sequencing Identifies Two Unique Macrophage Subsets in Control and Cisplatin-treated Mouse Cochleae
Cisplatin is a widely used anti-cancer drug that leads to the death of mechanosensory hair cells in the

cochlea (hearing organ), and their lack of regenerative capacity results in permanent hearing loss in adult cancer patients. During cisplatin treatment, the cochlea generates an active immune response that includes the activation of resident macrophages. Recent studies have suggested phenotypic heterogeneity within these macrophage subsets based on their expression markers and morphology in the developing cochlea. However, these macrophage subsets have not been definitively characterized in the adult cochlea, and their roles in response to cisplatin are poorly understood. Our lab previously developed a clinically relevant mouse model of cisplatin ototoxicity in which adult mice undergo three cycles of once-daily cisplatin injections for four days, followed by a 10-day recovery period. Here, we have used this mouse model to examine macrophage heterogeneity in the cochlea by performing single nucleus RNA sequencing (snRNAseq) of the whole cochlea from healthy adult mice and mice treated with cisplatin. The snRNAseq analysis identified two distinct subsets of cochlear macrophages, termed CMα and CMβ. CMα differentially expressed Tnfrsf11a, Lair1, and Stab1 genes, while CMβ differentially expressed H2-Aa (gene for MHCII), Cd74, and S100a4. Both macrophage subsets expressed common macrophage markers Cx3cr1 and Csf1r. To validate the CMα and CMβ subsets in the cochlea, we used heterozygous CX3CR1-GFP mice that expressed GFP in myeloid lineage cells and performed immunofluorescence staining to visualize CX3CR1-GFP and MHCII in cochlear sections harvested from healthy adult mice and cisplatin-treated mice. Visualization of CX3CR1-GFP and MHCII identified CMα as [CX3CR1-GFP+MHCII-] and CMβ as [CX3CR1-GFP+MHCII+]. Furthermore, quantification of CMα and CMβ showed that cisplatin differentially affected the numbers of the two macrophage subsets. CMα numbers remained unchanged throughout cisplatin treatment. In contrast, CMβ was significantly reduced in response to cisplatin, suggesting that CMβ is more susceptible to cisplatin than CMα. Together, identification of two transcriptionally distinct macrophage subsets in the cochlea may indicate the distinct properties and discrete roles of cochlear macrophages during cisplatin treatment and may illuminate clinical strategies to protect the hearing of cancer patients undergoing lifesaving cisplatin treatment.

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Identifying the transcriptional signatures of cocaine relapse
Relapse is an ongoing clinical problem, and there are currently no effective treatments to reduce the risk of relapse to psychostimulants like cocaine. Environmental cues previously associated with drug-taking can provoke craving and cause relapse long after cessation of drug use. These maladaptive cue-drug

associations are hypothesized to be encoded within specific patterns of neurons that are selectively activated by drug-related cues, termed neuronal ensembles. One strategy to combat relapse is to inactivate or weaken the persistent maladaptive drug-cue associative memories by targeting specific molecular mechanisms that occur only in the ensemble neurons. Our lab and others have shown causal roles for neuronal ensembles in relapse behaviors, but it is unknown which cell types comprise relapse ensembles and how they maintain this association at the molecular level. To address this gap, I developed a new single nuclei RNA-sequencing pipeline that allows sample multiplexing and enrichment of rare populations (ensembles are typically <5% neurons in a region). For these studies, I focused on the infralimbic subregion (IL) of the medial prefrontal cortex as it plays a critical role in reward processing and drug seeking behaviors. I hypothesize that cocaine-relapse ensemble neurons in the IL are heterogeneous in cell type with unique transcriptional signatures that support the long-lasting, maladaptive relapse memory. We trained rats to self-administer cocaine during twice daily 3 h sessions. Following 21 days of abstinence, we tested rats for cocaine-seeking (30 min, extinction conditions) or no test control and collected brains 3 h after the test (peak ensemble marker expression). We used fluorescence-activated nuclei sorting to isolate ensemble and non-ensemble nuclei as input for our new snRNA-seq technique. We identified that cocaine relapse ensembles comprise multiple cell types of excitatory and inhibitory neurons. Differential gene expression analysis revealed altered gene expression profiles that support memory maintenance, with enrichment in neuropeptides, receptors, and synaptic plasticity-related genes. Current work involves following up on key candidates with pharmacology and genetic approaches to assess a causal role for transcriptional changes in the cocaine relapse ensemble in persistent cocaine relapse during abstinence.

Lin Wang
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Neuroscience - Cellular, Molecular, and Glia
Spatial transcriptomic profiling of aging mouse brain
Aging is accompanied by cognitive deficits. Genes that contribute to this age-related cognitive decline have not been characterized. Understanding the molecular mechanisms involved in age-related cognitive decline will help identify ameliorative therapeutic targets and improve memory in aging individuals. Towards this direction, we designed a systematic spatial transcriptomics study of aging murine brains in both sexes. Our major goal in this study was to discover key genes that are differentially expressed during aging with spatial and near single-cell resolution. First, we aggregated all our sequenced cells, thereby recreating a typical hemisphere profile, like what might be observed with bulk RNA sequencing. We validated previously identified age-related genes in our dataset (C4b, Lyz2, Apod, Serpina3n, Gh) and further noted novel genes (Trem2, Vim, C1q, Gpnmb, Lgals3, Cd52) which monotonically increased in the mouse brain with age. Second, we identified 7 distinct transcriptional signatures which neatly segregated into clusters based on brain regions. Third, using differential gene expression analysis, we found that age-related transcriptomic changes were remarkably region-specific. Our results indicate aging signatures are most prominent in white matter fiber tracts. Furthermore, these strong aging markers are hallmarks of immune-response pathways suggesting an activated inflammatory response at fiber tracts. For example, we find strong expression of complement system genes such as C1q and C4b as well as Lyz2, Lgals3, and Trem2 which are expressed in activated and

disease-associated macrophage, microglia, and neutrophils. We further validated our results at the protein level by immunofluorescence and found that Galectin-3 (marker for white matter microglia and encoded by Lgals3) is specifically increased in fiber tracts in old mouse brains. IBA1 (marker for activated microglia), GFAP (marker for activated astrocytes) and SerpinA3N (marker for activated microglia and astrocytes) are also markedly upregulated in this region. Additionally, we find increased neutrophil numbers in the brain parenchyma of old mice by flow cytometry suggesting a breach in the blood brain barrier. Overall, our spatial omics and additional validation data reveal fiber tracts as a focal point of brain inflammation that can be targeted to combat age-related cognitive decline.

Roxan Stephenson
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Neuroscience - Cellular, Molecular, and Glia
Understanding the relationship between lipid dysregulation and Alzheimer's disease risk
Risk for late-onset Alzheimer's Disease (AD) is impacted by variants in apolipoprotein E (APOE), which codes for the brain's most abundant lipoprotein. Common variants in APOE (APOE2, APOE3, and APOE4) differ from each other at just 2 amino acid positions. While APOE4 is a primary genetic risk factor for late-onset AD, APOE2 allele is linked to lower incidence. APOE3, the common variant in the general population, is neutral with respect to AD risk. Despite this genetic correlation, the mechanisms by which APOE alters AD risk are poorly understood. Given APOE's role in lipid transport and the central role of lipids in cells, I hypothesize that APOE4 increases AD risk by transforming the cellular lipidome. Dr. Alois Alzheimer described lipid accumulation in glia as a key pathological hallmark in a report of the first named AD patient. Thus, I focused my work on microglia, the resident immune cells in the brain which respond to insults and clear debris via phagocytosis. Using human induced pluripotent stem cell (iPSC)-derived microglia, I observed that microglia accumulate lipids upon inflammatory activation. These excess lipids amass in fat storage organelles called lipid droplets (LDs). I explored how cellular lipid content relates to the vital microglial functions of cytokine secretion and phagocytosis. To do this, I used chemical modulators to increase or decrease cellular lipid burden. I found that neutral lipid synthesis is necessary for morphological changes and secretion of certain inflammatory cytokines (e.g., IL-6 and IL-1B) in response to microglia activation. When neutral lipid synthesis is reduced, and microglia cannot store them in LDs, proper phagocytosis and amyloid-beta clearance are impaired. I am now exploring whether LD catabolism is also essential for glial function. Further, I observed that microglia harboring the AD risk genotype, APOE4, accumulate more neutral lipids than those harboring the benign APOE3. To gain insights into how APOE alleles alter lipid homeostasis in microglia, I used liquid chromatography-mass spectrometry lipidomics on samples from an isogenic set of iPSC-derived microglia, harboring both risk and protective APOE genotypes. I am comparing these profiles to those found in samples from human patients to identify a lipid signature of risk or resilience. This study will further our understanding of how APOE alleles modulate AD risk, and the findings can be exploited to reveal novel targets for therapeutic benefit.

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Neuroscience - Cognitive and Behavioral Neuroscience

A state-based value model predicts motivation during a reinforcement learning task with token reinforcement

Reinforcement learning (RL) is a theoretical framework that describes how agents learn to select options that maximize rewards and minimize punishments over time. In many RL tasks, reward size and/or probability are varied, and animals learn to make choices to maximize the total reward received. We often make choices, however, to obtain secondary reinforcers (e.g. money, points) that can later be exchanged for primary reinforcers (e.g. food, drink). Although secondary reinforcers are highly motivating, little is understood about the computational and behavioral mechanisms that shape this motivation. In the present study, we have examined how monkeys learn to make choices that maximize fluid rewards through reinforcement with tokens. The monkeys learn through trial and error which visual images are associated with gaining tokens and which images are associated with losing tokens. Every 4-6 trials, tokens are exchanged for fluid rewards. On trials where both options lead to losing tokens, the monkeys abort more often and their reaction times get slower with learning. This important behavior reflects changes in motivational state but would be missed with traditional analyses and reinforcement learning models. There has been extensive work showing that motivational state has a neural basis and has critical effects on reinforcement learning processes. Thus, we have constructed a computational model to capture the learning, abort frequency, and reaction times to capture the motivational state of the animal in this tokens task. The model is a Markov decision process (MDP) model that calculates the state value of each moment within a trial, based on all the combined factors present in the tokens task (e.g. current number of accumulated tokens, task condition, task epoch, trials since last delivery of primary reinforcer, etc.). Choice behavior, reaction times, and abort frequency were found to be related to the state values and changes in state value during the tokens task. Furthermore, the model makes predictions for how neural responses can change on a moment by moment basis with changes in motivational state driven by changes in state value. Together, this task and model allow us to explore how traditional RL models do and do not capture learning from secondary reinforcers, which are typical in a real-world environment.

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Neural correlates of active vision serving goal directed naturalistic behavior

Primate vision is used heavily for actively sensing the environment for goal directed behavior. e.g., “How can I find a valued object in a cluttered environment?” This requires active vision involving continual sampling of the environment using eye, head, and body movements. This in turn requires cortical mechanisms to maintain stable perception of the external world despite changes in retinal stimulation due to rapid eye movements, blinks, and self-generated motion. Experimental approaches to study vision typically avoid the complexity of naturalistic behavior, for both practical and theoretical reasons.

Vision in humans and other primates is often conceived as being fundamentally feedforward, driven by retinal content and then modulated by behavioral influences, such as goal-directed attention or a perceptual judgement. This idea however may not extend well to natural conditions. A feedforward perspective suggests that neural activity will be interrupted by blinks, as reported in early visual areas. However, we found instead that most neurons in macaque face patches are immune to disruptive effects of blinks during continuous modes of active vision, and continue respond to structured visual input even during brief periods in which the eyes are closed. We then asked whether neural response regimes during the presentation of flashed images versus continuous video presentation will be comparable, as suggested by the conventional framework for visual processing. We used multivariate analysis to measure the range and general similarity of responses in neural representation space under the two visual presentation conditions. We found that, although population responses were highly consistent across trials within both conditions, the span of population representations were much broader in the image condition. The robustness and magnitude of this difference suggests that the neural responses in the primate brain are entrained in a qualitatively different mode during naturalistic vision than during the traditional discrete presentation of flashed images. Together, these findings may have far-reaching consequences for understanding and modeling primate vision and for applications like visual prostheses. Importantly, these findings suggest that it is critical to evaluate neurophysiological results and models obtained under conventional modes of visual testing vis-à-vis their relevance to everyday visual operations utilized during natural, goal directed behaviors.

Jonathan Chow
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Effect of peer-sex on operant responding for social interaction: Role of estrous and cycle and striatal dopamine
Preclinical models mimicking the human aspect of engaging with peers show that rats will choose such social interaction over taking addictive drugs, even after extensive drug-taking experience. Investigation into how operant social interaction functions as a reward indicates that it supports lever-pressing like other self-administered rewards and is affected by housing conditions and peer familiarity. However, these studies were conducted with peers of the same sex. Here we examined if peer-sex influences operant social interaction and the role that the estrous cycle and striatal dopamine may have in same- vs. opposite-sex social interaction. We trained rats (n=13; 7 females) to lever-press for access (15 s) to a same- or opposite-sex peer for 16 d (8 days/sex) and tracked females' estrous cycle. Next, we injected GRAB-DA and implanted optic fibers into the nucleus accumbens core and dorsal medial striatum. We then retrained the rats for social interaction for 16 d (8 days/sex) and then recorded striatal dopamine during operant responding for a peer for 8 d (4 days/sex). Finally, we recorded the assessment of economic demand for a same- or opposite-sex peer (10 days/sex). Male rats responded more when a female peer was present; there were no differences in responding in female rats. Estrous cycle had no effect on operant social interaction. Striatal dopamine signals during operant social interaction were dependent on the peer's sex in male rats. . Results suggest that striatal dopamine activity plays a role in operant responding for rewarding social interaction in same- vs. opposite-sex pairings. In future studies, we will determine the circuit mechanisms that underlie these observed sex differences.

Taylor Malone
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A consistent map in the medial entorhinal cortex supports spatial memory
The medial entorhinal cortex (MEC) is hypothesized to form a "cognitive map" of space for memory-guided navigation. MEC dysfunction leads to spatial learning and memory deficits in animals and humans and is associated with impaired spatial cognition in Alzheimer's disease (AD). However, the neural basis of the MEC's role in spatial learning and memory is poorly understood. Prior studies suggest that the spatial representation of the MEC changes during learning and is stabilized when spatial memory is established. However, these studies could not fully evaluate this hypothesis due to limitations of electrophysiology in reliably tracking neural activity over many days of spatial learning. The field also lacks studies disrupting the activity pattern of the MEC map and evaluating behavioral outcomes to establish a causal role of the map in spatial memory. Here, we utilized recent advances in in vivo two-photon microscopy to track the activity of hundreds of MEC neurons over multiple days and virtual reality to provide mice with reproducible visual cue-guided one-dimensional environments. Combining these techniques, we investigated the relationship between MEC activity and spatial learning performance while mice navigated and learned a novel environment for ten days. During successful learning, a spatially consistent MEC map gradually formed and then became stabilized. Specifically, neuronal activity in the MEC exhibited continuously improved inter-day and intra-day spatial consistency, increased specificity in representing salient environmental features, and increased accuracy in decoding the environment. c-Fos expression in the MEC of these mice increased in novel environments, suggesting the contribution of synaptic plasticity to shaping the consistent MEC map during learning. In contrast, mice with unsuccessful learning did not show the above features, indicating that a consistent MEC map is associated with effective spatial memory. Optogenetically disrupting the spatial consistency of the MEC map impaired memory-guided navigation in a well-learned environment, demonstrating the necessity of a consistent MEC map for spatial memory. Overall, our results reveal the establishment of a spatially consistent cognitive map in the MEC during successful learning and provide the first causal link between MEC map consistency and spatial memory. This study has built a foundation for further investigation on the role the MEC plays in spatial learning deficits in AD.

Zhewei Zhang
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Hippocampus is necessary for dopamine neurons to compute reward prediction errors when states are partially observable.
Dopamine (DA) neurons in the ventral tegmental area (VTA) signal reward prediction errors (RPEs), which reflect the discrepancy between the actual and expected value of outcomes. These RPEs depend critically on how a task is represented internally, a function likely dependent on hippocampus (HC). To examine the contribution of the HC to dopaminergic RPEs in the VTA, we recorded VTA DA neurons in rats with sham or neurotoxic lesions of the (ipsilateral) dorsal and ventral HC during a multi-block, odor-

guided choice task. In this task, odors signaled choices for rewards that varied in timing or number across blocks to induce RPEs. As expected, DA neurons' activity in sham rats increased to unexpected delivery of rewards and decreased to omissions. As well, their activity was adjusted to reflect the cues' different values in each block. By contrast, DA neurons in HC lesioned rats showed roughly intact responses to the cues but no longer signaled RPEs to the reward changes. To explain these phenomena, we built a reinforcement learning model with a hierarchical state space in a partially observable semi-Markov framework, where states were estimated based on observations and transitions between states. Upper-level states adjusted agent's belief on the current block through experience, while lower-level states described the process in each block in a trial-based manner. In the model, RPEs occurred to unexpected rewards at the start of each block when the block estimation did not match the reward outcome. With learning, these RPE signals diminished, and the RPE signals to the cues evolved to reflect their updated value, as observed in the sham rats. We simulated the effects of HC lesions by blurring transition between upper-level states, resulting in uncertain block estimations. Thus, the model heavily relied on observations rather than transition probabilities in choosing states. As a result, the model quickly adapted to reward changes by altering its estimated states to those from other blocks, which kept RPEs close to zero. Despite the blurry transitions, the lesion model still updated its estimation on the current block, leading to the evolution of RPE signals to the cues, as seen in the HC lesioned rats. These results are consistent with the hypothesis that HC represents the state space and helps construct a cognitive map, particularly when states are partially observable, and show that HC critically impacts the computation of RPEs by DA neurons in the VTA.

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Neuroscience - Developmental

Widespread transduction of the primate brain with prenatal delivery of adeno-associated viral vectors

Genome editing technologies extend our ability to dissect the genetic and molecular mechanisms guiding the development and function of nervous systems. In non-human primates (NHP) the development of transgenic animals is time-consuming, labor-intensive, and costly, limiting the options to study and manipulate the neural circuitry most relevant to human behavior. Recent work with replication-defective recombinant adeno-associated viruses (AAV) has shown promise for the widespread introduction of foreign or modified genes (i.e., transgenes) across the brain, providing safe and long-term access to defined cell populations. In the present study we developed a new method for efficient intrauterine AAV-mediated transduction of the NHP brain through ultrasound-guided injections in fetuses. We developed this method in the rat model and applied it the marmoset (Callithrix jacchus), a small monkey species whose sensory and cognitive specializations are shared with humans. In this straightforward, minimally invasive nonsurgical procedure, we target AAV injections into the fetal cerebral ventricular system by advancing a small needle through the abdominal and uterine walls, resulting in demonstrably low risk of maternal and fetal complications. At present we can routinely achieve dense and ubiquitous expression of reporters and other genes throughout the cerebral cortex and other structures within the central and peripheral nervous system. In rats, we have been developing approaches to induce gene expression in select neural subpopulations through a combination of molecular specificity and postconception timing of the injection. Carrying this work into marmosets, we

recently implemented an intersectional strategy, using an AAV-based nestin-dependent expression of Cre recombinase, to selectively limit opsin expression to late-born neurons. The method of prenatal injection enables the rapid onset of transgene expression starting in the womb and offers new experimental opportunities to dissect brain processes across lifespan in primates. Notably, this flexible method allows researchers to use a range of genetic approaches to study childhood and adolescent developmental processes that critically shape the emergence and maintenance of complex sensory, motor, and cognitive functions in humans.

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Neuroscience - Developmental

Elucidating the Additional Level of Dopamine Signal Modulation from D1R/D2R Co-Expressing Cells in the Basal Ganglia

Dopamine (DA) signaling is a crucial to cue for motor function that, when impaired, manifests motor deficits. DA acts on two dissociable neuronal populations which express either D1 or D2 receptors (D1R, D2R). In the striatum, D1R cells modulate the direct pathway to initiate movement while D2R cells modulate the indirect pathway to oppose movement. Though the expression and function of these segregated populations is well-characterized, less is known about a subset of cells that co-express D1R and D2R. These D1R/D2R-co-expressing cells show increased striatal expression in mice with 16p11.2 deletion, a mouse model of autism spectrum disorder. Interestingly, 16p11.2 mice display motor behavior deficits, suggesting a link between motor dysfunction and increased D1R/D2R co-expression. In this study, we investigated the role of D1R/D2R co-expressing cells in wildtype mice to elucidate potential mechanisms which underlie motor deficits in the 16p11.2 model. We first used double transgenic reporter lines (Drd1-tdTmto;Drd2-eGFP and Ai65D) combined with light sheet imaging to quantify D1R-, D2R-, and D1R/D2R-co-expressing populations in wildtype mouse brain. Results showed an enrichment of D1R/D2R co-expressing cells in ventral striatum, specifically in nucleus accumbens. Brain slice imaging of a reporter line, Drd2-Flpo;Drd1-Cre;FRTtdTom;LoxEGFP, revealed D1R/D2R cells project from the dorsal striatum to the globus pallidus externus, and from the ventral striatum to the ventral pallidum, a pattern consistent with D2R projections. Next, to understand how D1R/D2R cells contribute to the direct and indirect pathway output, we chemogenetically activated and inhibited D1R/D2R cells in the presence of either D1R or D2R agonists and measured effects on locomotor behavior in wildtype mice. We found activating and inhibiting D1R/D2R cells either blunted or enhanced, respectively, the effects of a D1R agonist, and vice versa for the D2R agonist, suggesting bi-directional modulation of the direct and indirect pathway. Thus, altering the activity of D1R/D2R cells affects overall motor circuit modulation and thereby impairs motor output. We will next examine the role of these cells in modulating other DA-associated behaviors like reward seeking and novelty recognition, both of which are sensitized by drugs of abuse and impaired in 16p11.2 mice. This research reveals the role of these cells in DA signaling and signifies the importance of these cells in 16p11.2 deletion.

