FARE2021WINNERS
Sorted By Study Section

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Biochemistry - General, Proteins, and Lipids
Nuclease free genome editing into Albumin using a promoterless AAV vector treats mice with methylmalonic acidemia (MMA)
MMA is a rare and heterogenous inborn error of metabolism most commonly caused by a deficiency of methylmalonyl-CoA mutase (MMUT). Patients suffer from frequent episodes of metabolic instability, severe morbidity, and early mortality. Elective liver transplantation has emerged as a treatment option for severely affected patients and can eliminate the potentially lethal metabolic instability associated with this disorder but is not without risk. Gene therapy has been explored in MMA mouse models as an alternative to transplantation. Conventional adeno-associated viral (AAV) mediated gene delivery was highly effective in the treatment of neonatal mice with severe MMA. However, treated mice experienced a significant loss in transgene expression over time and showed an increased incidence of hepatocellular carcinoma (HCC) caused by insertional mutagenesis. Conventional AAV gene therapy is limited by concerns of rapid transgene loss and potential genotoxicity with neonatal treatment. To preserve MMUT expression and minimize the potential of vector-related insertional mutagenesis after therapeutic gene delivery, we designed a promoterless AAV vector utilizing homologous recombination to achieve site-specific gene addition of human MMUT into the mouse albumin (Alb) locus. Neonates and juvenile MMA mice treated with the promoterless nuclease-free vector demonstrate continuous disease correction following treatment. The treated MMA mice display a constant increase of hepatic MMUT expression, which corresponds to increasing enzyme activity, continually reduced levels of disease-associated plasma biomarkers over time, a dramatic reduction of a disease associated plasma biomarker of mitochondrial dysfunction and improved weight gain. The number of transgene integrations into the Alb gene increased over time in treated mutants. Additionally, AAV corrected hepatocytes appear as distinct and widely dispersed clusters, which increased with size over time and is consistent with a pattern of clonal expansion. Treated mice have shown durable transgene expression for more than a year, and aged mice treated as neonates have not developed HCC (30 mice followed 13 to 26 months after treatment). The progressive clinical and biochemical improvement in the treated mice is consistent with an expansion of corrected hepatocytes, yielding a greater therapeutic benefit with time, and is accompanied by a predictable pattern of biomarker changes that will facilitate clinical translation.

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Vitamin E Treatment in NAFLD Reveals Novel Pathway: Oxidative Stress Drives De-Novo Lipogenesis Through S1P/S2P Proteases
Non-alcoholic fatty liver disease (NAFLD) is rapidly increasing in prevalence and is becoming a leading cause for liver transplantations in the US. The hallmark feature of NAFLD is the accumulation of hepatic lipids or steatosis. Steatosis and process linked to its development drive oxidative stress (OS), especially lipid peroxidation which in turn is linked to liver injury and inflammation. Vitamin E (alpha-tocopherol, aT), an antioxidant, improves liver injury but also decreases the upstream steatosis. This raises the question of how vitamin E, decreases steatosis and whether the process is controlled by oxidative stress. We designed a mechanistic clinical trial to understand the underlying mechanism of action of aT. We obtained paired liver biopsies, at baseline and early on treatment (week 4) from patients with NAFLD (n=20), and using a lipidomic approach, found that aT decreases hepatic de novo lipogenesis (DNL) . Furthermore, changes in hepatic DNL at week 4 predict the effect on steatosis later (week 24). Other pathways of hepatic fat accumulation (adipose tissue contribution, dietary sources, TG export from the liver) were not affected. In vitro, using Hep G2 and primary human hepatocytes, we confirmed the ability of aT to decrease DNL in 2 independent assays. We further found that aT lowers DNL by downregulating the proteases S1P and S2P, leading to decreased procession of the transcription factor SREBP-1 and impairing its nuclear translocation. We synthesized a vitamin E derivative, lacking the antioxidant effect (methoxy-alpha-tocopherol, m-aT) to tease apart whether the observed effect of vitamin E requires its antioxidant activity. m-aT failed to inhibit DNL which supports an involvement of OS in the process. To prove a direct role for OS in regulating SREBP-1 processing, we induced OS using cumene hydroperoxide (CHP). Indeed, CHP increased protein expression of S1P and S2P, SREBP-1 translocation, and resultant lipid accumulation, confirming a direct regulatory role of OS on SREBP-1 processing. Finally, we found that both lipid peroxidation markers (4-HNE protein adducts) as well as S2P protein are upregulated in livers of NAFLD patients, confirming the imporance of this pathway in vivo. In summary, we utilized aT to uncover a novel physiological role for OS in regulating hepatic DNL by modulating S1P and S2P, which in the context of NAFLD leads to a vicious cycle, where steatosis both drives and is driven by OS.