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Neuroscience - Developmental

Synaptotagmin β regulates neuropeptide release and circadian output in Drosophila

Neuropeptide signaling plays an important role in regulating a myriad of developmental, physiological, and behavioral functions throughout an animal's life cycle. Although much progress has been made in identifying the functional roles of a variety of neuropeptides, the molecular mechanisms controlling neuropeptide release remain largely elusive. The Drosophila ventral lateral neurons (LNvs) serve as a suitable system to study this fundamental question. LNvs produce pigment-dispersing factor (PDF), a neuropeptide well-known for its function in synchronizing of the central clock system and regulating the circadian output, such as the locomotor activity and sleep. Notably, despite the high level of pdf transcripts produced throughout the day, the amount of PDF peptides at the axonal terminal region of LNvs shows clear circadian oscillations, suggesting a mechanism that tightly controls the trafficking and release of PDF. Using cell-type-specific transcriptome analyses and genetic studies, we first examined a group of LNv enriched genes associated with vesicle trafficking and release. Our behavioral and anatomic data suggest that the Drosophila Synaptotagmin β (Sytβ), an atypical synaptotagmin enriched in peptidergic neurons, acts as a part of the control mechanism that regulates PDF release in LNvs. Similar as the pdf null mutants, sytβ knock-out flies show circadian behavior deficits, as well as abnormal distribution of PDF peptides. In addition, using the protein alignments of syt1 and sytβ, we identified that the C2B domain of sytβ mediates its inhibitory activity on PDF release. Using transgenic animals expressing two different chimeric Syt1-Sytβ proteins, our in vivo two photon imaging studies confirmed this model. Taken together, our genetic studies in the Drosophila system help us identify an inhibitory synaptotagmin that specifically controls neuropeptide release and physiologically regulates circadian output. This finding is potentially generalizable to other circuits and organisms and could help improve our understanding of the fundamental mechanisms underlying neuromodulation of behaviors and brain states.

Aijaz Naik
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Neuroscience - General

A Novel Behavioral Paradigm of Frustrative Non-Reward to Study Brain Mechanisms of Irritability-Like Behavior in Juvenile Mice

Emotion dysregulation is a symptom of many psychiatric disorders. Irritability, defined as proneness to anger that can reach a pathological extent, is a subtype of emotional dysregulation. Severe irritability is a defining symptom of disruptive mood dysregulation disorder (DMDD), a diagnosis in children of 6–18 years old. DMDD has long-lasting adverse outcomes, including high rates of school suspensions, hospitalizations, and suicide. Little is known about brain underpinnings of irritability, and treatment choices are limited and non-specific. Lack of behavioral paradigms to study irritability in model organisms is a major reason why the neuroscience of irritability is still in its infancy. Irritability typically manifests in the context of frustrative non-reward (FNR, frustration induced by omission of expected

reward). We therefore used FNR as a conceptual framework to develop a novel behavioral paradigm (Alternate Poking Reward Omission, APRO) to study the neural mechanism of irritability in juvenile mice that correspond to the age of children with DMDD. After APRO, mice were examined with a battery of behavioral tests to determine the effects of frustration and whole-brain c-Fos staining to map FNR-activated brain regions. Our results show that mice, regardless of sex, increased locomotion, and aggression towards conspecifics after APRO. There was no change in anxiety-like, depression-like, or non-aggressive social behaviors. FNR causes activation of 13 brain regions, including the prelimbic, ACC, hippocampus, dorsal thalamus, cuneiform nucleus, pons, and pallidum areas. Our co-activation network analysis shows that FNR makes the brain network adopt a more global processing mode. These results indicate that our novel FNR paradigm produces selective effect on motor activity and aggression, and that it alters the configuration of brain network. Its effects on behavior and brain network are similar to the observations made in children with severe irritability. This study lays the groundwork for future mechanistic studies of irritability and frustration that could potentially lead to more precisely tailored, innovative treatments for DMDD.

Daniel Zaldivar Perez
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Neuroscience - General
Resting state functional connectivity of face patch neurons during rest
Resting state fMRI (rs-fMRI) functional connectivity measures the coordination of spontaneous hemodynamic signals across the brain. It is widely accepted as a core noninvasive tool to infer the functional organization of brain in humans and animals, as it relies in the idea that areas showing coherent fluctuations are operationally functionally connected. Networks of functionally connected areas during rest are composed of the same areas engaged in task-based testing, such as during a motor action, cognitive operation or perceptual experience. However, as rs-fMRI is an indirect measurement of neural activity, measured through correlated dynamics in blood flow, its bearing on the structure and physiology of neural networks is imprecise. To what extent can the interaction of neurons in large-scale neural circuits be inferred from rs-fMRI measurements? In the present study we investigated this question using simultaneous local neural activity and fMRI signals across the brain of macaques. We measured local electrophysiological signals from two face patches, using these measures as seeds for analysis of brain-wide fMRI functional connectivity. Our results demonstrate some similarities, but also points of notable divergence between the electrophysiological and hemodynamic seeds in their relationship to fMRI fluctuations across the brain. For example, electrophysiological signals, including single-unit responses and high-frequency field potential responses, showed circumscribed, sometimes uni-hemispheric correlations across the brain. By contrast, the functional connectivity from fMRI seeds was broader and largely symmetrical across hemispheres. Further, electrophysiological seeds showed negative correlations with thalamic and brainstem neuromodulatory structures, whereas the fMRI seeds did not. These findings indicate that, while fMRI functional connectivity captures some aspects of the underlying circuitry, it may overestimate the breadth and bilateral symmetry of cortical interactions and may be insensitive to other types of whole-brain circuit interactions. The direct linking of local electrophysiological signals to whole-brain fMRI across the brain offers new avenues to study how local

neural signals contribute to the physiology of brain-wide neural circuits, both at rest and during behavior.

Miguel Arenivar
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Neuroscience - General
The role of somatostatin in the prefrontal cortex in shaping discrimination
The prefrontal cortex (PFC) is a neuronal hub critical for decision making, goal-directed behavior, and adaptive behavior. PFC circuits are tightly bound connections arising from various classes of cells. Disruption of this balance can lead to behavioral deficits that are relevant to psychiatric disorders, such as post-traumatic stress syndrome, schizophrenia, and/or depression. Somatostatin (SST)-expressing interneurons, a subset of inhibitory interneurons, contribute to excitation-inhibition balance of the PFC. Expression of SST has been canonically used as a genetic marker to define subclass of interneurons, and work has shown that they use the inhibitory transmitter GABA to influence PFC networks. However, little is known about the regulatory functions of SST, as a peptide neurotransmitter. Importantly, clinical studies have revealed decreased expression of SST in individuals suffering from the aforementioned mood and thought-related disorders. In this study, we used a combination of genetic and viral approaches, in-vivo calcium imaging, and behavior to determine the role that SST peptidergic transmission plays in PFC-mediated behaviors. I demonstrate that reducing SST expression through selective genetic ablation in the PFC impairs cued-fear discrimination. Furthermore, I used a novel genetically-encoded fluorescent SST receptor-based sensor to measure putative SST release in-vivo in the PFC during cued fear discrimination. I observed putative SST release during the acquisition phase, but not expression phase, of cued discrimination suggesting SST is critical for the learning of a behavior and not the expression of a learned behavior. Moreover, I found discrimination learning deficits in mice lacking SST in the PFC in other tasks including: an operant task, wherein mice press a lever to obtain appetitive rewards, sucrose preference, novel object recognition, and impaired nesting behavior. This suggests that SST peptide is critical for discrimination learning in both positive and negatively valanced tasks. Ongoing efforts are aimed at dissecting the role of acute SST signaling during different phases of discrimination learning and expression. Uncovering the dynamics of SST activity and subsequent SST neuropeptide release and how it shaped PFC-mediated behaviors, is a critical step in elucidating how SST shapes cortical circuits. These results also suggest that decreased SST expression in psychiatric disorders may contribute to cognitive deficits.

Sukanya Saha
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Neuroscience - General
Bioenergetic stress triggers Amyotrophic Lateral Sclerosis-like symptoms in mice
Optimum “bioenergetic balance” in the mitochondria, the powerhouse of neurons, is a key factor for proper brain functioning. The perturbance of this balance over time leads to a wide array of

neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS). ALS is a progressive, lethal neuromuscular disease characterized by degeneration of the cholinergic motor neurons (CMN), resulting in loss of voluntary movements, paralysis, and eventually death. Mitochondrial transcription factor A (Tfam) plays indispensable roles in regulating cellular bioenergetics by reducing oxidative stress and maintaining mitochondrial DNA and health. Here, we seek to elucidate the cause of neurodegeneration in ALS, which remains unknown, by studying the direct role of bioenergetic stress in its etiopathogenesis. For this, a new cholinergic Tfam-deletion mouse strain was developed (ChAT-cre;Tfam-/-, abbreviated as ChTf) with progressive loss of mitochondrial function in cholinergic neurons. In these ChTf mice, compared to their littermate controls, we observed a rapid decrease in body weights following 13.5 weeks of age. As they approach the 16th week of age, they show stooped posture and limited mobility in their home cages, potentially due to weakened muscle activity, and they resemble the predominant form of ALS (i.e. limb onset ALS). Behaviorally, this observation was reinforced in the rotarod test, when we found that ChTf mice showed persistent reduced motor performance and coordination age-dependently, exhibiting motor deficits with rotarod acceleration. In open field and Y-maze test, the ChTf mice did not show changes in exploratory behavior and memory. Further, the histological analysis of ChAT neurons revealed no damage to the basal forebrain and striatal cholinergic neurons. Together, the findings suggest the causality of bioenergetically stressed CMN in movement dysfunctions, implicating the potential damage of the CMNs in the brain stem and spinal cord. Most interestingly, the selective vulnerability of lower motor neurons revealed by equally applied bioenergetic stress bestows a newer angle to the susceptibility and treatment of CMN neurons in ALS. This indicates that differential bioenergetics is an important mechanism underlying CMN degeneration in ALS. Further, histopathology of the spinal cord, along with fiber photometry using ATP and cholinergic sensors, will validate the perturbed bioenergetic dynamics in multiple brain regions of this ALS model.

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Neuroscience - General

Domain-general and domain-specific neural evidence on experience and expectation of pain and aversive and pleasant tastes

Pain critically affects human physical and mental well-being, but we lack objective measures of pain. Brain imaging may provide potential biomarkers, but pain shares features and neural networks with other salient and aversive affective experiences. Whether a unique “pain matrix” functions distinctly from other salient experiences has been a long-lasting debate. Placebo effects demonstrate that expectations dramatically shape pain and clinical outcomes. Studies on expectation showed different neural substrates support modality independence (e.g. orbital frontal gyrus), modality dependence (e.g. sensory-specific cortex), and both (e.g. anterior insula and amygdala). However, we still lack direct evidence and a whole picture of domain-general and domain-specific neural mechanisms underlying pain and expectation, as compared to other salient aversive and non-aversive events (e.g. unpleasant or pleasant taste). To answer this question, we performed an fMRI experiment (n = 60) to investigate the neural mechanisms of expectancy and perception and applied cutting-edge approaches including multivariate pattern analysis based on machine learning, neural signature pattern comparison, and single trial fMRI analysis. We randomly assigned participants to receive noxious heat, unpleasant saline

solution, or pleasant sucrose solution during scanning (n = 20 per group). All participants underwent conditioning, in which one visual cue was followed by high-intensity stimulation and the other was paired with low-intensity stimulation. Then each cue was equally likely to be followed by stimulation of its conditioned intensity or a medium-intensity stimulus. Participants rated intensity and valence after each stimulus. Cues modulated intensity ratings for heat and salt but did not influence valence. Univariate and multivariate pattern analysis evidenced domain-dependent activations of high vs. low intensity for all three groups. Neural signature pattern comparison demonstrated pain-specific patterns for heat and shared negative affect patterns for heat and salt. Univariate and single trial analysis suggested domain-general activation in OFC at the stage of expectancy and experience for pain and salt. These findings 1) support the existence of the "pain matrix", and 2) provide direct evidence on the domain-general effect of expectation across aversive experiences. These attributions of pain should be considered when clinicians treat patients with acute and chronic pain.

Geoffrey Vargish
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Neuroscience - Neural Circuits

A novel, evolutionarily conserved inhibitory circuit selectively regulates dentate gyrus mossy cell function

The mammalian hippocampus supports spatial information processing and episodic memory encoding. Fundamental to this function is the ability to distinguish perceptually similar places or events, a process known as pattern separation. In the hippocampal circuit, this computation is performed by the dentate gyrus (DG). While granule cells (GCs), the main excitatory cell type in the DG, were canonically thought to enable pattern separation with their sparse activity profile and large cell numbers, recent evidence indicates that mossy cells (MCs), the other DG excitatory cell type, may play a critical role. In contrast to GCs, MCs are highly active and strongly innervate inhibitory interneurons (INs) in the DG. These divergent activity patterns coupled with MCs innervation of DG INs suggests that MCs drive disynaptic inhibition to actively shape GC sparseness, enabling pattern separation. However, the inhibitory circuits that regulate MC excitability and maintain high MC/low GC activity dynamics in the DG remain poorly understood. To address this, we used a combination of optogenetics, electrophysiology and in vivo 2-photon Ca2+ imaging to dissect inhibitory inputs to MCs and GCs. In mice, we identified a novel DG IN subtype that expresses vesicular glutamate transporter 3 (VGluT3) and has axonal arbors predominantly in the hilus, where MCs reside. Whole cell patch clamp recordings revealed that VGluT3+ INs preferentially innervate MCs over GCs while parvalbumin- and somatostatin-expressing IN subtypes were strongly biased to GC innervation. 2-photon Ca2+ imaging in awake, behaving mice corroborated these findings showing that chemogenetic activation of VGluT3+ INs significantly reduced in vivo MC activity but not GC activity. To probe the translational relevance of these unique inhibitory innervation patterns, we also evaluated inhibitory inputs to MCs and GCs in non-human primate (NHP) and human DG. Recordings of electrically evoked inhibition in both species, as well as optogenetically-evoked inhibition in NHPs using an IN-specific virus, revealed that inhibitory input to MCs, but not GCs, was significantly blocked by pharmacological activation of the cannabinoid receptor 1 (CB1), consistent with predominant VGluT3+ IN innervation. These findings establish that MCs and GCs have unique, evolutionarily conserved IN innervation patterns and suggest selective inhibitory circuits may be necessary to maintain DG circuit dynamics and enable pattern separation.

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Short-latency face preference in the primate superior colliculus depends on visual cortex
As social species, primates rapidly and preferentially detect and orient toward faces. This ability is crucial for interacting and communicating with others, and is exemplified by the fact that humans selectively move their eyes toward faces much faster than other stimuli. However, our understanding of face processing in the brain has been largely focused on the face-selective areas of the temporal cortex (the face patches), where the link to orienting behavior is not immediate. Moreover, primate newborns who have yet to develop organized cortical face-selective areas can still preferentially orient toward faces. This has led to the proposal of a potential subcortical route important for detecting and orienting toward faces, although the circuits are not known. Here, we show that the superior colliculus (SC), a vital subcortical structure for orienting, displays an extremely short-latency preference for faces. We recorded visually responsive neurons in the superficial and intermediate layers of the SC of two rhesus macaques while they passively viewed images presented in the neurons' visual receptive fields. We used 150 grayscale images of objects belonging to one of five categories that have been extensively used to test face processing in the temporal cortex: face, body, hand, fruit/vegetable, and human-made objects. Crucially, the 30 images comprising these categories were matched in their distributions of low-level features (RMS contrast, size, and power in three spatial frequency bands). This allowed us to determine how SC responses varied with object category in a manner that is independent of low-level visual features. We found that many SC neurons exhibited a preference for faces within 60ms of stimulus onset, well before neurons in temporal cortical face patches. Based on this short-latency visual activity, a linear classifier distinguished faces from other visual objects with accuracies of around 80%. Unexpectedly, inactivation of the lateral geniculate nucleus in the thalamus largely abolished SC visual responses, including face-related activity. Thus, contrary to expectations of a direct subcortical route for face processing, the short-latency SC preference for face stimuli depends on signals routed through the visual cortex. These results provide new insights into the primate visual system and suggest the existence of an unexpected circuit for rapid face detection.

Rachel Keith
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Neuroscience - Neural Circuits
Reduced long-term potentiation in the perirhinal cortex rather than the hippocampus mediates spatial learning impairments in the SCN2A mouse model of autism spectrum disorder
SCN2A codes for a subunit of the sodium channel Nav1.2, a protein critical to action potential initiation and backpropagation. In patients, haploinsufficiency of SCN2A increases susceptibility to autism spectrum disorder and cognitive impairments, including that of spatial learning. Yet, despite intense study, the brain areas, neural circuits, and mechanisms that result in this learning deficit remain unknown. We addressed this knowledge gap by studying the circuits and mechanisms which underlie spatial learning in mice haploinsufficient for Scn2a (Scn2a+/-). Using a Barnes Maze – a gold-standard

task to measure spatial navigation in mice – we revealed a spatial learning deficit in Scn2a+/- mice compared to wildtype littermates. Next, we used whole-brain light-sheet imaging, a cutting-edge technique, to quantify cFos immunoreactivity as a proxy of neural activity. Compared to wildtypes, 32 brain areas were significantly underactive in Scn2a+/- mice (19 cortical, 13 non-cortical). To test sufficiency of cortical Scn2a in spatial learning, we used an Emx1-Cre driver to restrict Scn2a loss to cortex and hippocampus; this was sufficient to recapitulate the spatial learning deficit and activity reduction in 17/19 cortical areas. We hypothesized these 17 cortical areas may act as a putative spatial learning circuit. Next, we used distinct CRE drivers to limit Scn2a loss to hippocampus and/or specific cortical layers. A key region was found: perirhinal cortex (PCtx), which integrates spatial and sensory information. We then examined long-term potentiation (LTP), a neural correlate of learning, in PCtx to ascertain the mechanism behind the spatial learning deficit and found an LTP deficit in Emx1Cre;Scn2a+/- mice. We hypothesized increasing neural activity in the underactive cortical regions may alleviate the LTP and spatial learning deficits. Indeed, expressing an Emx1Cre driven Gq-coupled DREADD in Scn2a+/- mice normalized brain activity, LTP, and spatial learning. We will next inject CRE-expressing virus into the PCtx of DREADD-floxed Scn2a+/- mice to determine if increasing PCtx activity alone is sufficient to rescue the LTP and learning deficits. If shown, PCtx dysfunction may serve as a key mediator of learning deficits in Scn2a+/- mice. Overall, these findings identify a neural circuit impaired by SCN2A haploinsufficiency, which may elucidate potential therapies for learning deficits associated with neurodevelopmental disorders.

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Neuroscience - Neural Circuits

Investigating the role of a recently identified midline thalamic projection to the area prostriata in defensive responses to aerial visual threats in mice

Survival in complex environments requires balancing metabolic needs with mitigating exposure to threat. In high threat-likelihood environments an organism may bias behavior away from foraging to minimize risk of threat exposure. As metabolic need increases, however, resolution of the need-state predominates, and foraging will be favored at the expense of an increased likelihood of encountering a threat. The paraventricular thalamus (PVT) is a critical nodal point for mediating metabolic homeostasis. Yet, how PVT balances resolution of metabolic need with guiding defensive responses to threat remains unclear. By combining molecular profiling of the mouse PVT with existing sequencing data from known thalamocortical cells, we recently identified a subpopulation of anterior PVT (aPVT) neurons that project to the area prostriata (APr), a limbic visual area whose function remains unknown. In both human, and non-human primates, APr is involved in the detection of rapidly moving peripheral objects. Based on its known tuning properties it has been proposed that this region may have evolved to guide defensive responses to threats arising from the periphery. Using the looming visual threat task in combination with in vivo fiber photometry we show that APr activity is increased following onset of the looming object. Chemogenetic inhibition of APr delayed escape behavior and reduced freezing to the looming stimulus, suggesting involvement in the detection of the visual object. Preliminary observations utilizing in vitro recording of APr neurons suggest excitation of aPVT fibers inhibits neuronal activity of APr cells. Follow-up experiments revealed conditions of metabolic stress (i.e., 24hr food restriction) delayed

escape behavior and prevented freezing to the looming stimulus. Using c-fos immunostaining as a proxy for neuronal activity we confirm that basal aPVT activity is increased following 24hr food restriction. These results are consistent with an interpretation that under conditions of metabolic stress, mice will inhibit threat responding in favor of foraging. Anatomical evidence positions the aPVT-APr pathway upstream of established mediators of visually evoked defensive behaviors, including the superior colliculus. That aPVT largely acts to inhibit APr, and APr is necessary for responding to visual threat, suggest a mechanism by which aPVT biases behavior away from threat responding and toward foraging when metabolic need is great.

Yiming Shen
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Neuroscience - Neural Circuits

Fever-like temperatures without immune activation alleviate cognitive deficits in the Scn2a autism mouse model

Autism spectrum disorder (ASD) prevalence is increasing – 1 in 54 children is diagnosed, and treatment options for its associated cognitive deficits are limited. Interestingly, 17 - 83% of ASD patients experience improved cognition during fever, a phenomenon called the 'Fever Effect'. Fever is considered an increase in body temperature (BT) beyond 38 degree and is usually induced by infection. It remains unclear whether cognitive improvements in ASD patients are facilitated by the thermal or immune component of fever. To test this, we newly developed a 'thermal fever' (TF) protocol to elevate mouse BT into fever range via exposure to infrared light. The TF protocol is combined with either the presence or absence of an immune challenge via a single intraperitoneal dose of the bacterial lipopolysaccharide (LPS) antigen. LPS alone did not elicit fever. In this study, we utilized mice haploinsufficient for SCN2A haploinsufficiency (Scn2a+/-), a well-validated model for ASD and intellectual disability. To assess cognition, Scn2a+/-mice and littermate controls performed a reversal learning test during exposure to the TF protocol, with and without immune challenge. Scn2a+/- mice made fewer errors during the task under TF, thus the thermal component of fever is sufficient to replicate the fever effect. To uncover the mechanism mediating improved behavior, we recorded evoked activity in cortical L2/3 pyramidal (PYR) neurons at increasing temperatures (30, 36, and 39 degree). Results revealed an enhanced spike probability at 30 and 36 degree, yet this normalized at 39 degree. We found that this spike probability normalization was associated with temperature-induced increases in potassium channel function in Scn2a+/- excitatory pyramidal neurons that elevated spike threshold. We next applied pimaric acid (a potassium channel opener), which decreased PYR neuron spiking in Scn2a+/- mice to wildtype levels at 36 degree. To confirm that this reduction in spiking improves cognitive function, we used an inhibitory DREADD to decrease cortical PYR neuron spiking during reversal learning. This intervention successfully resulted in normal reversal learning in Scn2a+/- mice. In sum, our results show the thermal component of fever is sufficient to improve cognitive impairments in Scn2a+/- mice, specifically by reducing abnormal spiking activity in cortical networks. These findings may lead to therapeutic strategies which reproduce the beneficial effects of fever in ASD.

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Neuroscience - Neurological and Neurodegenerative Disorders and Injury
Novel mechanisms of alcohol-mediated brain damage through the gut-brain axis: Protective role of mitochondrial aldehyde dehydrogenase-2
Mitochondrial aldehyde dehydrogenase-2 (ALDH2) metabolizes acetaldehyde, a major product of oxidative ethanol metabolism, into acetate. Hereditary ALDH2 gene mutation is found in approximately 35~40% of East Asians and ~8% of the rest of the world population. Absence of ALDH2 enzyme makes humans or mice more sensitive to alcohol (ethanol)-induced tissue damage, such as the gut and brain by accumulation of toxic acetaldehyde and other lipid aldehydes, including acrolein, which causes apoptosis and DNA damage. Yet the underlying molecular mechanisms of gut-associated brain injury is unknown. This study was aimed to investigate the molecular mechanisms of binge alcohol-induced gut and brain injury via gut-brain axis in Aldh2-KO mice. Age-matched young female Aldh2-KO and C57BL/6J wild type (WT) mice were exposed to three binge alcohol doses (4 g/kg/dose, oral gavage) at 12-h intervals. Serum, gut enterocytes, and brain tissues were collected from each mouse 1-h after the last alcohol dose. Intestinal tight junction and adherent junction (TJ/AJ) and apoptosis-related proteins were determined by immunoblots. Gut tissue sections were stained with H&E. Frozen mid-brain sections were analyzed by confocal microscopy to determine the rates of neuronal damage and inflammation, respectively. Binge alcohol exposure increased nitration of gut proteins. Immunoprecipitation followed by immunoblot analyses revealed that gut TJ/AJ proteins (Claudin-1, occludin, ZO-1, α-tubulin, etc.) were nitrated and ubiquitin-conjugated, leading to their proteolysis. The decreased levels of TJ/AJ proteins and increased apoptosis of gut enterocytes led to elevated gut leakiness, serum LPS (endotoxemia), ultimately brain injury, evidenced by increased Fluoro-Jade C and glial fibrillary acidic protein-positive cells in the hippocampus of the ethanol-exposed Aldh2-KO mice compared to those of the corresponding WT. Increased apoptosis and oxidative markers (cleaved Casp-3, PARP-1, Acrolein, CYP2E1, etc.) were observed in alcohol-exposed brain tissues of Aldh2-KO but not in WT mice. Confocal microscopy of alcohol exposed T84 colon cells also showed decreased intestinal TJ/AJ proteins with increased cell permeability to further support the mouse data. For the first time, these results show the novel mechanisms of alcohol-mediated gut and brain damage via the gut-brain axis and that ALDH2 is an important target for preventing neuronal tissue injury by alcohol and other toxic agents.

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Neuroscience - Neurological and Neurodegenerative Disorders and Injury
Studying NF-κB signaling during neuroinflammation in Alzheimer's Diseases in vitro using hiPSC-derived neural models
Alzheimer's Disease (AD) is characterized by the accumulation of extracellular amyloid beta (Aβ) and intracellular hyperphosphorylated tau (p-tau) proteins. Aβ plays a pivotal role in the progression of AD through its neurotoxic and inflammation effects. Nuclear factor kappa B (NF-κB) is a well-established inflammatory transcription factor that fuels neurodegeneration. This family consists of RelA, RelB, c-Rel,

NF-κB1 and NF-κB2. Depending on the cell type and/or combination of NF-κB subunits, the activation of NF-κB can play a dual role in either neuroprotection or neurodegeneration. Microglia are tissue-resident macrophages in the brain and their primary function is to protect the brain from pathogens and clear cellular debris including Aβ. The activation of NF-κB signaling and consequent release of cytokines and chemokines from microglia can lead to chronic inflammation in AD. We reported that ex vivo microglia consisted of two subpopulations distinguished by morphology and motility. Most cells formed a tight cluster, termed “clustered”. The other subpopulation of microglia with smaller cell size and high motility, was termed “free-roaming”. In aged microglia, the composition of clustered versus free-roaming subsets was shifted toward a higher prevalence of free-roaming cells where c-Rel is expressed and the canonical NF-κB signaling is more sustained. In this study, we developed in vitro neural models, using human induced pluripotent stem cell-derived neurons from healthy donors of various age groups and co-culturing them with microglia from NF-κB knock-in mice expressing fluorescently labeled endogenous c-Rel subunit (mScarlet-c-Rel). Live cell imaging of purified microglia upon Aβ incubation indicated variability of Aβ uptake timing and amounts. Moreover, in the neuron/microglia co-culture, Aβ phagocytosis is mostly carried out by microglia. A small fraction of microglia clears most of the Aβ. Deeper analyses of single-cell c-Rel time series data for Aβ phagocytosing microglia may reveal NF-κB dynamics which might be the coding language to convey specific information and enable appropriate responses. This new approach allows us to explore local and distal NF-κB activation in two subpopulations of microglia induced by Aβ. The findings of this research will shed light on how microglia and neurons communicate and coordinate in response to various immunological assaults including Aβ and p-tau.

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Neuroscience - Neurological and Neurodegenerative Disorders and Injury
How prion disease disrupts neurotransmission in the limbic system, an insight into physiological and molecular changes underlying the disease's behavioral and psychiatric symptoms.
Prion diseases are neurodegenerative diseases caused by misfolding of normal prion protein (PrPC) into pathological isoforms called prions. The disease is associated with cognitive decline and behavioral and psychiatric symptoms, which are directly linked to neurodegenerations in the limbic system, including the hippocampus, amygdala, and hypothalamus. Little is known about how the disease alters neuronal communication in the limbic regions; improving this knowledge would facilitate the development of therapeutic interventions. We used mouse models of prion disease to assess how the dysfunction of neurotransmission in these limbic regions evolved throughout the disease. We intracerebrally inoculated mice expressing wild-type PrPC with or without mouse-adapted prions and assessed the neuronal communication of the limbic regions in ex vivo brain slices starting at 50% of the disease progression to terminal stage disease (TSD). We used multi-electrode arrays to assess basal neurotransmission by stimulating slices with increasing electrical stimulation strengths. We then stimulated the slices with high-frequency stimulation to measure neuroplasticity, a neuro-electrophysiological correlate of cognition and mood. We found that the disease significantly altered both basal neurotransmission and plasticity in the limbic regions from as early as 50% of TSD, with these dysfunctions becoming robust after 60% of TSD. Notably, plasticity was significantly reduced in the

hippocampus and hypothalamus while increased in the amygdala, correlating with poor cognition and sleep with heightened fear and depression symptoms of disease. Consistent with the functional changes, the synaptic markers MAP2 (dendric spines), synaptophysin (pre-synapse), and post-synaptic density 95 (post-synapse) were reduced in the hippocampus and hypothalamus, while increased in the amygdala. Further immunofluorescence analysis revealed that the disease appeared to cause synaptic terminals in the amygdala to collapse and enlarge while retracting in the other regions. This explains the plasticity difference between the amygdala and other regions. Overall, we observed abnormal neurotransmission in the limbic system early in the disease, associated with mice displaying mild symptoms such as reduced nesting, a rodent psychiatric symptom. Our findings are important observations for future studies to interrogate the underlying molecular mechanisms of behavioral and psychiatric symptoms in prion diseases.

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Neuroscience - Neurological and Neurodegenerative Disorders and Injury
Multiomic characterization of the local impact of FTD/ALS-associated RNA binding proteins
Measuring up to a meter in length, highly polarized neurons must respond rapidly to distal stimuli; to accomplish this, neurons transport mRNAs and translate the encoded proteins in situ. This local translation adds an additional level of quality and temporal control, enabling rapid and specific responses to stimuli. Interestingly, defects in mRNA transport have been identified in several neurodegenerative diseases, including frontotemporal dementia and amyotrophic lateral sclerosis (FTD/ALS). Further, many FTD/ALS-associated mutations are found in RNA binding proteins (RBPs). These mutations alter stress granule formation, membraneless organelles that sequester mRNA and prevent translation upon exposure to exogenous stress. While the same RBPs are found in mRNA transport granules, their role in these granules is not well understood. To determine which types and how many transcripts are transported, we identified the local transcriptome in neurons derived from induced pluripotent stem cells (iPSCs). We grew iPSC-derived neurons on microporous membranes which retain cell bodies on the top while allowing axons to grow through to the underside of the membrane and performed RNA-sequencing on each fraction. We identified thousands of axon-localized transcripts, including transcripts encoding all ribosomal proteins and neurodegenerative disease-associated transcripts. As loss of RBP function has been implicated in FTD/ALS pathogenesis, we used CRISPR interference to knock down (KD) FTD/ALS-associated RBPs TDP-43, FUS, and hnRNPA1 to determine how loss of function affects mRNA transport and the local proteome of neurons. In exploring the differential effects of RBP KD on transcriptomic signatures, we found that TDP-43 KD induced the largest changes in the axonal transcriptome, with some transcripts changing compartment in the KD. We observed only modest changes in the local transcriptome in hnRNPA1 and FUS KD. However, we found that two related proteins, hnRNPA3 and TAF15, were upregulated in both compartments in hnRNPA1 and FUS KD neurons, respectively. Increased family member protein expression coupled with the modest changes in axon transcriptomics suggest hnRNPA3 and TAF15 may be compensating for hnRNPA1 KD and FUS KD. In conclusion, we have shown that protein family members compensate for RBP KD in mRNA transport, suggesting that increasing the expression of these family members may improve mRNA transport defects in FTD/ALS pathogenesis.

Paul Lafosse
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NIMH
Neuroscience - Sensory
Filtering of extraneous input patterns by visual cortex measured with single-cell stimulation
Brains comprise millions of neurons with highly complex patterns of connectivity, yet the brain readily coordinates widespread changes in neuronal activity to produce sensation and guide behavior. It is difficult to untangle the overarching rules of these changes – how input is transformed into output – because of not only feedforward and feedback influences, but also how dense local connections between neighboring cells impact individual cell activity. While individual cell input-output functions have been measured in vitro or in anesthetized animals, it is not well understood how typical levels of ongoing network activity in awake animals shapes these transformations. To investigate these local network influences, precise control of individual neuron activity is needed. To address this, we measured the input-output functions of excitatory neurons in primary visual cortex (V1) of awake mice using a combination of in vivo two-photon calcium imaging and cell-specific two-photon optogenetic stimulation during spontaneous conditions – that is, in the absence of visual stimuli – and during vision. By providing a fixed optogenetic input to cells (N=367) across conditions, we are able to reconstruct the average input-output function of these neurons. We find that as cells are suppressed below their spontaneous firing rates (due to feedforward visual input into the broader V1 network), they produce smaller, non-zero responses to fixed optogenetic input. On the other hand, as cells increase their firing rate due to visual input, responses to fixed optogenetic input remain unchanged before eventually saturating for the largest degree of visual drive. Overall, the average input-output function of V1 neurons is characterized by a supralinear regime for low input followed by a substantial linear regime before response saturation. Moreover, we find that cells sit just above the supralinearity on average during spontaneous conditions. These results suggest the network may attenuate inputs unrelated to the ongoing visual stimulus (i.e. inputs arriving to visually-suppressed cells) to effectively amplify relevant inputs to the network during vision. Taken together, this work proposes a new role of ongoing activity in densely-connected cortical networks of awake animals: to enhance sensory processing of external stimuli by selectively filtering out extraneous features of sensory input.

Raiza Hardy
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NCCIH
Neuroscience - Sensory
Deciphering the contribution of mechanosensation to dexterous behavior
Walking, eating, and reaching and grasping a small object are routine actions that require the combined function of the somatosensory and motor systems. A key component for the execution of successful dexterous movements is our sense of proprioception, or body positioning, which is essential for posture, balance, reflexes, and motor coordination. Proprioceptive information is initiated and transduced by sensory afferents, termed proprioceptors, that are activated by mechanical forces exerted onto skeletal muscles and tendons. Another key component of dexterous behavior is gentle touch, which allows us to rapidly and accurately adjust our grip to hold and manipulate objects and regulate our steps in response

to information about terrain surface when walking. Recent studies have identified a crucial role for the mechanosensitive cation channel, Piezo2, in mediating proprioception and touch. Notably, our group demonstrated that humans with PIEZO2 loss-of-function mutations display uncoordinated movements, are unable to feel vibration, lack tendon reflexes, and have significant impairments in gentle touch detection and skilled reaching. Moreover, studies in rodents closely paralleled the human findings and revealed that mice lacking Piezo2 in certain afferent populations experienced proprioceptive defects, including hind limb coordination deficits. In the present study, we begin to dissect the relative contribution of mechanosensation in distinct classes of gentle touch neurons and proprioceptors during execution of dexterous behaviors. Using mouse genetics, we ablated Piezo2 from genetically defined proprioceptors and touch neurons, and characterized how Piezo2 in these neuronal cell types contributes to specific aspects of innate and learned dexterous movements. We have found that mice lacking Piezo2 specifically in proprioceptors have improper gait and demonstrate difficulties in learning and performing operant conditioning tasks, such as lever pressing or nose poking. Furthermore, in vivo imaging of dorsal root ganglia revealed that molecular defined proprioceptors respond to natural stimuli, including muscle stretch and contraction. Our study is starting to elucidate the role of mechanosensation in proprioceptors and touch neurons for the execution of dexterous movements and holds a therapeutic promise for individuals affected by PIEZO2 loss-of-function or other patients with somatosensory loss.

Rosa Lafer-Sousa
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Neuroscience - Sensory

Behavioral detectability of optogenetic stimulation of inferior temporal cortex varies with the visibility and size of concurrently viewed objects.

Artificial perturbation of neural activity in the visual system is known to alter visual perception, yet the precise nature of these effects remains unclear. Clarifying the perceptual nature of these perturbations is essential for bridging the causal gap between neuronal activity and vision as a behavior, and for the development of visual prosthetics for patients with severe visual impairments. We have previously demonstrated that macaque monkeys' ability to behaviorally detect a subtle optogenetic impulse delivered to their inferior temporal (IT) cortex depends on some characteristics of the images viewed at the time of stimulation. This raises an intriguing question about the phenomenological nature of the perceptual event induced by stimulation: Does stimulation of the same neural population induce a consistent perceptual event, independent of the concurrently fixated image, that is more or less difficult to detect due to figure-ground effects? Or does stimulation induce a variable perceptual event depending on the concurrent visual input? To tease apart these two interpretations, we tested how diminishing the visibility of the visual input affected detection of the cortical event. In one experiment visibility was diminished by reducing the contrast, saturation, and spatial frequency of the objects viewed during stimulation. In a second experiment visibility was diminished by reducing object size. If cortical stimulation evokes a consistent perceptual event, it should be similarly if not more easily detected when the onscreen images are less visible. Two monkeys were implanted with LED-arrays over a region of their central IT cortex transduced with the depolarizing opsin C1V1. In each trial an image was displayed on the screen for 1s. In half of trials, randomly selected, an LED was turned on for 200ms

halfway through image presentation. The animal was rewarded for correctly identifying whether the trial did or did not contain cortical stimulation. Attenuating objects' visibility by diminishing their contrast, spatial frequency, and saturation significantly decreased detection performance, as did reducing their size. These results show that identical stimulation impulses induce variable perceptual events depending on the visibility of the objects viewed at the time of stimulation. The findings carry significant implications for the design and interpretation of perturbation studies, and for the development of visual prosthetics.

Santosh Kumar
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Neuroscience - Sensory
Anatomical and functional communication between vagal sensory neurons and pancreatic islet β-cells in mice
Brain and the gastrointestinal tract communicate via complex bidirectional processes. This higher order communication includes, but is not limited to, the parasympathetic (via the vagus nerve) and the sympathetic (via the prevertebral ganglia) arms of the autonomic nervous system. The role of the autonomic nervous system in metabolism and associated disorders is poorly understood due to insufficient understanding of the neural pathways that communicate with internal organs. Vagal sensory neurons (VSNs) are known to innervate the brain and the peripheral organs. Moreover, VSNs regulate the parasympathetic autonomic nervous system (PANS) via integrated bidirectional communication between the brain and the peripheral organs. However, the anatomical and functional details of these communications are unclear and, hence, are the focus of my research. My preliminary experiments using monosynaptic tracing in combination with RNAscope methods suggest the existence of molecularly diverse types of VSNs innervating pancreatic islet β-cells in mice. More specifically, I find that the VSNs anatomically connected to insulin producing β-cells express cocaine- and amphetamine-regulated transcript (CART) in addition to several other important genes. Further, I observed that CART-positive axons densely innervate the pancreatic islets, which is notable given that CART-deficient mice exhibit impaired β-cell function. The preliminary results on the functional aspects of this vagal CART neuron − islet β-cell circuit suggest a role in glucose homeostasis and food intake that depend on the metabolic state of the mice. Specifically, via chemogenetics approaches in combination with automated glucose telemetry, I find that activation of vagal CART neurons modifies blood glucose levels. Further, optogenetics stimulation of vagal CART neuron terminals in the brainstem alter food consumption. Delineating the vagal sensory complex-endocrine pancreas communications will unravel a central–peripheral neuronal circuitry vital to glucose homeostasis and metabolic disorders.