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CUL3-KLHL4 regulates cytoskeletal-based cell fate decisions during neurodevelopment
Embryonic development is a complex process requiring tightly coordinated cell-fate decisions to ensure proper lineage maintenance and differentiation. To control these fate choices, stem cells frequently make use of the post-translational modification ubiquitylation, an essential process required for intracellular protein degradation and signaling. The specificity of ubiquitylation is conferred by E3 ligase enzymes that recognize particular substrates to catalyze ubiquitin transfer. CUL3-BTB E3s are a class of ubiquitin ligases that play crucial roles during neurodevelopment and are dysregulated in neurological diseases; however, their underlying mechanisms have remained largely elusive. Using a genotype-first approach to query rare disease databases for missense mutations in genes encoding for CUL3 substrate adaptors, we identified two patients presenting with neurodevelopmental phenotypes with hemizygous missense variants in KLHL4. Supporting that these variants are pathogenic, we find that KLHL4 depletion leads to defects in neuroectodermal differentiation in human embryonic stem cell models and during chick development. To understand how KLHL4 regulates neural differentiation, we employed proteomic and biochemical approaches. These revealed that KLHL4 interacts with the GIT-PIX-PAK signaling module, a key regulator of cytoskeletal dynamics during neurodevelopment. Strikingly, we found that KLHL4 variants found in patients lead to the loss of interaction with either the CUL3 catalytic subunit or the GIT-PIX-PAK signaling complex. Importantly, similar to reduction of KLHL4, depletion of components of the GIT-PIX-PAK module resulted in aberrant neuronal differentiation of human embryonic stem cells. Taken together, our data demonstrates that ubiquitylation activity by CUL3-KLHL4 is required for signaling through the GIT-PIX-PAK cytoskeletal signaling axis for proper neuronal development and, if reduced, leads to neurodevelopmental disease.

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A Patient-derived Glioblastoma Model in a Three Dimensional Bioprinted Neurovascular Unit tissue: Moving towards in Tissue Personalized Medicine
Glioblastoma (GBM) is a fast-growing, aggressive brain cancer with high heterogeneity of subpopulations. The prognosis for patients with high-grade GBM remains extremely poor, thus, there is

a tremendous medical need for more effective treatments. Using three dimensional (3D) biofabrication technologies, including tissue bioprinting, we have successfully established a 3D neurovascular unit (NVU) tissue in a multiwell plate format. The biofabricated NVU includes human GFP-tagged brain endothelial cells, brain pericytes, and astrocytes. A ring-shaped bioprinted structure of vasculature with an inner circular-shape drop of mCherry expressing GBMCs was created in each well to facilitate the quantification of vascular morphology and GBM invasion and growth, respectively. Time dependent vascular angiogenesis in the presence or absence of GBMCs was monitored using a high content fluorescence imaging system. In the tissues without the inclusion of the GBMCs, we confirmed vasculogenic and angiogenic activities occurring for two weeks after bioprinting. The total length of angiogenetic vessel gradually increased with time, along with deposition of brain relevant extra cellular matrix components, like collagen IV, laminin, and fibronectin, demonstrating formation of a 3D neurovascular unit. When introducing the GBMCs into this NVU model, we observed cancer cell infiltration into the outer ring vascular area through the branched vessels. Moreover, the microvascular structure in contact with the GBMCs area collapsed, even though the endothelial cells remained viable and proliferated inside the GBM area. The GBM-microvessel interaction observed is similar to that described in human GBM pathological condition, which showed an abnormal and collapsed microvascular morphology. The single cell RNA sequencing and anti-cancer drug treatments are now being used to study changes in cell populations in the tissue as well as finding potential druggable targets driving cancer cell growth in these tissue models. For the future work, neuronal cells will be included in the tissue model to achieve a better physiological relevance of the neurovascular tissue. This GBM in-a-tissue model will ultimately provide extensive opportunities to investigate patient-specific pathology and to find a personalized drugs for patients.