Ashley Nilson
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Neuroscience - Therapeutics and Translational Research
Identification and Optimization of Selectivity and Pharmacokinetic Stability of a Novel D2 Dopamine

Receptor Antagonist for the Treatment of Schizophrenia

Schizophrenia is a devastating neuropsychiatric illness that is the 15th leading cause of disability worldwide and is characterized by positive (hallucinations, delusions, paranoia), negative (flat affect, decreased motivation) and cognitive symptoms. All FDA-approved antipsychotic medications antagonize the D2 dopamine receptor (D2R), which is the mechanism of action for their efficacy in treating schizophrenia. Unfortunately, all current antipsychotics interact with numerous other receptors leading to off-target side effects such as sedation, weight gain, and diabetes, among others. We recently identified a novel D2R-selective antagonist, MLS6916, from a high throughput screen that when counter-screened against 168 receptors using a functional assay, a saturating concentration of MLS6916 only antagonized the D2R, and to a lesser extent, the D4 dopamine receptor (D4R). This unprecedented selectivity profile suggests that MLS6919 would exhibit few, if any, off-target side effects if employed as a therapeutic. Despite its promise as a drug lead, MLS6916 exhibited poor metabolic stability when assayed using rat liver microsomes. Thus, to chemically optimize this scaffold and explore its structure-activity relationships, more than 100 analogs were synthesized to identify modifications that improve its metabolic stability. All analogs were evaluated for D2R, D3R, and D4R activities using radioligand binding and beta-arrestin recruitment functional assays. Lead compounds were identified that exhibited both high affinity for the D2R and were selective versus the D3R and D4R. Notably, we identified analogs that exhibited high metabolic stability in human liver microsomes and improved metabolic stability in mouse/rat liver microsomes. We further determined the pharmacokinetic profiles of the most promising analogs in mice as there are good behavioral models for predicting antipsychotic efficacy in rodents. After injecting 30 mg/kg i.p., the analogs exhibited half-lives of 5-6 hr in both plasma and brain. Importantly, the analogs exhibited 1:1 brain-plasma ratios with max concentrations >10 uM indicating excellent brain penetration. In summary, we have identified lead drug candidates with exceptional D2R-selectivity, excellent metabolic stability in human liver microsomes, and sufficient metabolic stability in mice to conduct behavioral studies. Studies investigating the antipsychotic activities of these analogs in mouse models are currently being pursued.

Lauren Henry
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NIMH
Neuroscience - Therapeutics and Translational Research

CALM-IT: Feasibility, reliability, validity, and clinical relevance of a novel mobile application probing inhibitory control

Background: The identification of brain-based mechanisms underlying psychopathology is critical to developing efficacious interventions. Inhibitory control, or the ability to modulate prepotent behavioral responses, is mediated through established neural circuitry and deficits are associated with psychopathology. However, existing tools for assessing inhibitory control are repetitive, time intensive, expensive, and laboratory based, posing barriers to broad dissemination. We examined the utility of "CALM-IT," a novel, gamified inhibitory control task based on the Go/No-Go paradigm delivered via mobile application. In four aims, we investigated the (1) feasibility, (2) reliability, (3) validity, and (4) potential clinical relevance of CALM-IT. Methods: We recruited 148 youth (56.2% male, 8-18 years) from the community, including typically developing youth and youth with primary diagnoses of disruptive mood dysregulation disorder, attention deficit hyperactivity disorder [ADHD], and anxiety. At home,

youth completed CALM-IT on a mobile device across two sessions approximately one week apart. We operationalized inhibitory control performance during CALM-IT as d', the standardized difference between hits versus false alarms. Higher d' indicates better performance. In the laboratory, youth completed four canonical inhibitory control paradigms (Anti-saccade, AX Continuous Performance, Flanker, and Stop Signal tasks) from which we calculated a latent factor of inhibitory control. In addition, parents and youth completed dimensional measures of irritability, ADHD, anxiety, and depression. Results: Aim 1. Approximately 99.30% of CALM-IT levels produced complete and usable data. On average, youth hit 90.82% of targets, demonstrating task engagement. Aim 2. Good consistency emerged between the two CALM-IT sessions (d' ICC=.85). Aim 3. Greater inhibitory control as measured by CALM-IT d' was associated with greater latent inhibitory control (r=.38, p<.001). Aim 4. Consistent with hypotheses, lower inhibitory control as measured by CALM-IT d' was associated with higher levels of parent-reported (r=-.21, p=.02) and youth-reported (r=-.22, p=.02) irritability and parent-reported ADHD symptoms (r=-.23, p=.01). CALM-IT d' was not associated with anxiety or depression symptoms, demonstrating specificity of effects. Conclusion: Taken together, findings provide preliminary support for CALM-IT feasibility, reliability, validity, and clinical utility.

Seungmi Ryu
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Neuroscience - Therapeutics and Translational Research

Modeling chemotherapy-induced peripheral neuropathy using an iPSC-derived human dorsal root ganglia organoid model for high-throughput drug screening

Chemotherapy-induced peripheral neuropathy (CIPN) is the most common dose-limiting adverse effect of chemotherapy, resulting in numbness, hypersensitivity, and pain. CIPN affects the quality of life of over 60% of chemotherapy patients. However, there is a lack of advanced models for screening CIPN, as most of our current drug testing platforms are either based on monolayer cell culture or animal models. To address this, we developed a 3D in vitro human dorsal root ganglia (DRG) organoid model from induced pluripotent stem cells (iPSC) to better assess the effect of chemotherapeutic drugs on CIPN. The DRG is a crucial organ in the peripheral nervous system (PNS) for pain perception, transmitting sensory information from peripheral organs to the central nervous system. To establish a foundation for our project, we first generated 3D human DRG organoids (DRGOs) and confirmed proper differentiation with immunohistochemistry and flow cytometry. Next, we profiled the cellular subtypes within the DRGOs through single-cell sequencing and western blot analyses. These analyses revealed the presence of diverse sensory neuron subtypes (i.e. mechanoreceptor, proprioceptor, and nociceptor) along with glial subtypes (i.e. satellite glia and Schwann cells) commonly present in the DRG. Electron microscopy further confirmed the myelination of axons by Schwann cells, which is important for rapid conduction of action potentials along the axons, but often challenging to model in vitro. We then investigated the effect of PNS glial cells on DRG function. The presence of PNS glial cells significantly enhanced neurite growth and electrophysiological activity in the DRGOs, which we confirmed via microelectrode array and calcium activity imaging assays. These results highlight the importance of proper induction and interaction between glial and neuronal cells for the establishment of a fully functioning DRG model system. Lastly, we demonstrate the application of DRGOs in high-throughput screening for compounds that induce CIPN. Using a set of neuropathy-inducing chemotherapeutic drugs, we tested the dose-

dependent effect of these drugs on DRGOs, looking at changes in neurite length and ATP production. Overall, our study demonstrates that DRGOs can serve as a physiologically relevant 3D model for high-throughput drug screening and investigation of CIPN, therefore providing a more accurate and predictive model for drug safety assessment.

Changyou Shi
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Omics - Genomics/Transcriptomics
Remodeling of the enhancer landscape induces an immune response in aged muscle stem cells
Skeletal muscle aging is characterized by significant reduction of muscle mass and strength, due in part to the loss in number and function of muscle stem cells (MuSCs). The transcriptional networks and epigenetic changes (histone modifications, 3D genome structure etc.) that confer diminished regenerative function in MuSCs with age are only partially understood. In this study, we use an integrative genomics approach to profile transcriptome, epigenome, and 3D genome architecture in isolated MuSCs from young (2-4 months), old (20-24 months), and geriatric (28-32 months) mice. From bulk transcriptomics data, MuSCs display an elevated immune response and quiescence exit signature during aging. Specifically, we found many inflammatory cytokine genes (Ccl2, Cxcl10, Il6, Cx3cl) are highly induced in aged MuSCs, likely revoking proliferative arrest. In contrast, notch pathway (Notch2, Hey1, Heyl, Calcr) and upstream collagen V genes (Col5a1 and Col5a3) are downregulated during aging leading to quiescence exit. Together, these changes may contribute to premature activation and decline in MuSC number and function with age. To further elucidate the epigenomic basis for these transcriptomic changes, particularly the elevated immune response with age, we performed two assays: (1) assay for transposase-accessible chromatin coupled to sequencing (ATAC-seq) to determine chromatin accessibility genome-wide, and (2) cleavage under targets and release using nuclease (CUT&RUN) with H3K4me1 and H3K27ac antibodies to capture enhancer landscapes in young and old MuSCs. A Hidden Markov Model-based chromatin state analysis showed active enhancers marked by both open chromatin and H3K27ac/H3K4me1 dual modifications are highly enriched in old MuSCs. Concordantly, a greater number of super (large) enhancers were identified agnostically by the algorithm Rank Ordering of Super-Enhancers (ROSE). We also noted a strong enrichment of H3K27ac at both super enhancers and typical (small) enhancers. Finally, using the high-throughput chromosome conformation capture technique HiC, we found that these new age-related enhancers loop over to immune genes driving the elevated immune response observed in old MuSCs. Overall, in this study, we show that aberrant transcriptomic changes during aging such as upregulation of immune genes can be driven by distinct changes in the non-coding genome to affect MuSC function.

Liping Yang
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NCI-CCR
Omics - Genomics/Transcriptomics
Elucidating novel immune checkpoint receptor-ligand interactions using a robust CRISPR/CAS9 activation

platform

Background: Immune checkpoints are receptor-based signaling molecules that regulate the immune system to maintain self-tolerance and are exploited by tumor cells to evade immune surveillance. Hence, immune checkpoint inhibitors such as antibodies against Cytotoxic T-Lymphocyte-Associated Antigen 4 (CTLA4), Programmed Cell Death-1 (PD-1) and Programmed Cell Death-Ligand 1 (PD-L1), and immune checkpoint stimulators such as agonistic antibodies against 4-1BB (CD137, tumor necrosis factor receptor superfamily 9) have become important cancer therapeutics. However, further development in this area is hindered by the fact that many checkpoint family members still lack known binding partners, due in part to the challenging nature of receptor deorphanization. Methods: Here, we exploited CRISPR/CAS9-guide mediated gene activation (CRISPRa) to create a library of activator cells where each cell expresses on average a single transmembrane encoding gene. To optimize bait binding affinity, we compared protein A-, anti-HIS-mAb- and streptavidin-linked magnetic beads armed with various bait ectodomain proteins fused to Fc, 6xHIS or biotin, respectively. To further improve sensitivity, we performed serial enrichments using magnetic-activated cell sorting, expanding the sorted cells in culture between each enrichment. Moreover, magnetic beads bound to both bait and FITC were used in flow cytometry to monitor enrichment. Genes enriched in target cells were identified using next-generation deep sequencing. Results: Using CTLA4 ectodomain as bait we were able to identify CD80 and CD86, its two known binding partners, as the top hits in our screen. Furthermore, PD-L1 and 4-1BB were able to identify their known binding partners, PD-1 and 4-1BB ligand, respectively. Streptavidin-bait coated magnetic beads consistently displayed more robust enrichment than protein A or anti-HIS beads in multiple screening campaigns. Interestingly, after the 3rd round of enrichment, we also identified a novel cell surface binding partner of 4-1BB and validated the interaction using flow cytometry, ELISA and surface plasmon resonance analysis. Furthermore, the novel binding partner was also able to modulate 4-1BB-induced NF-kappa B signaling pathway. Conclusion: This cell-based CRISPRa system provides a robust and sensitive platform to explore uncharacterized immune checkpoint receptor-ligand interactions, which is essential to develop the next-generation of immune checkpoint therapeutics.

Mary Makarious
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Omics - Genomics/Transcriptomics

Genome-wide Association Identifying Novel Etiological Insights Associated with Parkinson's Disease in African and African Admixed Populations

Background: African and African admixed populations have greater genetic diversity and unique linkage disequilibrium patterns, making them ideal for studying complex genetic traits, such as Parkinson's disease (PD). Previous PD research has focused largely on European populations, leading to significant gaps in knowledge about PD genetics in traditionally excluded, underrepresented populations. This study aimed to conduct the first genome-wide assessment of PD genetics in African and African admixed populations to fill this gap and inform future clinical trials. Methods: The study analyzed genetic data from 197,918 individuals (1,488 PD cases, 196,430 controls) of African and African admixed ancestry. We identified population-specific risk factors, differential haplotype structures and admixture, coding and structural genetic variations, and polygenic risk profiling. Short- and long-read whole genome

sequencing analyses were conducted to identify any coding or structural variants underlying the GWAS signal. Findings: The study identified a novel African-specific genetic risk factor for PD and age at onset at the GBA1 locus. This risk factor was rare in non-African/African admixed populations and was found to mediate PD risk via expression quantitative trait locus (eQTL) mechanisms. While previously identified GBA1-associated disease risk were via coding mutations, here we suggest a novel functional mechanism consistent with a decreasing trend in glucocerebrosidase activity levels. We also characterize polygenic risk profiling and highlight the utility of the African and African admixed risk haplotype substructure for future fine-mapping efforts. Interpretation: By conducting the first genome-wide assessment of PD genetics in these populations, the study dissects differences in risk and age at onset, characterizes known genetic risk factors, and identifies a novel disease mechanism via decreased GBA1 activity levels. This work represents a valuable resource for pioneering research and sheds light on molecular mechanisms involved in PD. Deciphering causal and genetic risk factors in all these ancestries will help determine whether interventions, potential targets for disease-modifying treatment, and prevention strategies that are being studied in the European populations are relevant to the African populations. The present study represents a valuable resource for pioneering research within the Global Parkinson's Genetics Program (GP2) and beyond.

Sumit Mukherjee
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Omics - Genomics/Transcriptomics

Alternations of RNA splicing in MAPki-resistant melanoma and predicting patient's treatment response using the expression profiles of altered transcript isoforms

The development of resistance to MAPKi treatments in a substantial number of melanoma patients presents a significant clinical challenge, as it is associated with cancer relapse and reduced patient survival rates. The clinicians still face significant limitations in identifying beforehand which patients will respond to treatment and which will not. Despite multiple studies aimed at comprehending the molecular mechanisms responsible for intrinsic resistance, little is still known about why some patients do not respond to MAPki treatments. Further, the mutations and gene expression patterns observed in resistant tumors do not provide enough information to distinguish between individuals who will respond and those who will not respond to MAPKi treatment. In cancer cells, alternations of RNA splicing can contribute to drug resistance, as it can alter the expression of drug targets or activate alternative signaling pathways that promote the survival of the cancer cells in the face of drug-mediated damage. For example, some cancer cells can splice out exons that encode drug-binding domains, rendering the drugs ineffective. Further, the cancer cells can splice into exons that encode truncated, non-functional proteins that interfere with drug activity. Despite the critical role of alternative splicing in drug resistance, no previous studies have investigated the landscape of altered transcripts in MAPKi-resistant tumors and their associated functional consequences. We obtained three publicly available RNA-seq datasets comprising a total of 178 melanoma patients who had undergone MAPKi treatment and were categorized as either responders/sensitive or non-responders/resistant. By utilizing various computational methods, we have identified a common set of differentially expressed transcript isoforms with potential functional impact in resistant tumors, which can’t be detected from the traditional differential gene expression analysis. Additionally, we are currently working on developing a machine

learning-based predictor to determine whether the expression of these altered transcript isoforms can accurately differentiate between responders and non-responders to MAPKi treatment. To summarize, our study has identified the landscape of altered transcript isoforms in MAPKi-resistant tumors, indicating that targeting transcript isoforms specific to resistant tumors may be a promising strategy for developing precision oncology-based treatments for melanoma.

Jungeun Lim
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Omics - Metabolomics/Proteomics
Black - White differences in prospective serum metabolites and biochemical pathways associated with prostate cancer risk in the COnsortium of METabolomics Studies (COMETS)
Prostate cancer incidence and mortality rates are substantially higher in Black men as compared with White men. Although prospective studies have examined metabolites in relation to prostate cancer risk, it is unclear whether and which metabolites and biochemical pathways may explain the racial disparity. To address this question, we examined metabolomic profiles of prostate cancer risk by race within the COnsortium of METabolomics Studies (COMETS). Meta-analysis of eight prospective cohort studies including 2,060 prostate cancer cases and 2,350 controls was conducted using the multivariable logistic regression model in COMETS Analytics v2.0, the qqman package of R 3.6.3, and random effects models (STATA16.0). Among a total of 1,452 harmonized metabolites, 911 metabolites measured in at least 3 studies were included in this meta-analysis. Overall, 15 metabolites including 4 amino acids, 4 cofactors and vitamins, 3 lipids, 2 peptides, 1 nucleotide, and 1 xenobiotic were significantly associated with prostate cancer risk at P<0.05 including L-cystine (1-SD OR=0.77; P=0.009) and indoxyl sulfate (1-SD OR=0.88; P=0.002). In the race-stratified analysis, lipids including pimeloylcarnitine/3-methyladipoylcarnitine (1-SD OR=1.29; P-value=0.0003) and LysoPA(16:0/0:0) (1-SD OR=1.15; P-value=0.0003) were predominantly and positively associated with prostate cancer risk in White men. In Black men, 39 metabolites including 11 amino acids were significantly associated with prostate cancer risk. Amino acids related to tryptophan, lysine, and phenylalanine metabolism were inversely associated with prostate cancer risk; e.g., the strongest metabolite being alpha-N-phenylacetyl-L-glutamine (1-SD OR=0.81; P=0.003) which is produced by Clostridial anaerobic bacteria in the gut. Some lipids related to fatty acid metabolism were associated with higher prostate cancer risk in both Black and White men (e.g., docosahexaenoylcholine in Black men and 3-hydroxybutyroylglycine in White men). Alpha-tocopherol (vitamin E) was inversely associated only in White men (1-SD OR=0.84; P=0.026). There may be racial differences in prospective serum metabolites and biochemical pathways associated with prostate cancer risk that require consideration in future multi-racial studies. To our knowledge, this is the first metabolomic meta-analysis of prostate cancer risk which provides evidence of biochemical etiologic differences relevant to the Black-White prostate cancer disparities.

Maria Mironova
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Omics - Metabolomics/Proteomics

Postprandial changes in plasma proteome in non-alcoholic fatty liver disease

Background: Non-alcoholic fatty liver disease (NAFLD) is the most prevalent liver disease globally. A subset of subjects with NAFLD can further develop non-alcoholic steatohepatitis (NASH), a progressive condition at risk of liver-related morbidity and mortality. It is still unclear what drives the progression of steatosis to NASH. Although energy supply is key to the pathogenesis of NAFLD, most studies focus on the outcomes of long-term energy imbalance, while the acute alterations of metabolism postprandially are relatively unstudied. We have previously identified unique postprandial alterations in the plasma lipidome in subjects with NAFLD. We hypothesized that similar unique alterations will be seen in the plasma proteome. Methods: single-center prospective study. Subjects with NAFLD and healthy controls were fed a standardized liquid meal. Plasma samples were obtained in the fasting state, and 2 and 4 hours after the meal. 1,317 unique proteins were quantified using the SomaScan assay. Descriptive statistics and repeated measures ANOVA were performed in RStudio. Results: 34 subjects with NAFLD and 7 controls were included in the study and as expected, significantly differed in BMI, ALT, AST, HbA1c, fasting glucose and insulin, and triglycerides (p<0.05). There were significant differences in postprandial temporal patterns between groups for several plasma proteins: haptoglobin, albumin, leptin receptor, C-C motif chemokine 16 (CCL16) and 23 (CCL23), hepcidin, cathepsin B and two serpins, protein Z-dependent protease inhibitor (ZPI) and C1 esterase inhibitor (C1IN). The postprandial changes in haptoglobin, albumin, hepcidin and C1IN were blunted in subjects with NAFLD compared to controls. The temporal patterns of CCL16 and CCL23 showed opposing directions of change between NAFLD and healthy subjects. Conclusions: We observed in NAFLD an altered postprandial pattern in several proteins; interestingly all belong to the class of acute phase reactants and are related to the innate immune response. We hypothesize that liver injury leading to NASH occurs due to postprandial oxidative stress, endothelial dysfunction, or microbial translocation. The blunted postprandial response in subjects with NAFLD may suggest impaired compensatory mechanisms in NAFLD and greater susceptibility to injury. This is the first study in humans exploring postprandial changes in the plasma proteome in NAFLD.