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Structure and engineering of the homodimeric Mango-IV fluorescent RNA
Of the 6.4 billion base pairs composing the human genome, no more than 3% of this information is translated into proteins while no less than 75% is transcribed into RNA. Fluorescent proteins revolutionized cell biology by functioning as genetically encodable fusion tags for observing protein interactions and localization in live cells. The motivation of this work is to develop and characterize genetically encodable fluorescent RNA tags with functions analogous to fluorescent proteins. These chemical tools have the potential to unlock the spatial organization and interaction networks encoded by the majority of the genome. The combination of in vitro evolution and microfluidic fluorescence sorting has produced approximately 12 fluorescent RNAs (7 distinct families) that activate the fluorescence of a small molecule upon binding. Of these RNAs, Mango-IV is optimal at imaging RNA fusions in both fixed and live mammalian cells. To understand how Mango-IV achieves superior activity in cells, we determined the Mango-IV crystal structure complexed with its cognate fluorophore, TO1-Biotin. The structure reveals a domain-swapped homodimer with two independent fluorophore binding pockets. This architecture is unique amongst fluorescent RNAs and highlights the diversity of RNA folds attainable by combined selection protocols. Structure-guided analyses indicate that the Mango-IV core has a relaxed fluorophore specificity, and a tendency to sample multiple conformations in the binding core. Based on Mango-IVâ€™s domain-swapped structure and ability to enhance fluorescence of spectrally diverse small molecules, heterodimers between Mango-IV and the fluorescent RNA iSpinach were designed and constructed to optimize association and spectral overlap of fluorophores. These heterodimers were demonstrated to perform fluorescence resonance energy transfer (FRET) between their respective RNA-activated fluorophores, YO3-Biotin and DFHBI-1T. This work advances fluorescent RNA technology towards multi-color imaging with the purpose of understanding RNA coexpression and colocalization in live cells.

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Carcinogenesis

Inhibition of protein arginine methyltransferase 5 ameliorates MYC-driven hepatocellular carcinoma
Liver cancer is one of the leading causes of cancer-related deaths worldwide, with incidence and death rates increasing by about 3% per year in western countries. Hepatocellular carcinoma (HCC) is the most common liver cancer, representing up to 90% of all primary hepatic malignancies. Current first-line treatment for HCC is limited to the kinase inhibitors sorafenib and lenvatinib, but resistance is a matter of course. Thus, there is an urgent need to develop new agents for HCC early detection and treatment. MYC, a basic helix-loop-helix (bHLH)-leucine zipper transcription factor with broad effects on various biological processes, is frequently dysregulated in human HCC. In the current study, HCC was induced in mice with hepatic-specific disruption of Myc (Myc-dHep) and control mice (Myc-floxed) by administration of diethylnitrosamine (DEN). Hepatic-specific Myc disruption suppressed HCC tumorigenesis. Dynamic LC/MS-based metabolomics analysis of urine samples from Myc-dHep and Myc-floxed mice revealed that dimethylarginine, especially symmetric dimethylarginine (SDMA), were increased in HCC in a MYC-dependent manner. We found that protein arginine methyltransferase 5 (Prmt5) was a novel direct target gene of Myc. Consistent with the mouse data, the level of SDMA was increased in the urine samples from HCC patients compared with healthy people. MYC and PRMT5 were both increased in human liver tumor specimens compared with adjacent non-tumor tissues. Administration of GSK3326595, a PRMT5 inhibitor in trials for the treatment of non-Hodgkinâ€™s lymphoma and solid tumors, to human MYC-overexpressing transgenic mice that spontaneously develop HCC, suppressed the growth of liver tumors as monitored by serial magnetic resonance imaging (MRI) scanning. Flowcytometry and immunofluorescence staining showed a surprising and unexpected increased lymphocyte infiltration in the GSK3326595-treated liver tumors compared with liver tumors from vehicle-treated mice, which may contribute to enhanced anti-tumor immunity and decreased tumor growth. Collectively, this study suggested that PRMT5, encoded by a MYC target gene, could be a new drug target for HCC treatment.