Laura Kammel

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NIEHS

Oncology - Development and Metastasis

Circadian disruption induces breast cancer-permissive estrogen receptor transcriptional program in hormone sensitive mammary epithelium

The International Agency for Research on Cancer classified night shift work, or shift work involving circadian disruption, as “probably carcinogenic to humans” based on rodent and human breast cancer studies. However, despite this established risk association and the known physiological effects of disrupted light-dark cycles, the mechanism linking circadian disruption with mammary carcinogenesis remains poorly understood. Because abnormal estrogen receptor (ER) signaling drives most breast cancers, I asked if circadian disruption alters the estrogen-ER transcriptional signature of ER sensitive mammary epithelium. I first crossed an ER reporter strain with a mouse model that lacks the core circadian clock gene Bmal1 (Bmal1KO) and which is arrhythmic in constant darkness. Then, I ovariectomized and entrained ER-reporter+ wt and Bmal1KO mice to 12:12 light-dark conditions before

transitioning them to constant darkness. After confirming arrhythmic behavior, I acutely stimulated mice with estradiol to induce an estrogen response and isolated mammary cells enriched for expression of the ER reporter and an epithelial cell marker by FACS. Single-cell RNA sequencing indicated that most captured cells were classified by the established mammary epithelial hormone-sensing (HS) lineage. Strikingly, I found that wt and Bmal1KO cells occupied largely distinct HS subclusters. Further analysis revealed differential enrichment of estrogen-induced genes, such as Stc2 and Areg, in Bmal1KO- and wt-enriched clusters, respectively. Surprisingly, Bmal1KO-dominant clusters also showed enrichment of ER cooperating pioneer transcription factors Gata3 and FoxA1. While necessary for normal mammary gland development, Gata3 and FoxA1 are thought to promote breast cancer-specific ER transcriptional programs during the transition from healthy to cancerous epithelium by increasing ER chromatin accessibility. Finally, clusters predominant in Bmal1KO cells also showed enrichment of the RNAPII scaffolding complex Ctr9, which has been shown to maintain global chromatin occupancy of ER and RNAPII in ER-positive breast cancer cells in the presence of estrogen. Together, my work shows that circadian disruption alters both the ER-induced response and the expression of factors that regulate global accessibility of ER-target genes in estrogen sensitive mammary epithelium. We propose that this altered ER transcriptional program could induce a cell state permissive of oncogenic transformation.

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Oncology - Development and Metastasis

SOX9 is a key component of RUNX2-regulated transcriptional circuitry in osteosarcoma

Osteosarcoma (OS) is a prevalent type of bone cancer that primarily affects children and adolescents, and the 5-year survival rate is 25% with recurrent or metastasized tumors. Unfortunately, the standard care of OS has not advanced significantly, and there is no FDA-approved targeted therapy and immunotherapy for OS. Therefore, there is a urgent need to develop new therapeutic targets to reduce side effects and improve the prognosis for OS patients. The lack of targetable genetic alterations in OS suggests that transcriptional and epigenetic events are involved in the etiology of this deadly cancer type. Thus, understanding of the master transcriptional circuitries is essential for developing novel therapeutical strategies for OS. We found that RUNX2, a transcription factors (TFs), is critical for the survival of OS cells, and it is highly expressed in OS cells and tumors. However, the transcriptional network related to RUNX2 in OS remains largely unclear. Using an integrated multi-omics approach including RNAseq, ChIPseq, and proteomics, I identified a dozen of transcription factors in the RUNX2-regulated transcriptional circuitry. Among these factors, I focused on SOX9 as a direct target of RUNX2 and assessed its function to promote OS cell survival by employing CRISPR-Cas9-mediated knockout. The depletion of SOX9 induced apoptosis in vitro and reduced OS tumor growth in vivo. To identify SOX9 downstream targets, I used RNAseq, ChIPseq, pathway analysis, and gene set enrichment analysis and found that SOX9 activated the transcription of MYC, a downstream target of RUNX2. To further explore the SOX9-regulated network in OS, BioID was carried out to identify the interactome of SOX9. Interestingly, SOX9 binds to RUNX2, suggesting a transcriptional network involving SOX9, RUNX2, and MYC. In addition, I identified the chromatin factor JMJD1C as a novel binding partner of SOX9, and the proximity ligation assay (PLA) confirmed the interaction between SOX9 and JMJD1C in OS cells. Also, JMJD1C depletion caused reduction of OS xenograft tumors growth, and phenocopied SOX9 depletion.

From these result, I suggested that SOX9 and JMJD1C interact to promote OS growth. Because inhibitors of JMJD1C are being developed, my results reveal a possible targeting node regulated by SOX9 in the RUNX2-regulated transcriptional circuitry and are likely to contribute to developing targeted therapy for OS to overcome the current limitation in clinical trials.

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Oncology - Development and Metastasis
Tumor necroptosis-mediated shedding of cell surface proteins promotes metastasis of breast cancer by suppressing anti-tumor immunity
Necroptosis is a form of regulated necrosis and is executed by Mixed Lineage Kinase domain-Like protein (MLKL). MLKL is engaged in triggering the rupture of cell plasma membrane. MLKL activation also leads to the protease, A Disintegrin And Metalloproteases (ADAMs)-mediated ectodomain shedding of cell surface proteins of necroptotic cells. As the rupture of plasma membrane and the shedding of cell surface proteins of necroptotic cells in tumor results in the release of many tumor factors into the tumor microenvironment, it has been suggested that tumor necroptosis affects tumor progression through modulating the tumor microenvironment. However, the exact mechanism by which tumor necroptosis promotes tumor metastasis remains elusive. Here, we report that the ectodomain shedding of cell surface proteins of necroptotic cells is critical for promoting breast tumor metastasis because the soluble, shedded cell surface proteins inhibit the anti-tumor activity of T cells. (1) We found that blocking tumor necroptosis by MLKL deletion in two preclinical breast cancer models led to the dramatic reduction of tumor metastasis to the lungs and that the anti-tumor activity of tumor infiltrating and peripheral blood T cells was significantly elevated in MLKL null tumors and tumor-bearing mice. (2) Importantly, the increased anti-tumor activity of T cells in MLKL null tumors/mice is a key cause for the reduced metastasis as the depletion of CD8+ T cells completely restored the levels of metastasis in the mice with Mlkl KO tumors. (3) Interestingly, the levels of some soluble cell surface proteins including E-cadherin that are known to promote metastasis are also dramatically reduced in MLKL null tumors/mice. (4) Administration of ADAMs inhibitor reduces the levels of soluble cell surface proteins in WT tumors/mice and leads to the elevated anti-tumor activity of both tumor infiltrating and peripheral blood T cells and the dramatic decrease of metastasis. (5) Finally, we showed that E-cadherin/Killer cell Lectin-like Receptor subfamily G member 1 (KLRG1) is the major pathway for necroptosis-mediated suppression of the anti-tumor activity of T cells and the promotion of metastasis. Hence, our study reveals a novel mechanism of tumor necroptosis-mediated promotion of metastasis and suggests that tumor necroptosis and necroptosis-activated ADAMs are potential targets for controlling metastasis.

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Oncology - Epidemiology and Surveillance
Sex differences in the association of ionizing radiation exposure and lung cancer death in the US

Radiologic Technologists Cohort

Is there a higher risk of radiation-induced lung cancer in females compared to males? The gold standard cohort, the Life Span Study of Japanese atomic bomb survivors, suggests females are at greater risk. Although findings from this Japanese wartime population may not be generalizable to a healthy US population, NASA relies on these results, estimating a greater risk of lung cancer from space radiation in females than males if missions are equitably assigned. Using findings from US-based occupational cohorts to update risk projection models is essential. However, most US-based cohorts are limited by small numbers of females and inadequate data on smoking, which may bias the radiation-lung cancer relationship. The US Radiologic Technologists cohort includes over 110,000 workers with occupational radiation exposures, is 75% female, and has smoking information for 98% of the cohort. The US Radiologic Technologists cohort provides an opportunity to update US cancer risk projection models with US-based, sex-specific lung cancer risk estimates. US radiologic technologists who responded to a baseline questionnaire in the 1980s-1990s were included in lung cancer mortality analyses through 2021. Annualized, cumulative individual radiation doses to the lung were estimated from 1916-1997, and we captured smoking behavior from questionnaire responses. We used Poisson regression to estimate excess relative risk (ERR) of lung cancer mortality per 100 milligray (mGy), adjusting for age, year of birth, sex, baseline smoking status (ever/never/former), total pack-years, and years since quitting smoking (for former smokers). There were 555 lung cancer deaths among 25,894 male and 1,128 among 80,183 female technologists. Across sexes, we found no evidence of increased lung cancer mortality risk with increasing occupational radiation dose [ERR/100 mGy: 0.04, 95% confidence interval (CI): -0.11, 0.19]. Sex-specific findings showed that the male ERR decreased with dose while the female ERR increased (male ERR/100 mGy: -0.14, 95% CI: -0.26, -0.12; female ERR/100 mGy: 0.18, 95% CI: -0.07, 0.43; p-sex difference: 0.06). We did not observe increased risk of lung cancer mortality with increasing radiation dose overall. There was some evidence for sex-specific differences in dose-response pattern, with females at a greater, albeit imprecise, risk of radiation-induced lung cancer than males. Our results could inform future updates to NASA lung cancer risk projection models.

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Residential proximity to carcinogenic industrial air emissions and breast cancer incidence in a United States-wide prospective cohort

Purpose: To evaluate whether residential proximity to air emissions of industrial carcinogens, both singly and in combination, is associated with breast cancer incidence. Methods: Using the United States (US) Environmental Protection Agency's Toxics Release Inventory, we estimated the 10-year annual average air releases of 26 known or probable carcinogens within 3, 5, and 10 km of Sister Study participants' baseline residences (n=50,343, 2003-2009). We used Cox proportional hazards regression to estimate adjusted hazard ratios (HR) and 95% confidence intervals (CI) for the association between ambient concentrations of each individual compound and incident breast cancer. To assess mixtures, we applied an exposure continuum mapping (ECM) framework to identify latent mixture profiles via a self-organizing map and assessed whether these profiles were related to incident breast cancer by estimating a joint exposure-response function with generalized additive models. Results: During follow-

up (mean=11.6 years), 4,282 breast cancer cases were diagnosed. The exposure prevalence for compounds emitted within 3-km of participants' residences ranged from <1%-15%. For individual compounds, HRs for the association between quantiles of emission levels and breast cancer were largely null, except for cadmium, vinyl chloride and asbestos (e.g., asbestos 3-km HR-(>median vs. no exposure)=2.61, 95% CI: 1.37-5.09). Our application of ECM identified 49 profiles that explained 79% of the variance in emission patterns observed within 3-km of participant residences. Profiles revealed that relatively high levels of exposure to several compounds were rare (<1%), and most participants resided in locations characterized by low emissions patterns. Estimation of a joint exposure-response surface indicated that breast cancer was not significantly related to changing patterns of the composition of emission mixtures (p=0.31). Conclusions: Preliminary results suggest that breast cancer incidence may be related to emissions of certain individual industrial carcinogens, particularly asbestos. Examination of complex exposure scenarios revealed that high simultaneous exposure to many compounds was rare, and that our identified latent patterns of these emissions were not associated with breast cancer.

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Genetically Predicted Telomere Length and Risk of Multiple Primary Cancers in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial

Objectives: With increases in the life expectancy, the incidence of multiple primary cancers (MPC) has risen in the U.S., but its etiology is not well understood. Telomeres are important in cell division and senescence and critical for chromosomal stability. Rare mutations leading to excessive telomere length (TL) elongation have been found in families with MPC, and longer genetically predicted leukocyte TL has been associated with increased risk of some cancers. This study sought to examine whether genetically predicted TL contributes to the risk of developing MPC. Methods: We conducted a cohort study among 110,562 participants genotyped with a high-density SNP array in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial. A total 22,879 individuals were diagnosed with a single primary cancer (SPC) and 4,355 were diagnosed with MPC, defined as two or more confirmed primary cancers at different sites, during the 15-years follow-up. Using previously identified TL variants, we calculated polygenic risk scores (PRS) and estimated hazard ratios (HRs) and 95% confidence intervals (CIs) for the association of PRS with MPC within each ancestry using a Cox model, adjusting for potential confounders. Ancestry-specific results were meta-analyzed using a fixed effects model. Results: Longer genetically predicted telomere length was associated with an increased risk of both SPC (Ptrend=4.3x10-12) and MPC (Ptrend=5.3x10-15); however, the association was stronger for MPC (HR=1.91, 95% CI: 1.63-2.24) than SPC (HR=1.29, 95%CI: 1.21-1.39). Cancer survivors in the highest quintile of TL were discovered to have a 1.3-fold increased risk of developing a second cancer compared to the lowest quintile group (p=0.002). Higher risks were observed for MPC among nonsmokers (HR=2.59, 95%CI: 1.98-3.39) compared to smokers (HR=1.55, 95%CI: 1.27-1.89, Phet=0.003). Heterogeneity was observed by cancer histology with longer predicted TL being associated with higher risk of MPC with one or more cancers being a lymphoid malignancy (HR=2.41, 95%CI:1.73-3.36), sarcoma (HR=1.83, 95%CI: 1.52-2.21), or adenocarcinoma (HR=1.74, 95%CI:1.48-2.04), but little or no association with myeloid, small cell, or

squamous cell cancer. Conclusions: Our study demonstrates that higher genetically predicted TL is associated with an increased risk of developing multiple primary cancers and suggests a potential role for genetically predicted TL in assessing MPC risk among cancer survivors.

James Shamul
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Oncology - Therapeutics and Translational Research

Multiscale biomaterials-enabled glioblastoma stem cell-targeted therapy with microRNA

Glioblastoma (GBM) is the most lethal of all brain tumors in adults, causing ~10,000 deaths each year in the United States. Even with standard GBM treatment, there is a 7.2% 5-year survival after initial diagnosis, and greater than 95% of GBM patients experience tumor recurrence due to the drug resistance and tumorigenicity of unresected cancer stem cells (CSCs). Unfortunately, there are currently no methods to reliably isolate true GBM CSCs (GSCs) capable of self-renewal and multi-lineage differentiation. This has limited the success of targeted therapies that address GBM drug resistance and recurrence. MicroRNAs (miRs) are small ribonucleic acids that have been shown to play crucial roles in regulating GBM drug resistance and stemness. However, miRs are very sensitive to RNases that exist in body fluids, cannot passively cross the cell plasma membrane, and are difficult to be delivered specifically into the cytosol where they perform their function. The goal of this work is to address the aforementioned challenges by using multiscale biomaterial strategies to isolate true GSCs and deliver GSC-specific miRs intracytosolically as a modality against GBM drug resistance and recurrence. First, we employed high-throughput microfluidics-generated early embryo-like core-shell hydrogel microcapsules to encapsulate one single patient-derived GBM cell in the nanoliter core of each microcapsule for culture to isolate true GSCs. MiR-sequencing identified a set of miRs that are differentially expressed in GSCs compared to conventionally-cultured, heterogenous GBM cells. The miRs were encapsulated inside nanoparticles to provide a pH-triggered endosomal escape for highly efficient delivery, specifically into the cytosol of GSCs to inhibit their tumorigenesis. Lastly, the GSC-targeted therapy in combination with the clinically-used GBM chemotherapeutic drug temozolomide (TMZ) demonstrated highly efficient inhibition of GBM recurrence in vitro. Nanoparticle-encapsulated scrambled miRs were also evaluated to confirm specificity of the selected miR targets in all experiments. Animal studies will be performed to investigate in vivo GBM tumor destruction and recurrence inhibition using the miR-loaded nanoparticles and TMZ in combination. Collectively, this will be the first work to take advantage of multiscale biomaterials for developing a true GSC-targeted therapy with miRs that combats GBM drug resistance and recurrence, the major causes of GBM patient death.

Sanjay Pal
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Oncology - Therapeutics and Translational Research

STING Agonist and Extracellular Matrix Scaffold Therapeutic Tumor Vaccine Induces Antigen-Specific Anti-Tumor Immunity

Extracellular matrix (ECM) scaffolds prepared from decellularized tissues are widely used in surgical oncology to repair traumatic deficits caused during tumor resection surgery. Implanted biomaterials trigger a host immune response that have been leveraged enhance vaccine delivery by attracting leukocytes to vaccine depots. However, it's not known whether the pro-healing immune response to highly collagenous ECM scaffolds can enhance cytotoxic anticancer immunity. Here, we have engineered a decellularized small intestinal submucosa (SIS) ECM scaffold-assisted cancer vaccine to determine whether the Type 2 biased ECM immune response can synergize with CD8 T cell priming. SIS-ECM was cryogenically milled into injectable particles and infused with one of three adjuvants that activate different immune pathways – MPLA- TLR-4 agonist, c-diAMP (CDA)- STING agonist, and GM-CSF- a myeloid differentiation cytokine. SIS-ECM scaffolds with adjuvant were injected in C57BL6 mice for H&E and immunofluorescent staining. SIS-ECM alone attracted large numbers of leukocytes, primarily macrophages (F4/80+) and antigen presenting cells (CD86+). Total leukocyte number increased with adjuvants: 3.4-fold MPLA, 2.2-fold CDA, and 2-fold GMCSF after 2 weeks. We used an in vivo Cytotoxic T-lymphocyte (CTL) assay to determine functional cytotoxicity by delivering the model antigen ovalbumin with each ECM scaffold vaccine formulation. Our findings indicate that the SIS-ECM, co-delivered with CDA, could induce potent cytotoxic immunity, resulting in more than 95% specific killing. Moreover, we observed only temporary alterations in critical ECM immune responses, essential for wound healing, such as the expression of Il4 cytokine. We used the EG.7-ovalbumin murine lymphoma tumor model to test a 2-dose course of SIS-ECM scaffold vaccine with CDA and were able to cure more than 50% of established tumors compared to 0% if CDA and ovalbumin were delivered with saline controls. Surviving mice treated with SIS-ECM assisted vaccines had long-term anti-tumor memory on tumor rechallenge 230 days later. Subsequent immune depletion studies showed CD8 T-cells are a necessary effector cell population responsible for anti-tumor immunity. This study has shown that the ECM scaffold pro-healing immune environment is synergistic with cancer immunotherapy and can be a promising biomaterial scaffold for cancer vaccines.

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Oncology - Therapeutics and Translational Research

Exploiting metabolic vulnerabilities through pharmacological inhibition of NAD production as a potential therapy for neuroblastoma

Neuroblastoma is the most common pediatric extracranial solid tumor. As high-risk patients have a poor prognosis with an overall survival rate of less than 50%, novel therapeutic strategies are critically needed. To address this need, we performed a high-throughput drug screen and identified nicotinamide phosphoribosyltransferase inhibitors (NAMPTis) as highly active against neuroblastoma cells compared to other cancer cell types. NAMPT is the rate-limiting enzyme in the nicotinamide adenine dinucleotide (NAD) salvage pathway, which cancer cells preferentially utilize to generate NAD, a key co-factor that plays essential roles in energy metabolism, DNA repair, gene expression, and redox homeostasis. In this study, we investigated the translational potential and mechanistic effects of NAMPT inhibition in preclinical neuroblastoma models. Using 3 different NAMPTis in a panel of 10 neuroblastoma cell lines (including 2 neuroblastoma PDX-derived lines), we confirmed that a majority of these models were highly responsive to NAMPTis. Notably, we observed complete growth inhibition in most of the models

at doses of less than 3.2 nM of OT-82, a clinically relevant NAMPTi with a more favorable toxicity profile than earlier generation NAMPTis. Addition of nicotinamide mononucleotide, the product of NAMPT, rescued cellular proliferation, confirming on-target activity of OT-82. Moreover, we observed dose-dependent depletion of intracellular NAD in cells treated with OT-82. As NAD is a co-factor necessary for energy metabolism, we next measured activity of glycolysis and oxidative phosphorylation (OXPHOS) via biochemical and extracellular flux analyses. With OT-82 treatment, we observed decreases in glucose consumption and lactate production in most models, as well as decreases in OXPHOS in some cell lines. Since these processes generate ATP, we also assessed ATP levels, which decreased in all models with OT-82 treatment. Finally, an in vivo pilot study testing OT-82 in an orthotopic neuroblastoma PDX model demonstrated antitumor activity, thus we are expanding these studies to analyze additional in vivo neuroblastoma PDX models. Together, these data show that neuroblastoma is susceptible to disruption of the NAMPT pathway and suggest that NAMPTis have translational potential as a novel therapy for neuroblastoma patients.

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Megacomplex: Revealing a Novel Mechanism of Drug Resistance to Antiestrogen in Epithelial Ovarian Cancer

Epithelial ovarian cancer (EOC) is a prevalent ovarian cancer type developing in the outer layer of the ovaries that is often diagnosed at an advanced stage, resulting in a 30% five-year survival rate. The antiestrogen Fulvestrant (ICI) shows promise in inhibiting estrogen receptor alpha (ESR1) positive breast and ovarian cancers, but drug resistance is the common challenge, likely due to ESR1 upregulation and mutation. We discovered a novel drug resistance mechanism of ICI in ESR1-positive EOC as well as an approach to overcome the resistance. Our previous RNA-seq analysis of high-grade versus low-grade EOC predicted the regulatory role of transcription factors (TFs) ESR1, PITX1, RARA, FOXA1, TFAP2C, and BHLHE40 in ovarian tumorigenesis. Current ChIP-seq data of the TFs, MED1, H3K27Ac and H3K27Me3 in 5 ovarian cancer cell lines suggested clustering of factors to form a regulatory complex. Not reported before in EOC, we identified 1.3MDa megacomplex from the nuclear lysate of estrogen-responsive PEO4 cell line utilizing size exclusion chromatography, and confirmed the presence of ESR1, PITX1, RARA, FOXA1, and TFAP2C by Western blot. We compared the sensitivity of ESR1 in the megacomplex from ICI treated cells to estradiol (an ESR1 agonist) treated cells as a control. To our surprise, ESR1 was insensitive to ICI in the megacomplex but sensitive in non-megacomplex fractions. We further confirmed that unlike cytoplasmic ESR1, nuclear ESR1 was degraded minimally by ICI. Treatment with the BRD4 inhibitor JQ1 (which inhibits TFs clustering), inhibited nuclear occupancy of ESR1 leaving it mostly in the cytoplasm. The combination of JQ1 with ICI not only stopped nuclear occupancy of ESR1 but also degraded those molecules present in the cytoplasm, thus breaking the ESR1 signaling circuit responsible for tumor cell proliferation and the challenges of targeting. Moreover, cell counting and viability measurements revealed a combined inhibitory effect of JQ1 plus ICI over vehicle, estradiol, ICI or JQ1 alone treatments. Utilizing mass spectrometry and ChIP-seq, we are further exploring molecular environment in the megacomplex surrounding ESR1 which provides resistance to ICI. We conclude, ESR1 in megacomplex is resistant to ICI and sensitized by combining ICI with JQ1 thus providing a novel drug

resistance mechanism. Our findings may also help in explaining similar therapeutic drug resistance in other cancers and diseases.

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Nanobody tethering of ligands to GPCRs promotes highly biased agonism

To date, 35% of FDA approved drugs are directed towards G-protein coupled receptors (GPCRs), the largest family of cell surface proteins in humans. One such GPCR, the parathyroid hormone receptor-1 (PTHR1), is a validated target for the treatment of osteoporosis. Like many GPCRs, PTHR1 activated by ligand signals through multiple intracellular pathways including those mediated by Gs and arrestin. The pharmacological concept of biased signaling (functional selectivity) describes the ability of select ligands to preferentially activate one receptor-mediated signaling pathway over others. The development of biased ligands holds significant implications for the development of drugs with fewer side effects. Antibodies (Abs) are renowned for high selectivity. Whether receptor-targeting Abs can assist with the generation of biased agonists is unknown. Further, the development of GPCR-targeted Abs remains challenging. Camelid single-domain Abs (or nanobodies, Nbs) have been shown to target epitopes in GPCRs not accessible to conventional Abs. However, it remains difficult to identify Nbs that can directly activate GPCR signaling. We applied a unique methodology developed in our lab (see below) to link PTHR1-binding Nbs with synthetic ligands that directly modulate GPCR function to provide semi-synthetic conjugates that overcome this hurdle. Recombinantly produced Nbs were labeled site-specifically with click chemistry handles using the enzyme Sortase A. We used click chemistry to link Nbs with a weakly active PTHR1 agonist (PTH1-11). I then evaluated the signaling profiles of these Nb-PTH1-11 conjugates through two major signaling pathways: Gs/cAMP (Glosensor assay) and arrestin recruitment (bioluminescence resonance energy transfer assay, BRET) in HEK293 cells. In cAMP assays, conjugation of PTH1-11 to a PTHR1-binding Nbs increases potency by 10- to 1000-fold. Nb-PTH1-11 conjugates, but not PTH1-11, induced cAMP responses that persisted for up to 1 h after ligand washout. In contrast, Nb-PTH1-11 conjugates displayed negligible recruitment of arrestin2 relative to comparator ligand PTH1-11, which was fully active. Our findings show that Nb PTH1-11 conjugates are highly selective for the Gs pathway in contrast to the PTH1-11, which signals through all PTHR1-engaged pathways. The platform described here has the potential to advance our understanding of diseases associated with PTHR1 signaling by enabling selective modulation of a specific pathway.

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Novel mechanisms underlying gut leakiness and systemic endotoxemia in promoting liver fibrosis via the gut-liver axis and the mechanistic protection of melatonin.

Over 1.5 billion people worldwide have chronic liver diseases including fibrosis, which can progress to

cirrhosis and liver failure. However, the causal role and mechanisms of gut leakiness in liver fibrosis are poorly understood, and there is no FDA-approved drug to treat liver fibrosis. In this study, we aimed to investigate the causal role of protein acetylation in promoting gut leakiness, endotoxemia, and liver fibrosis via the gut-liver axis and the prevention by melatonin (MT), a safe agent recognized by FDA, against gut leakiness and liver fibrosis. Sprague-Dawley rats were pretreated with MT followed by thioacetamide (TAA) twice a week to induce liver fibrosis or vehicle (Control) for 1, 2, or 4 weeks. Our results showed that TAA caused hyperacetylation of gut and liver proteins via selective suppression of Sirtuin 1 (SIRT1) deacetylase, but not other SIRT isoforms, leading to gut leakiness, endotoxemia, and liver fibrosis, where these changes were prevented by MT. Immunoprecipitation followed by immunoblot revealed that gut tight and adherent junction (TJ/AJ) proteins were acetylated and degraded via ubiquitin-dependent proteolysis. Histological and biochemical analyses confirmed the decreases in these TJ/AJ proteins with increases in enterocyte apoptosis, intestinal deformation, and serum endotoxin at 1 week, while liver fibrosis and injury markers (fibrosis-related cytokines, serum ALT and AST, and cell apoptosis) were markedly elevated 2 and 4 weeks after TAA exposure. Mass-spectral proteome and immunoblot analyses showed that decreased SIRT1 caused hyperacetylation and aberrant expression of liver proteins (e.g., NF-kB, FoxO1, p53, etc.) involved in inflammation, metabolism, and apoptosis. Pretreatment with MT ameliorated all these changes at 1, 2, and 4 weeks. Notably, acetylation-related factors (acetyltransferase, acetyl-CoA, and NAD+/NADH) were unchanged. TAA-exposed gut- and liver-specific Sirt1-KO mice also showed markedly decreased gut TJ/AJ proteins with elevated enterocyte apoptosis, endotoxemia, liver fibrosis markers, and hyperacetylation of gut and liver proteins compared to those of WT mice. Overall, this study showed the novel mechanisms of the decreased SIRT1-mediated protein hyperacetylation promoting gut leakiness and liver fibrosis and the protective role of melatonin through the gut-liver axis. These findings can also explain the important role of gut leakiness with decreased TJ/AJ proteins in many other disease states.

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Protein Structure/Structural Biology
Structure and function of an RNA chaperone complex containing misfolded RNA
Although RNA chaperones assist correct RNA folding and can detect and refold misfolded RNAs, relatively little is known as to how they recognize their RNA targets and carry out their functions. A major RNA chaperone in eukaryotic cells is the La protein, which binds all newly synthesized RNA polymerase III transcripts and assists their correct folding, maturation and ribonucleoprotein (RNP) assembly. In Xenopus oocyte nuclei, La is found complexed with the Ro60 protein and misfolded pre-5S rRNAs. As Ro60 is a ring-shaped protein that binds the 3' ends of misfolded RNAs in its central cavity, the La/Ro60/misfolded pre-5S rRNP may represent an intermediate in RNA refolding. Earlier studies have shown that the N-terminal domain (NTD) of La binds RNA 3' ends. However, while the La C-terminal domain (CTD), consisting of an RNA Recognition Motif (RRM) and an intrinsically disordered C-terminus, is required for chaperone activity, the mechanism by which it functions is unknown. To address this question, we reconstituted the La/Ro60/misfolded pre-5S rRNP and used cryo-electron microscopy to solve the structure at 2.8 $\mathring{A}$ resolution. In the structure, the single-stranded 3' end of the misfolded RNA passes through the Ro60 cavity and interacts with the La-NTD. A long helical linker between NTD and

CTD of La wraps around the side of the Ro60 ring, positioning the La-CTD near a misfolded stem of the pre-5S rRNA. A helix of the RRM motif inserts through a gap between RNA loops and the misfolded stem, and part of the intrinsically disordered C-terminus becomes structured to form an extended alpha helix. Consistent with the structure, electrophoretic mobility shift assays showed that the isolated La-CTD binds misfolded pre-5S rRNA and that the presence of the disordered C-terminus is crucial for this interaction. Remarkably, this interaction was specific for misfolded RNA, as the La-CTD has a significantly lower affinity for correctly folded pre-5S rRNA. We also demonstrated that the misfolded pre-5S rRNA binds Ro60 first and then interacts with La to form the full RNP. Together with our structure, this result suggests that Ro60 carries out the initial recognition of misfolded RNAs. We propose that Ro60 binding presents the misfolded pre-5S rRNA in a way that makes the misfolded region more accessible to the La-CTD. Our work reveals how the La CTD interacts with misfolded RNA and supports a model in which Ro60 and La function together to salvage misfolded RNA precursors.

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Protein Structure/Structural Biology
Stepwise lipid transport mechanism of MFSD2A revealed by cryo-EM
Omega-3 fatty-acid docosahexaenoic acid (DHA) is a key component of cellular membranes in the central nervous system (CNS) and essential for neurological functions. In human cerebral cortex, DHA contributes to around 25 percent of total fatty acids. Deficiency of DHA results in various CNS-related disorders, including memory deficits, dyspraxia, and dyslexia. However, our body cannot synthesize DHA, and the major source of DHA in the brain is obtained from the diet and transported across the blood-brain-barrier in the form of lysophosphatidylcholine (LPC)-DHA by transporter MFSD2A. The detailed translocation mechanism of LPC-DHA across the lipid bilayer is still elusive. Here, by using single particle cryo-EM, we revealed the structure of Danio rerio MFSD2A (59 kDa) in a complex with a FAB in the inward-open conformation with ligands bound to amphipathic pockets between the N- and C-terminal domains of the transporter at 2.9 angstrom resolution. Multiple MFSD2A structures with individual ligands located at unique positions along the translocation pathway were further identified through a specialized 3D classification approach. These structures elucidated the LPC-DHA translocating trajectory along the transporter cavity, where a 70-degree rotation of the LPC headgroup followed by translation of the ligand towards the cytoplasmic exit occurred after the initial lipid flipping step. It has been reported that mutation of residue S166 in human MFSD2A is associated with decreased DHA transport to the brain and severe microcephaly. Our structure showed that the corresponding residue S160 in Danio rerio was found in the translocation pathway coordinating the LPC headgroup, which confirmed its key role in mediating lipid translocation. In summary, this study depicted a step-by-step lipid translocation of MFSD2A and provided a blueprint for the design of the delivery strategies for amphipathic drugs across the blood-brain-barrier.

Riley Metcalfe
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Protein Structure/Structural Biology
Structure and regulation of full-length human leucine-rich repeat kinase 1
The two human leucine-rich repeat kinases (LRRKs), LRRK1 and LRRK2 are large and unusually complex multi-domain kinases which serve to regulate fundamental cellular processes, in particular serving as master regulators of membrane trafficking. Near-uniquely, the LRRKs contain both a kinase domain and Ras-like GTPase domain in the same polypeptide chain, along with several repeat domains and scaffolding domains. Both LRRKs are implicated in human disease. Specifically, LRRK1 is implicated in bone development, with inactivating mutations in LRRK1 causing a bone disease, osteosclerotic metaphyseal dysplasia (OSMD), while kinase-activating mutations in LRRK2 are associated with Parkinson's disease. Despite their biological significance, both LRRKs have proved to be challenging structural targets, due to their size (~230-280 kDa, ~2000-2500 residues), heterogeneity, flexibility, and low recombinant expression levels. Recent experimental structures of LRRK2 in a variety of states have begun to provide structural detail into this family of proteins, however the structure and exact molecular mechanisms regulating the activity of LRRK1, similarities and differences among the family members remain unclear. Here, we report a cryo-EM structure of the LRRK1 monomer, and a lower-resolution cryo-EM map of the LRRK1 dimer. The monomer structure, in which the kinase is in an inactive conformation, reveals key interdomain interfaces which serve to control kinase activity through linking the kinase domain and the Ras-like GTPase domain, which we have further validated experimentally using both biochemical kinase assays and in vitro cellular experiments. We have additionally examined the structural basis of LRRK1 regulation by two of its regulatory partners, the kinases PKC and EGFR. LRRK1 is structurally distinct compared to LRRK2, particularly in the position of the leucine-rich repeats relative to the kinase domain. Through analysis of the experimental cryo-EM data, we observed structural dynamics in the leucine-rich repeats in LRRK1, which we speculate may have a role in controlling substrate access to the LRRK1 kinase. Overall, our results provide new structural insights into the human LRRKs for understanding the physiology and pathology of these proteins and provide insight into the regulation mechanisms used by these unique and unusually large multi-domain kinases.

Sudhaker Dharavath
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Protein Structure/Structural Biology
Cooperative unwinding of RNA duplex by DEAD-box helicase DDX3X, a new paradigm of division of labor
RNA-binding protein DDX3X is a member of the DEAD-box helicase (DDX) family that regulates ATP-dependent RNA processing and metabolism by unwinding short double-stranded (ds) RNAs. It is composed of an N-terminal domain (N, containing a nuclear export signal), two RecA-like domains (D1D2, the helicase core), and a C-terminal domain (C, containing a low complexity region). Playing important roles in cancer progression and HIV-1 infection, DDX3X is an attractive target for novel anti-cancer and anti-AIDS therapeutics. Previous functional and structural information showed that three DDX3Xs unwind dsRNA in a cooperative manner, the D1 and C domains are involved in oligomerization, and the N domain is dispensable. A division of labor model was proposed in which two loading protomers and one unwinding protomer act in concert. However, this hypothetical model has not been

fully verified. Here, we report two crystal structures along with functional data of site-directed mutagenesis and Hill cooperativity analysis, shedding lights on the division of labor mechanism. One of our structures is determined for D1D2 in complex with ADP and dsRNA at 2.4-Å resolution and the other is for D1D2-C. The complex structure is composed of two D1D2 cores, two ADP molecules, and one RNA duplex. Comparative analysis of this and previous structures suggests that residue E186 is essential for D1-D1 interaction. Accordingly, we create mutant proteins D1D2E186A and D1D2-CE186A for Hill coefficient cooperativity analysis along with wild-type D1D2-C. The Hill coefficients for the unwinding and ATPase activities of D1D2-C are 2.8 and 2.0, respectively, indicating a cooperation between three D1D2-C protomers, including one loading protomer, which does not hydrolyze ATP, and two unwinding protomers, which hydrolyze two ATP molecules. These data represent a new paradigm of the division of labor mechanism. Furthermore, Hill coefficients for the ATPase activity of D1D2E186A and D1D2-CE186A are 1.2 and1.3, respectively, indicating that the E186A mutation abolishes the interaction between the two D1 domains. Our D1D2-C structure is also determined at 2.4-Å resolution, in which the C domain is disordered most likely due to the absence of RNA. These new structures and results of functional assays will be presented and discussed in detail along with previous information. Structural analysis of unwinding assembly by small-angle X-ray scattering, X-ray crystallography, and cryo-EM is in progress.

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Psychology and Psychiatry

Relationship between pupil-linked arousal and procedural motor learning

Background: Acquiring a new motor skill engages repetitive practice, a phenomenon referred to as procedural motor learning which is important in neurorehabilitation of patients with neurological disorders and in the practice of sports and music. Pupil linked arousal is known to reflect a variety of cognitive states. It is unknown if pupil-linked arousal systems could influence procedural motor learning. In this study, we asked whether trial-by-trial changes in pupil size could be predictive of trial-by-trial procedural motor learning progression. Materials and Methods: 24 subjects were trained on a procedural motor learning task that involved repetitively typing a sequence of keypresses (4-1-3-2-4) as fast and as accurately as possible with their non-dominant hand over 36 trials (10 s each) interspersed with rest intervals (also 10 s each). On the next day, the subjects were tested on the same sequence and several unpracticed sequences. Pupil diameter data were collected before (baseline1), during, and after (baseline2) the learning task on Day 1, and during testing on Day 2 using an EyeLink 1000 Plus eye tracker. Skill was defined as the correct sequence typing speed. Improvements in skill were assessed during actual practice (micro-online gains) and during inter-trial rest intervals separating practice periods (micro-offline gains). Total learning was defined as the sum of micro-online and micro-offline gains. Trial-by-trial subject-normalized pupil diameter data were used to predict these three early learning outcome measures using a linear regression model. Results and Discussion: On average, subjects reached 95% of peak performance by practice trial 11 (early learning) on Day 1, which was maintained until the end of the practice session. Pupil diameter was larger during practice periods compared to inter-trial rest intervals and baselines (1-way ANOVA, post-hoc Tukey test, p < 0.05). During early learning, pupil diameter predicted micro-online gains (r = 0.65), micro-offline gains (r = 0.72), and total learning (r = 0.74), with correlations significantly higher than those obtained during

baselines (1-tail paired t-test; p < 0.05). On Day 2, pupil diameter predicted performance for practiced but not unpracticed skill sequences. Conclusion: Pupil size predicted skill learning progression on a trial-by-trial basis. These results suggest that pupil-linked arousal systems contribute to early procedural skill learning in humans.

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An Experimental Study of the Effects of Cognitive Fatigue on Youth's Energy Intake: Interaction of Cognitive Fatigue with Reported Dietary Restraint

Resource-based models of disinhibited eating posit that the depletion of a person's cognitive resources (cognitive fatigue) is an impetus for increased energy intake. Dietary restraint (DR), or the attempted reduction of energy intake, may further diminish available cognitive resources and exacerbate disinhibited eating when a person is cognitively fatigued. Studies support this model among adults who report DR, but it is unknown whether this pathway is relevant for youth. Therefore, we investigated if DR impacted the effects of cognitive fatigue on energy intake in youth. Using a randomized crossover experimental design, youth completed a two-hour cognitive fatigue condition (a demanding computer task) and a two-hour control condition (watched movies) on separate days within a 31-day period. Immediately following the conditions, participants were instructed to "eat until you are no longer hungry" and presented with a ~10,000 kcal buffet-style meal, from which their energy intake was measured. DR in the past month was measured via a validated interview and dichotomized: "no restraint" and "any restraint". We conducted a mixed-model ANCOVA with energy intake (kcal) as a repeated measure, experimental condition (fatigue vs. control) as a within-subject factor, and DR as a between-subjects factor. Models were adjusted for height, fat mass (%), lean mass (kg), sex, race/ethnicity, condition order, and days between visits. Among 98 youth (age 13.0±2.6y; BMI% 60.1± 29.0; 47% Female; 47% non-Hispanic White) the main effects of condition (p=.10) and DR (p=.45) on intake were not significant; but the DR x condition interaction significantly predicted intake (p=.004). Youth with DR had greater intake following the fatigue condition (EMM ±SE, 1085.9±101.3) compared to the control condition (874.7±111.44; p=.004). Intake did not differ between fatigue (1053.8±48.0) and control (1081.8 ± 52.8) conditions among youth without DR (p=.41). As hypothesized, cognitive fatigue induced greater intake only among youth with DR. However, the differential energy intake pattern between youth with and without DR was driven by the DR group's relatively lower intake following the control condition, not a relatively greater intake when cognitively fatigued. Studies using more nuanced assessments of DR and disinhibited eating are needed to elucidate how cognitive fatigue affects DR attempts, and whether it also promotes disinhibited eating among youth with DR.

Liangchen Liu
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Radiology/Imaging/PET and Neuroimaging

Fully-automated Segmentation of Lymphoma with Anatomic Priors

Lymphoma is a hematopoietic malignancy that can affect people of all ages. Positron Emission Tomography (PET)/Computed Tomography (CT) is the primary imaging method for assessing lymphoma because PET is sensitive to lymphoma regions with high Standardized Uptake Value (SUV) while CT preserves anatomical structures. Precise and accurate segmentation of lymphomatous lesions on PET/CT scans is pivotal for assessing prognosis and treatment response. However, the segmentation of lymphoma is challenging due to its highly variable patterns. Additionally, some normal organs such as the heart, kidneys, and bladder may have high SUV responses that mimic the distinct appearance of lymphoma. Thus, it is crucial to incorporate knowledge of anatomy during the pre and post processing stages of this multi-modality task. We developed an automatic lymphoma segmentation deep learning model with anatomic knowledge priors. The model uses a multi-modal nnUnet as the backbone. The method uses two critical anatomic priors: locations of structures that normally exhibit high SUV and augmentation with scans from patients without lymphoma. The dataset consisted of 46 whole body PET/CT scans from patients with lymphoma and 33 scans without. An experienced radiology resident labeled the scans with help of a semi-automated AI labeling tool. The labels were verified by a nuclear medicine physician. Segmentation quality metrics for the proposed method and alternative publicly available baseline methods (patch-based 3D-UNet and naïve nnUNet) were computed. The Dice score, Jaccard score, precision, sensitivity, and specificity for our method were 0.48±0.32, 0.36±0.26, 0.8±0.41, 0.41±0.3, and 1.00±0.0 compared to 0.46±0.31 (p=NS), 0.34±0.25, 0.86±0.34, 0.40±0.34, and 1.00±0.0 for naïve nnUNet and to 0.33±0.16 (p=NS), 0.20±0.11, 0.24±0.12, 0.63±0.17, and 1.00±0.0 for patch-based 3D-UNet. The use of the AI model reduced the time required for segmentation by 55.7% compared to the reference manual segmentation. The mean Total Metabolic Tumor Volume (TMTV) calculated using the AI-predicted labels was not significantly different from those calculated using the manual labels (P = 0.153). This indicates that AI-based segmentation was comparable to manual segmentation. Our method was much faster and just as accurate as manual segmentation. It provided a small numerical improvement compared to the baselines. With further development, incorporating anatomic priors may benefit lymphoma segmentation.