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Carcinogenesis
Loss of tumor suppression by Dlx3 is associated with poor prognosis in human SCCs and tumor promotion in mice
Squamous cell carcinoma (SCC) is the second most frequent skin cancer. Development and progression of cutaneous SCC is known to be regulated by traditional oncogenic and tumor suppressive proteins (e.g., RAS, FYN, p53); however, new evidence suggests that homeoproteins can act as cancer modulators through regulation of proliferation, migration and survival. We have shown that the DLX3 homeoprotein regulates keratinocyte proliferation and differentiation through p53 targets and cell cycle exit, suggesting that Dlx3 may also play a role in tumorigenesis. We carried out a human clinicopathologic analysis of DLX3 expression in 121 skin SCC and 6 benign or intermediate skin tumors (verruca vulgaris, actinic keratosis and Bowenâ€™s disease). Statistical correlation analysis showed that tumors of increased pathologic stage and grade had significantly diminished levels of DLX3 protein expression. Kaplan-Meier analysis of overall survival (OS) and progression free survival (PFS) revealed statistically significant differences in both metrics between patients with high DLX3 expression and those with low

DLX3 expression. We then used a murine two-stage dimethylbenzanthracene (DMBA)/12-O-tetradecanoylphorbol 13-acetate (TPA) skin tumorigenesis model to gain insight on Dlx3 function in vivo comparing Dlx3 knockout (Dlx3KO) to wild-type (WT) littermate mice. In the two-stage skin carcinogenesis model, the Dlx3KO mice presented with significantly quicker and higher number of tumor formation compared to WT mice. It is widely accepted that treatment with tumor initiator (DMBA) does not produce tumors without a chemical promoter (TPA). To assess if absence of Dlx3 function confers a â€œpromotedâ€ phenotype, we next examined the effect of DMBA alone on Dlx3KO skin. In Dlx3KO mice, tumors began to appear at ~16 weeks after DMBA treatment whereas none of the WT mice developed tumors during the entire time frame of the experiment (~31 weeks). Our study showed that a single DMBA application was sufficient to produce tumors in Dlx3-deficient skin. To understand the molecular mechanism by which Dlx3 loss leads to tumor development, we performed transcriptome analysis (RNA-seq) of tumor and skin tissue from our mouse model. Our preliminary analysis indicates ERBB2/EGFR as a key downstream mediator of Dlx3 function. Taken together, data from human and mouse model system supports a tumor suppressive function for Dlx3 in skin.