Qingyu Chen
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Trustworthy artificial intelligence for healthcare: accessible, robust, and automated diagnosis of age-related macular degeneration

Age-related macular degeneration (AMD) is the leading cause of blindness in developed countries. To date, over 90 million Americans are at high risk to have AMD; by 2040, 288 million patients are projected to have AMD worldwide. Based on clinical features, it is classified into early, intermediate, and late stages. There is a critical need to provide a timely diagnosis of AMD in early/intermediate stages to delay its progression which leads to loss of vision or blindness. However, research shows that up to 50% of cases of AMD remain undiagnosed or are diagnosed too late for treatment – representing a significant disease burden. Artificial Intelligence (AI)-assisted disease diagnosis has achieved remarkable progress over a decade; this has the potential to support clinical decision-making. However, despite remarkable advances, recent literature points out that less than 15% of the studies evaluated the

performance of AI systems with clinicians and quantified its impacts on clinical workflows. The generalizability and usability of AI systems, therefore, remain largely unknown. In this work, we propose a novel AI framework for the detection of AMD, evaluate its performance with 88 clinicians, and conduct a user study with 24 clinicians to quantify the efficacy of AI-assisted AMD diagnosis in clinical workflows. First, the framework includes multi-modal (detection from single or multiple image modalities), multi-task (training different tasks simultaneously to improve generalizability), and multi-attention (improving ensembled feature representation) operations. In addition, to provide the final prediction of AMD severity only, it also provides the predictions of AMD-related risk factors such as drusen sizes and pigmentary abnormalities to improve the interpretability. Second, the model was compared with 88 clinicians on an independent sample of 200 patients. The model shows better overall performance (with an accuracy of 0.67 vs 0.58) with high AUC in the detection of large drusen (0.94) and pigmentary abnormalities (0.93). Third, a head-to-head comparison of 24 clinicians from 15 institutes with and without AI assistance shows that the average diagnostic accuracy and efficiency improved by 20% and 50% respectively with AI assistance. Overall, this work demonstrates the successful development and comprehensive evaluation of a novel AI method that enables accessible, robust, and automated AMD diagnosis.

Yixuan Wu
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Radiology/Imaging/PET and Neuroimaging
Realistic digital phantoms for prostate ultrasound and photoacoustic imaging
Background: Photoacoustic (PA) imaging and ultrasound tomography (UST) are two emerging modalities for cancer detection. Both can provide functional and quantitative parameters and are compatible with conventional ultrasound (US). Specifically, PA imaging maps out hallmarks of malignancy based on angiogenesis and hypoxia, or targets at other biomarkers by using exogenous contrast agents. UST quantifies speed of sound (SoS) and acoustic attenuation to enable more comprehensive diagnosis. Currently both modalities are understudied for prostate cancer (PCa). Most existing PA and UST platforms are designed to image breasts in full angles in a transducer-lined water container, which are impractical to image prostate due to prostate's deep anatomy surrounded by bones. This imposes a great challenge to light illumination, image acquisition, and limited angle reconstruction. Further development requires simulations to validate algorithms and apparatus that are not possible through experiments. Therefore, realistic US and PA digital phantoms are in great need to guide practical system designs. Methods: Fresh ex vivo whole prostate specimens with biopsy-confirmed cancer were extracted from radical prostatectomy. To acquire SoS and attenuation, specimens were 3D-scanned in full angles in on a UST imaging platform. The anatomical information was acquired from open-source CT and MRI atlas data in the pelvic area, where urinary bladder, rectum, anal canal, penile bulb, neurovascular bundles, femoral heads, prostate, and seminal vesicles are delineated. The optical absorption, optical scattering, anisotropy, density, SoS, and acoustic attenuation of the structures were acquired from literature according to the tissue type. We then replaced the prostate in the original atlas data with the cancerous ex vivo prostate. Results: Prostate specimens were collected from 80 patients in total. Together with 19 anatomical atlas data, data augmentation is possible so that 1520 combinations of digital phantoms are achieved. Each digital phantom is composed of four sub-phantoms, including

optical absorption and optical scattering mainly for PA imaging and SoS and density mainly for US imaging. Conclusion: By combining SoS and attenuation information acquired from ex vivo prostate UST images and anatomical information acquired from CT/MRI atlas in the pelvis area, realistic digital phantoms for prostate US and PA imaging are obtained.

Ryan Marquardt
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Reproductive Biology

The Serum Response Factor-Myocardin Pathway is Essential for Female Reproductive Function

The success of pregnancy relies on delivery of an embryo to the uterus via the oviduct and subsequent support from differentiated endometrial stromal decidual cells in the uterus. Disruption of these processes can lead to a dangerous ectopic pregnancy or implantation failure, which is responsible for 75% of recurrent pregnancy losses. Progesterone (P4) signaling through the nuclear progesterone receptor (PGR) regulates both oviductal embryo transport and differentiation of myofibroblast-like early decidual cells by inducing the transcription of PGR target genes and modulating inflammation; however, the transcriptional cofactors it requires are not fully defined. We identified serum response factor (SRF) as a potential PGR coregulator based on enrichment of its DNA binding motif in PGR chromatin binding intervals in human and mouse uterine tissues. Further, the SRF coactivator myocardin (MYOCD) is P4 responsive, leading us to hypothesize that the SRF-MYOCD pathway collaborates with PGR to enable oviduct and decidual function. We therefore employed the Pgr-Cre mouse model to drive targeted deletion of Srf or Myocd in the Pgr-expressing cells of the oviduct and uterus. Pgrcre/+; Srff/f (Srfd/d) females were sterile in six months of breeding, and Pgrcre/+; Myocdf/f (Myocdd/d) females were subfertile. At gestation day (GD) 3.5, all embryos from controls were in their uteri, Srfd/d mice retained all embryos in their oviducts, and Myocdd/d mice retained an average 32% of embryos in their oviducts. At post-implantation, Myocdd/d mice displayed a comparable number of morphologically normal implantation sites to the number of embryos that reached the uterus, demonstrating functional decidualization. In contrast, Srfd/d uteri were unable to undergo artificially induced decidualization. In addition, knocking down SRF in human endometrial stromal cells (HESC) before inducing in vitro decidualization resulted in a blunted response. Transcriptome analysis of both SRF-deficient HESC and P4-treated Srfd/d mouse uteri revealed dysregulation of myofibroblast-associated genes and a hyperinflammatory, progesterone-insensitive gene expression environment. These results demonstrate the necessity of SRF-MYOCD for normal oviductal embryo transport and of SRF alone for endometrial stromal decidualization. Further, they suggest that SRF cooperates with PGR to enact anti-inflammatory and myofibroblast differentiation programs in female reproductive tissues required for fertility.

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Reproductive Biology

Somatic cell fate specification and separation in the fetal ovary

Proper differentiation of somatic cell types in the fetal ovary lays the foundation for future ovarian function in the adult. Understanding how each cell type is established is crucial in developing methods to intervene in ovarian diseases caused by cellular dysfunction in women. The different cell types in the ovary include germ cells, supporting cells, and interstitial cells. While germ cells are specified outside of the fetal ovary, supporting cells and interstitial cells originate from a common somatic progenitor within the fetal ovary. One of the critical gaps in the field of ovarian biology is how interstitial cells are specified apart from the supporting cells during ovarian development. To address this question, we performed single-cell mRNA sequencing of mouse ovarian cells at the beginning of ovary formation. We discovered two major somatic populations, one positive for the nuclear receptor Nr2f2, and the other positive for the nuclear receptor Nr5a1. To investigate the differences between these two populations, we performed differential gene expression analysis and identified that the two cell populations express different components of an important signaling pathway, the Notch pathway. Specifically, the Nr2f2+ population expresses the Notch receptor Notch3 and the downstream transcription co-activator Maml2, whereas the Nr5a1+ population expresses high levels of the Notch antagonist Numb. This suggests that the Notch pathway is active in the Nr2f2+ population, and conversely, is repressed in the Nr5a1+ population. To test whether Notch signaling is involved in somatic cell fate specification, we performed lineage tracing experiments by labeling early Notch-active cells in the fetal ovary. We found that Notch-active cells co-express NR2F2 at the beginning of labeling, and that these cells become interstitial cells in adult ovaries. In conclusion, our results support a model in which active Notch signaling in Nr2f2+ cells specifies the interstitial cell lineage and separates them from the Nr5a1+ supporting cell lineage at the beginning of ovary formation.

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RNA Biology - Coding RNAs

Investigating RNA tailing by noncanonical terminal nucleotidyl transferases and their function in C. elegans fertility

The regulation of RNA stability and translation is critical to reproduction and development. Tailing is the addition of nucleotides to the 3' end of RNA molecules in an untemplated manner. It is mediated by terminal nucleotidyl transferases (TENTs) and is a determinant of RNA biology. Historically, long poly(A) tails have been associated with stable and highly translated transcript. However, new high-throughput sequencing methods show the incorporation of U- and G- residues in mixed tails and suggest that nucleotide composition in addition to the length determines tail function. RNA tails in the germline and early embryo are particularly dynamic, yet our understanding of their function and requirement in oocytes is incomplete. I propose the use of traditional genetics in combination with new sequencing and biochemical approaches to characterize RNA tails in oocytes, identify maternal targets of TENTs, and investigate their role in oogenesis. We are currently investigating two C. elegans orthologues of the vertebrate GLD-2, a noncanonical TENT that specializes in poly(A) tailing: gld-2 and gldr-2. GLD-2 is expressed throughout the germline and is required for germline differentiation; knockout of gld-2 shows a complete absence of oocytes. Our imaging of a tagged GLDR-2 reveals a robust expression in the germline as well, but gldr-2 null animals remain fertile. Due to significant homology, we are also exploring genetic interactions between gld-2 and gldr-2 in the germline. The auxin-inducible degron

(AID) system was also used to conditionally deplete their expression in the germline and determine their physiological function in oocytes. Next, we will assess oocyte production and quality in conditional mutants, monitoring oocyte number, morphology, and fertilization as well as the rate of viable progeny. To accurately resolve maternal RNA tail length and composition in C. elegans, we have adapted a novel direct cDNA sequencing method (Nano3P-seq). We observe significant tail heterogeneity. In the absence of GLDR-2, preliminary data also shows a decrease in the average length of mixed tails (from 46nt to 34nt) and a dramatic loss of U residues. This project will explore the molecular regulation and the physiological implications of RNA tailing in the oocyte. Long-term, this will provide a comprehensive RNA perspective to our understanding of oocyte viability and quality, as well as aid in the development of assisted reproductive technologies.

Chunmei Shi
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RNA Biology - Non-coding RNAs
DROSHA REGULATES IGF2 EXPRESSION INDEPENDENT OF MICRORNAS IN WILMS TUMOR
The nuclear RNase III enzyme DROSHA interacts with its cofactor DGCR8 to form the Microprocessor complex (MC), which initiates microRNA (miRNA) maturation by cleaving hairpin structures embedded in primary transcripts. Recurring mutations of miRNA biogenesis factors are documented in a variety of cancers, highlighting the role which miRNAs play in tumorigenesis. Interestingly, mutations in the MC are detected in a much higher frequency than mutations in the rest of miRNA machinery in Wilms Tumor, strongly suggesting a miRNA-independent role of the MC in Wilms Tumor. To identify genes regulated by MC independent of the miRNAs, we generated a set of isogeneic HEK293T cells in which DROSHA, DGCR8, and DICER were knocked out individually or in combination. We find that Insulin-like growth factor 2 (IGF2), a growth-promoting gene strongly associated with Wilms tumorigenesis, is upregulated in both DROSHA/DICER and DGCR8/DICER Double Knockout (DKO) cells, but not in DICER KO cells. Expressing Wild-Type DROSHA, but not its catalytic mutant or the hotspot mutant detected in Wilms Tumor patients, rescued the level of IGF2 in DROSHA/DICER DKO cells. These results indicate that the DROSHA inhibits IGF2 level independent of miRNAs. IGF2 resides in a gene cluster with H19, which encodes a long noncoding RNA (lncRNA). The expressions of IGF2 and H19 are coupled due to gene imprinting of this genetic locus. Interestingly, H19 is known to be cleaved by MC, suggesting that MC regulates IGF2 via H19. To test this, we sought to block Drosha's cleavage on H19 and check if the effect of IGF2 upregulation is abolished. By using CRISPR, we knocked out the Drosha cleavage site embedded in H19 in HEK-wild type, Dicer-KO, and Drosha-KO cells. IGF2 level is upregulated in HEK-wild type and Dicer-KO, but not in Drosha-KO cells. This indicates that Drosha cleavage of H19 is essential for Drosha's regulation on IGF2. Our findings nominate a new mechanism underlying the pathology associated with the DROSHA mutations in Wilms Tumor. It also suggests a novel crosstalk between miRNA biogenesis and gene imprinting.

Karrie Spain
Other

NCI-CCR
RNA Biology - Non-coding RNAs
Mapping functional microRNA target sites in vivo by RNA editing
MicroRNAs (miRNAs) are master post-transcriptional regulators, governing the expression of more than 60% of protein coding genes. Through partial base-pairing, miRNAs guide the RNA-induced silencing complex (RISC) to target mRNAs, down-regulating their expression post-transcriptionally. Dysregulation of miRNAs has been linked to human diseases such as cancer, highlighting the importance of understanding miRNA regulation in cells. A wide array of prediction tools was developed to establish the target repertory of a given miRNA. However, these tools not only have a high false-positive rate but also fail to predict known functional targets. Biochemical approaches such as cross-linking Immunoprecipitation (CLIP), are used to experimentally identify transcripts directly bound by miRNA-programmed RISC. However, these approaches require a large amount of starting material, which restricts their application to in vitro cultured cells. Furthermore, true targets of miRNAs are degraded and therefore depleted in transcripts recovered in the immunoprecipitation, leading to false-negative predictions. To overcome these challenges, we hypothesize that altering target sequences via a novel fusion protein with an AGO binding domain and an RNA editor would allow for the identification of targets because those modifications would be visible in sequence readouts. This method would serve to protect against false identification while permitting studies in settings with only overexpression of a fusion protein. We predict a fusion protein with the RNA-binding portion of TNRC6 (T6B), which halts degradation, and ADAR2, an adenosine deaminase, which leads to A-to-I edits, will support this hypothesis. To test this hypothesis, we generated a set of fusion proteins with T6B and ADAR2. Using co-immunoprecipitation and deep sequencing, we confirmed that this fusion protein associates with endogenous AGO2 and is able to mark a reporter bearing known miRNA target sites with A-to-I editing. Currently, we are testing whether this method can be used to identify novel miRNA target sites in vivo. If successful, this method would allow the identification of miRNA targets in physiological relevant settings where cell numbers are limited.

Sharan Malagobadan
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RNA Biology - Non-coding RNAs
Pathogenic Dicer RNase IIIb Hotspot Mutation leads to Dysregulation of Argonaute Strand Selection
Dicer is an RNase III enzyme that plays an essential role in microRNA (miRNA) biogenesis by processing hairpin-shaped miRNA precursors (pre-miRNA) into mature miRNA duplexes. Depending on the 5'end-thermostability, either the 5p- or the 3p-arm of the pre-miRNA joins Argonaute proteins (AGO) to form an RNA-induced silencing complex (RISC), while the other strand is degraded. The mature miRNA strand that remains associated with AGO is called the guide strand while the one discarded is called the passenger strand. Accordingly, mature miRNAs are designated as 5p- or 3p-miRNAs, depending on which strand is used. Dicer mutations are implicated in cancer progression, as evidenced by the DICER1 syndrome, a tumor predisposition syndrome that increases the risk of pediatric and certain adult cancers. A key question in DICER1 syndrome research is how DICER1 hotspot mutations alter miRNA production. Previous studies have shown that the Dicer RNase IIIb domain hotspot mutation impairs 5p-miRNAs production while leaving 3p-miRNAs unaffected. However, these findings were based on

overexpression systems on a Dicer-null background, which do not accurately represent physiological conditions for miRNA biogenesis and degradation. As such, the overall impact of the mutation on the endogenous miRNA landscape and its targetome remains unclear. To address this, we engineered P19 mouse embryonic carcinoma cell line using CRISPR to mimic the pathogenic DICER1 syndrome mutations to elucidate its functional implication. Our results of miRNA profiling with northern blot and deep sequencing confirm the loss of mature 5p-miRNAs and retention of 3p-miRNAs in the mutant cell line as expected. Surprisingly, we also observed that misprocessing by the mutant Dicer leads to downstream dysregulation of AGO strand selection for the 3p-miRNAs. Notably, 3p-miRNAs that were originally passenger strands of abundantly expressed 5p-miRNAs were highly upregulated and loaded into AGO. We further demonstrate the functionality of the upregulated passenger 3p-miRNAs using AGO2 immunoprecipitation, reporters assay, and mRNA-seq, suggesting a potential role in DICER1 tumor pathogenicity. Thus, our study indicates that Dicer RNase IIIb hotspot mutation not only causes loss-of-function for 5p-miRNAs as initially believed but also has a functional impact from the dysregulated 3p-miRNAs.

Dominik Reichert
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Stem Cells - General and Cancer
Mutation Specific Cell Autonomous Phenotypic Differences in iPSC derived RPE from Ciliopathy Patients
The primary cilium is an antenna-like organelle extending from the cell and vital for the development and function of all cells in the human body. Mutations in ciliary genes are associated with a large group of syndromically heterogenous developmental disorders - ciliopathies. Retinal Degeneration (RD) is one of the most common phenotypes among all ciliopathies and yet heterogenous in presentation. Primary cilia are important for the development of photoreceptors (PR), the light sensing cell of the eye and the adjacent Retinal Pigment Epithelium (RPE), a polarized monolayer required for proper functioning of PR. To reveal the effect of RPE defects on RD in ciliopathies, we asked how different ciliary mutations in RPE contribute to cell autonomous defects. Here, we generated induced pluripotent stem cell (iPSC)-derived RPE (iRPE) from patients with various ciliopathy-associated mutations (BBS1, BBS10, BBS16, CEP290, LCA5, MYO7A, PRPF31), that affect cilia structure and function differently. All patient iPSC lines differentiated into RPE, showed junctional integrity as measured by transepithelial resistance, and expressed RPE maturity markers. Visual inspection of patient iRPE monolayers showed abnormal cell morphology, indicating loss of epithelial phenotype. To quantify morphometry changes, we used an artificial intelligence (AI)-based image analysis software (REShAPE) that allows evaluation of single cell shape. REShAPE revealed differential effects of ciliary mutations on iRPE shape metrics. Ultrastructural analysis via SEM and TEM revealed sparse and malformed apical microvilli of patient iRPE, suggesting improper maturation of iRPE with different mutations and further confirmed the loss of epithelial phenotype. Structural abnormalities of mitochondria were found in TEM, validated with immunofluorescence staining, and further indicated disparate effects of different mutations. To better understand how cilia-associated mutations differentially affect RPE and to find common molecular targets among different mutations we are performing metabolic analyses and RNA-seq. This work introduces ciliopathy-associated cell autonomous disease models and provides insight into how ciliary mutations differentially cause RD. Our work to discover common molecular targets in cells derived from

different patients will help find mutation-agnostic gene and drug-based therapy approaches, addressing the current absence of treatments for ciliopathy linked RD.

Jingxin Feng
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Stem Cells - General and Cancer
Transplantable adult bone marrow hemogenic endothelial cells generate multilineage hematopoietic progenitors
During mouse development, hematopoietic stem cells (HSC) that originate from hemogenic endothelial cells (ECs) through a process of endothelial-to-hematopoietic transition are generally thought to sustain adult hematopoiesis. However, it remains unknown whether adult endothelial cells retain hemogenic potential. We used in vivo genetic lineage tracking and analysis of single-cell RNA sequencing to evaluate the presence of hemogenic ECs in the adult mouse. Our analysis identifies a subset of bone marrow-resident, adult ECs that spontaneously produce CD45+ hematopoietic cell precursors and mature blood cells. These hemogenic ECs are transplantable into adult host mice and contribute to recipient hematopoiesis. Blood cells from adult and developmental endothelial-to-hematopoietic transition physiologically home to peripheral tissues, where they similarly contribute to the inflammatory response and elimination of bacterial pathogens. Thus, our results identify a previously unrecognized bone marrow-derived population of engraftable adult hemogenic ECs that generates hematopoietic progenitors and functional mature blood cells, suggesting that such population represents a potential new source of hematopoietic cells.

Susannah Shissler
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Stem Cells - General and Cancer
Investigation of adult thymic epithelial cell progenitors using Foxn1 lineage tracing
The development of T cells is dependent on the thymus and thymic epithelial cells (TEC). Cortical (c)TEC facilitate early T cell development and positive selection and medullary (m)TEC coordinate negative selection and enforce central tolerance. Significant heterogeneity is evident within TEC compartments, but the relationships between these subtypes. Enhanced understanding of TEC progenitor-successor relationships will inform regeneration in the elderly and immunocompromised. Whereas cTEC and mTEC derive from bipotent progenitors during fetal ontogeny, progenitors in the adult – bipotent or lineage-restricted – remain to be better identified. The transcription factor Foxn1 is required for formation of the thymus. Postnatally, Foxn1 is known to regulate genes involved in TEC functions such as antigen processing and presentation. We hypothesized that adult TEC progenitors express Foxn1. To test this hypothesis, we developed a Tamoxifen-inducible, fate mapping system under the control of Foxn1 (Foxn1-Cre-ERT2). Preliminary results demonstrate that induction of Foxn1-Cre-ERT2 in adult mice efficiently fate maps cTEC as well as both MHC-Hi and MHC-Lo mTEC populations. The proportion of labeled TEC persists long term (out to 20wk) in all TEC compartments, including the MHC-Hi subset of

mTEC that is known to be a short-lived population. The stable, long-term labeling of the MHC-Hi mTEC compartment indicates that the progenitor that maintains this population expresses Foxn1 in adult mice and is traced in our system. To determine whether single progenitors are bipotent or lineage restricted, we utilized the Confetti reporter system and Ce3D full-lobe imaging to assess the clonal contribution to TEC compartments in situ. Preliminary data from 5 days and 20 weeks post-induction show the presence of small medullary clonal clusters specifically in late time points. Additionally, whereas single, fluorescently labeled Aire+ cells are present at early timepoints, fluorescently labeled Aire+ cells were only detectable in clusters at later timepoints. We are currently pursuing TEC subset markers to determine the composition of these clonal clusters and nuclear markers to calculate clonal burst size. Our experiments will thus characterize mTEC progenitors that express Foxn1 and are labeled in Foxn1-Cre-ERT2 mice, and will clarify lineage relationships between these labeled TEC progenitors and their putative downstream progeny.

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NIA
Stress, Aging, and Oxidative Stress/Free Radical Research
B cells from old mice produce lower affinity antibodies than B cells from young mice upon immunization
Vaccination is the most successful strategy to reduce morbidity and mortality from viral infections to date, but the efficacy of vaccination is greatly reduced in the elderly. To improve the protection of the old from novel infectious diseases, such as COVID-19, it is essential to understand how immunity changes with age. Our lab has found that B cells change with age and have altered functionality even when the rest of the immune system is young. We found that naïve B cells from old mice produce less specific antibody than naïve B cells from young mice after immunization, which models what happens in humans after vaccination. In this study, we investigated the participation of old B cells in the germinal center reaction, which is a process responsible for increasing the quality of antibodies during an immune response. Using single-cell B cell receptor sequencing (scBCR-seq), we characterized the antibodies produced by young or old B cells. We found that while the old B cells were participating in the germinal center reaction by mutating their B cell receptors (BCRs), they were making different antibodies than young B cells. We produced these antibodies recombinantly to study their affinity for the immunogen using biolayer interferometry and found that old BCRs had lower affinity than young BCRs. We conclude that the reduced serum antibody response from old B cells is due to the production of lower quality antibodies from the germinal center reaction. Our hypothesis is that old B cells have a lower threshold of BCR affinity required for activation, thus allowing lower affinity antibodies to prevail during selection.

In support of this hypothesis, we have found that old B cells have higher levels of BCR signaling both before and during the germinal center reaction. Our ongoing work will investigate the molecular mechanism that causes this age-related phenomenon. Understanding this process will guide the development of vaccine additives that can improve antibody responses and thus protection from disease in the elderly.

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Stress, Aging, and Oxidative Stress/Free Radical Research

Senescent cells produce and respond to extracellular matrix proteins such as THBS3 to implement a pro-survival EMT-like program

Cells facing sublethal damage implement a complex dynamic program known as senescence, entering a state of indefinite growth arrest and active secretion of a range of molecules. This latter trait, referred to as the SASP (senescence-associated secretory phenotype), mostly comprises soluble molecules such as cytokines and growth factors. However, several pro-fibrotic extracellular matrix (ECM) proteins are also known to be produced during early senescence, although their role remains poorly understood. Previous findings in our lab indicated that integrin signaling is key for favoring senescence over apoptosis upon cellular stress by promoting an Epithelial-to-Mesenchymal Transition (EMT)-like program. Since most integrin ligands are ECM proteins, we wondered whether such proteins could reinforce the pro-survival phenotypes associated with an EMT-like program in senescent cells. To investigate that hypothesis, we performed a screen using a library of small interfering (si)RNAs capable of silencing each of the 53 integrin ligands expressed in human WI-38 lung fibroblasts, and triggered senescence by exposure to sublethal etoposide levels. Importantly, we found that 16 of these proteins significantly promoted senescence and survival over apoptosis at early stages of the damage response. These ECM candidates included collagens (COL1A2, COL4A1, COL5A1, COL5A2, COL6A1, COL10A1, COL15A1, COL16A1, COL22A1, and COL24A1) and laminins (LAMB2, LAMB3, and LAMC2), along with MFGE8, THBS3, and VCAM1 proteins. Among them, THBS3 was the ECM protein most capable of both promoting senescent cell viability and implementing an EMT-like program. We observed that senescent cells robustly increased THBS3 levels early into the response (24 h after etoposide treatment), and that silencing THBS3 reduced by 50% the viability of WI-38 cells in conditions leading to cell senescence. In sum, we have found that senescent cells produce and respond to ECM proteins such as THBS3 to promote an EMT-like program and favor survival over apoptosis in response to damage and stress. Importantly, these findings suggest that the presence of certain ECM proteins influences the balance between senescence and apoptosis in response to injury, possibly favoring the accumulation of damaged senescent cells in tissue decline processes such as aging or fibrosis.

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Stress, Aging, and Oxidative Stress/Free Radical Research

Senescence-Associated Decrease in Ribosome Content Prevents uORF Bypass During the Integrated Stress Response

Senescence is a permanent state of cell cycle arrest that occurs in response to sub-lethal genomic damage and is expected to serve as a guard against tumorigenesis. However, the accumulation of senescent cells has been associated both with decreased longevity and several aging-associated diseases. These findings have prompted a "senocentric" view of aging, in which the selective clearance of senescent cells may serve as a therapeutic approach toward ameliorating conditions of aging. To investigate new avenues for therapeutic intervention of senescence, we conducted a tandem-mass tag (TMT) based mass spectrometry screen of senescent, cycling, and quiescent cells to identify proteomic changes specific to senescence. We identified that senescent cells have unique increases in proteins related to ER function suggestive of integrated stress response (ISR) activation. However, we found that while senescent cells had substantial increases in eIF2α phosphorylation (eIF2α-P), indicative of ISR activation, they did not express the downstream factor ATF4 normally increased by the ISR. The expression of ATF4 is linked to eIF2α-P levels by a mechanism of upstream ORF (uORF) bypass, wherein increased eIF2α-P reduces the rate of translation initiation and thus promoting scanning of the 40S ribosomal subunit over an inhibitory uORF in ATF4 mRNA. We used ribosome sequencing to determine the state of ATF4 translation in senescence and found that ribosome footprints of senescent cells were mainly located in the uORFs, indicating bypass was not effective. We also saw that senescent cells had much lower ribosome footprints in total. We thus hypothesized that a global decrease in ribosomes would suppress the effects of eIF2α-P by effectively reducing the demand for eIF2α. Consistent with this, we found that transfection of ATF4 mRNA produced little protein senescent cells that was rescued by mutation of the uORFs. We further explored senescent resistance to uORF bypass by subjecting senescent cells to proteotoxic and starvation stressors, both of which also showed little ATF4 expression despite high eIF2α-P in senescence. Our results show that translation is highly perturbed in senescence and offers a new strategies for senolytics to exploit both reduced ribosomes and a less effective ISR. Furthermore, our results on ISR resistance may broadly apply to other cases where ribosome content has decreased such as ribosomopathies like Diamond-Blackfan anemia.

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Diverse viral small RNAs in geographically distinct Aedes aegypti: implications for vector competence

Aedes aegypti mosquitoes are key vectors of arthropod-borne (arbo) viruses such as Zika, dengue (DENV), and yellow fever. However, not all A. aegypti are equally able to transmit arboviruses in the field. Why this is remains poorly understood. Previous studies showed that A. aegypti haplotype differences were associated with major differences in their abilities to support virus infection. However, little is known how the mosquito virome is involved. Throughout their lifetime, mosquitoes are infected with insect-specific viruses (ISVs) that may impact arbovirus transmission dynamics, perhaps by mediating RNA interference (RNAi), the mosquito's main antiviral immune mechanism. Virus-specific small RNAs (sRNA) generated by RNAi are indicative of an active infection; we therefore hypothesized that sRNA sequencing in A. aegypti of distinct geographic origins would capture ISV diversity on a global scale. We sequenced sRNAs in A. aegypti across the Americas – in the southern and West coasts of USA,

across Mexico, and in Recife, Brazil. These populations were chosen based on their varying abilities to transmit DENV. Overall, ISV sRNAs were highly diverse. We captured sRNAs from more than 15 unique ISVs and found that most mosquitoes harbored 5-10 different ISVs at a time. Although some ISV sRNA families defied geographic boundaries – such as those from Phasi Charoen-like and Humaita-Tubiacanga viruses – others were more restricted. For example, Anphevirus and Verdadero virus sRNAs were present in mosquitoes in the southern US and Poza Rica, Mexico but were absent from those further south. This suggested that some ISVs display geographic structure. Our data correlated with another study showing that A. aegypti from Poza Rica or Tapachula, populations separated by a neovolcanic axis, displayed significant differences in their abilities to transmit DENV. We therefore infected both mosquito strains with DENV2, and fed a subset uninfected cell culture as controls, and are repeating small and total RNA sequencing to compare ISV and DENV2-specific sRNA abundances and to perform whole transcriptome analyses. Future work comparing ISV sRNAs in mosquitoes across Asia and Africa is also underway. Our study highlights the diversity of ISVs sRNAs across A. aegypti populations and demonstrates how sRNA profiling sheds light on virome responses. Understanding ISV-arbovirus interactions is important because ISVs could be exploited to curb arbovirus transmission in the field.

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Unveiling mosquito antiviral immunity mediated by hemocytes
Mosquito-transmitted viruses emerge frequently and are a major threat to public health worldwide. The Aedes aegypti mosquito is the primary arboviral vector in tropical and subtropical regions, while anopheline mosquitoes are major vectors of human malaria parasites. Intriguingly, although in some areas malaria vectors encounter humans infected with arboviruses, anopheline mosquitoes are not vectors of major arboviral infections such as dengue, zika or chikungunya. This suggests some mosquito species may be mounting more robust antiviral responses than others. In insects, hemocytes mediate cellular innate immune responses, but their role in limiting mosquito viral infections is still unclear and has been controversial. Some studies have suggested that in Aedes aegypti hemocytes support viral replication and can promote dissemination and persistence of infection. In contrast, Drosophila hemocytes have been shown to control viral infection by using viral RNA as a template to generate viral DNA (vDNA) through reverse transcription. In turn, vDNA serves as a template for de novo synthesis of non-infectious vRNA that are delivered systemically by hemocyte exosomes, providing an antiviral defense to other tissues before they are infected. This project aims to understand the role of hemocytes

on viral dissemination in different mosquito species. We compared the immune response of Aedes aegypti and Anopheles gambiae to infection with Eilat virus. Our data indicate that viral replication is 43 times higher in Ae. aegypti than An. gambiae when both are infected by injection with the same number of viral particles. Interestingly, while An. gambiae hemocytes produced the vDNA in response to infection, as reported for Drosophila, vDNA could not be detected in Ae. aegypti hemocytes. We are currently investigating how An. gambiae hemocytes generate vDNA. We identified a specific hemocyte population in An. gambiae that generates large amounts of vDNA and is absent in Ae. aegypti. We are currently silencing several putative reverse transcriptase in An. gambiae to identify the enzyme(s) mediating vDNA synthesis, and to determine the role of hemocyte vDNA in antiviral immunity. Overall, this study provides valuable insights into the mechanisms underlying mosquito vectorial capacity, and the role of hemocytes from susceptible and resistant species on arboviral disease transmission.

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Virology - General

MUTATIONS OUTSIDE INTEGRASE LEAD TO HIGH-LEVEL RESISTANCE TO INTEGRASE INHIBITORS

Second-generation integrase (IN) strand transfer inhibitors (INSTIs) are highly potent antiretroviral compounds that exhibit a high genetic barrier to resistance. Recent clinical studies concluded that some INSTI-treated individuals experience virological failure in the absence of resistance mutations in IN. To elucidate INSTI resistance mechanisms and pathways, we performed long-term passaging of lab-adapted and primary viral isolates using human T-cell lines or primary PBMCs over nearly one year with an escalating concentration of the INSTI dolutegravir (DTG). Independent of viral subtype and coreceptor usage, HIV-1 became resistant to DTG by sequentially acquiring mutations in Env and Gag-nucleocapsid (NC) in the absence of resistance mutations in IN. The NC mutations selected with DTG clustered in the zinc-finger domains and conferred modest (3-5- fold) resistance to INSTIs but not to the RT inhibitor. We show that one of the HIV-1 mutants, 7XEnv, containing seven Env substitutions exhibits faster-than-WT replication and resistance to multiple classes of ARVs, with the fold resistance being ~2-logs higher for INSTIs than for other classes of drugs. HIV-1 Env mediates viral entry via cell-free infection or cell-cell transmission. We demonstrate that viral transmission of 7XEnv through cell-cell contact is more efficient than that of WT, resulting in a higher multiplicity of infection (MOI) and reduced sensitivity to DTG. However, despite this highly efficient cell-cell transfer capacity, the 7XEnv mutant exhibits impaired cell-free viral infectivity and fusogenicity. Interestingly, viral infection using VSV-G-pseudotyped viruses over a range of MOIs revealed that INSTIs are more readily overwhelmed by high MOI than RT inhibitors. 7XEnv exhibits higher binding affinity for CD4 relative to WT but reduces CD4-induced conformational rearrangement of the Env trimer, which is necessary for viral entry in cell-free infection. Overall, these findings demonstrate that a combination of mutations in Env and NC can confer high-level resistance to INSTIs in the absence of IN mutations. The Env mutations overcome inhibition by INSTIs through increased MOI mediated by highly efficient cell-cell transfer. These results advance the understanding of how HIV-1 can evolve resistance to ARVs in the absence of mutations in drug-target genes and provide new insights into the contribution of cell-cell transfer to viral replication and drug resistance.

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Virology - Pathogenesis/Therapeutics

A novel model of Nipah virus neurologic disease in Syrian hamsters

Nipah virus (NiV) is a highly pathogenic virus that has been associated with outbreaks in Malaysia, Bangladesh, and India with mortality rates up to 75%. NiV infection causes fatal respiratory and neurologic disease. Approximately 60% of survivors suffer from long-term neurologic sequelae or late onset and relapsed encephalitis. There are currently no approved treatments or vaccines for Nipah virus infection, and experimental treatments are tested in animal models skewed towards the development of lethal respiratory disease rather than neurologic disease. The pathogenesis of neurologic disease is complex and cannot be studied in existing animal models. Here, we investigated a Syrian hamster (Mesocricetus auratus) model to mimic acute and chronic NiV neurologic disease in humans by treating them with a suboptimal dose of the antiviral drug remdesivir. Hamsters were challenged intranasally or intraperitoneally with 10^6 TCID50 of NiV, strain Bangladesh. At this dose, 25% of intranasally-challenged animals did not show disease signs, 25% fatal respiratory disease, and 50% neurologic signs. Comparatively, in intraperitoneally challenged animals the infection was 100% lethal by 6 days. To decrease the incidence of respiratory disease and potentially increase survival with neurologic manifestation, animals were administered either 15mg/kg, 20mg/kg or 25mg/kg remdesivir intraperitoneally for 7 days, starting at 1-day post inoculation (dpi). Treatment with remdesivir resulted in a delay in time to death by 3-10 days in the intranasally challenged group but only 1-2 days in animals challenged intraperitoneally. Interestingly, the delay in death shifted the disease manifestations from primarily respiratory to neurologic signs. Higher Nipah viral loads were detected in the brains compared to the lungs. Histopathologic examination of the brain showed moderate to severe meningoencephalitis primarily restricted to the cerebrum. NiV antigen was detected in endothelial cells, neurons, and microglia/macrophages. Furthermore, infectious virus was detected in the brain until 18 dpi in hamsters treated with remdesivir. We are further optimizing the challenge doses and genotype to establish a reliable, reproducible model to study the molecular basis of Nipah virus neurologic disease and enable testing of therapies in preventing or curing NiV neurologic disease.

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Detection of envelope-dimer epitope-like broadly protective antibodies in dengue-immune children in the Philippines following vaccination and natural infection

The dengue vaccine, Dengvaxia, has been described as inducing antibody-dependent enhancement (ADE) in dengue-naive individuals. There is an urgent need to develop an effective dengue vaccine that elicits protective, cross-reactive neutralizing antibodies (Abs) like envelope-dimer epitope (EDE) Abs, which target quaternary epitopes on the E protein dimer and neutralize dengue viruses 1-4 (DENV1-4) without triggering ADE. Here, we investigated natural infection and vaccine-induced cross-reactive neutralizing Abs in children with prior DENV infection histories in a longitudinal vaccine cohort in Cebu,

Philippines. In total, 1,214 children remained unvaccinated while 1,782 children received a single dose of Dengvaxia in June of 2017 during a mass vaccination campaign. Serum samples were collected one month before and one year after the vaccine campaign. We selected a random subset of n=223 polytypic DENV-immune children to measure baseline status and change in EDE-like Abs due to natural infection and vaccination. Samples were tested by Plaque Reduction Neutralization Test (PRNT) with mature DENV1-4 virions and a Blockade-of-Binding (BOB) assay to detect Abs that prevent EDE Ab C8 from binding a DENV2 E protein dimer. An IgG ELISA against the DENV1-4 E protein monomer and DENV2 E protein dimer were also performed on the same participants for comparison to the mature PRNT and BOB. Both vaccinated and naturally infected groups had high levels of EDE C8-like Abs. We also observe a significant increase in Ab level in both groups across all assays, but C8-like Abs had a stronger correlation to the mature PRNT in naturally infected compared to vaccinated populations, indicating differences in Ab quality. These results highlight the presence of EDE-like Abs that could possibly protect against the four DENV serotypes and provide an insight for future vaccine candidates.

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Mapping the dynamic host-pathogen interactions in JC virus infection and disease
Introduction: Progressive multifocal leukoencephalopathy (PML) is an often-fatal demyelinating disease of the central nervous system, due to reactivation of JC virus (JCV) in the context of severe immune dysfunction. Pathophysiology of JCV infection is complex, and many open questions remain regarding how the virus maintains latency, reactivates, and specifically infects glia. Next generation sequencing (NGS) technologies provide the tools to comprehensively survey host-pathogen interactions longitudinally and create a detailed map integrating JCV intra-host genomic evolution, host cell function, and host immune response. Experimental Methods: Fresh frozen brain sections from two patients who died of PML were analyzed. Ante-mortem clinical MR imaging was used to identify four regions of interest for transcriptomic characterization, including leukocortical junction, edge of an active PML lesion, center of a lesion, and normal appearing white matter. Reads were aligned to the annotated human and the JCV genomes (CY AB038249.1). Results: Regions of active infection, including leukocortical junction, lesion edge and lesion center, have increased numbers of JCV-infected cells compared to normal appearing white matter. Most highly infected cells cluster together and away from healthy cells indicating distinct molecular phenotypes due to infection status. Highly infected cells have less diverse host transcriptional profiles and are more likely classified in S- or G2M-phase, consistent with viral cooption of host cell transcriptional machinery. A minority of infected cells still cluster with healthy cells, primarily oligodendrocytes, neurons, and myeloid cells, possibly depicting earlier stages of infection or phagocytosing myeloid cells. Preliminary differential gene expression analysis highlights that pathways associated with innate immunity and antigen presentation are enriched in infected cells compared to uninfected cells. Conclusions: We have established an initial workflow for MRI-guided tissue sampling and single cell sequencing that can effectively capture changes in viral and host biology during JCV infection. Current challenges include interpretation of variability observed between patients and optimization of ROI selection. Next steps are to increase sample numbers and complete

histopathologic validation of ROIs. Advancing our understanding PML pathogenesis will be critical to develop novel therapies for this devastating disease.