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Overexpression of hTLX3 in association with mutant IL7RÎ± is sufficient to generate T-ALL in vivo and to transform thymocytes in vitro
Mutations of the IL-7Ra chain occur in approximately 10% of pediatric T-cell acute lymphoblastic leukemia cases. While we have shown that mutant IL7Ra is sufficient to transform an immortalized thymocyte cell line, mutation of IL7Ra alone was insufficient to cause transformation of primary T cells, suggesting that additional genetic lesions may be present contributing to initiate leukemia. Studies addressing the combinations of mutant IL7Ra plus TLX3 overexpression indicates in vitro growth advantage, suggesting this gene as potential collaborative candidate. Furthermore, patients with mutated IL7R were more likely to have TLX3 or HOXA subgroup leukemia. We sought to determine whether combination of mutant hIL7Ra plus TLX3 overexpression is sufficient to generate T-cell leukemia in vivo. Double negative thymocytes were isolated from C57BL/6J mice and transduced with retroviral vectors containing mutant hIL7R plus hTLX3, or the genes alone. The combination mutant hIL7R wild type and hTLX3 was also tested. Transduced thymocytes were cultured on the OP9-DL4 bone marrow stromal cell line for 5-13 days and accessed for expression of transduced constructs and then injected into sublethally irradiated Rag-/- mice. Mice were euthanized at onset of clinical signs, and cells were immunophenotyped by flow cytometry. Thymocytes transduced with muthIL7R-hTLX3 transformed to cytokine-independent growth and expanded over 30 days in the absence of all cytokines. Mice injected with muthIL7R-hTLX3 cells, but not the controls (wthIL7R-hTLX3 or mutIL7R alone) developed leukemia approximately 3 weeks post injection, characterized by GFP expressing T-cells in blood, spleen, liver, lymph nodes and bone marrow. Furthermore, leukemic mice had increased white blood cell counts and presented with splenomegaly. Phenotypic analysis revealed a higher CD4-CD8- T cell population in the blood, bone marrow, liver and spleen compared in the mutant hIL7R + hTLX3 mice compared with mice injected with mutant IL7R alone indicating that the resulting leukemia from the

combination mutant hIL7R plus hTLX3 shows early arrest in T-cell development. Taken together, these data show that oncogenic IL7R activation is sufficient for cooperation with hTLX3 in ex vivo thymocyte cell transformation, and that cells expressing the combination muthIL7R-hTLX3 is sufficient to trigger T-cell leukemia in vivo.

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Centriole motility and subcellular position requires Pericentrin-Like-Protein and Kinesin-1
Centrosomes are the major microtubule organizing center (MTOC) of the cell. They comprise a pair of centrioles surrounded by a matrix of proteins term the pericentriolar material. Through microtubule nucleation they organize the mitotic spindle, cilia and flagella. To fulfill these functions, centrosomes

must be motile to achieve proper positioning within the cell. Typically, movement is thought to be governed by the activity of microtubule motors acting on the microtubules anchored at the centrosome. In some cell types, inactive centrioles lack PCM and microtubules. Inactive centrioles must be motile as their intracellular positioning is critical for asymmetric cell division and sensory cilia formation. Despite this the mechanism by which inactive centrioles are able to move around the cell is poorly understood. Here we investigate how inactive centrioles move in interphase cells. Using live cell imaging, we show that centrioles move on microtubules. We then show, through a targeted genetic screen that this occurs in a manner involving Dynein and Kinesin-1. Importantly, structured illumination microscopy revealed Kinesin-1 localizes to centrioles in Drosophila cells. We identified the centriole protein Pericentrin like protein (Plp) as necessary for centriole movement in interphase cells. Through yeast-2-hybrid analysis coupled with an in vivo interaction assay we mapped direct protein-protein interactions between Plp and Kinesin-1. A secondary yeast two hybrid screen then enabled us to isolate specific mutations which disrupt the interaction between these two proteins and assay function in a developmental context. Our data support a model where Plp acts as an adaptor that links the centriole to motor proteins; facilitating centriole movement. In this work we propose the first detailed mechanism of how centrioles can move independently of their role as an MTOC, in the context of developing tissue.

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Cell Biology - Cell Cycle and Division
Senolytic therapy suppresses radiation enhanced tumor growth
Radiation therapy is a commonly used curative treatment modality for cancer patients. Radiation-induced normal tissue injury is a well-described phenomenon that that can limit the radiation dose patients can receive. Even in the absence of moderate or severe injury, radiation results in increased normal tissue stem cell senescence which can result in the release of pro-inflammatory, pro-angiogenic, and mitogenic molecules secreted by senescent cells, collectively known as the senescence associated secretory phenotype (SASP). Although it is known that the SASP can further propagate normal tissue injury, the effects on tumor in the setting of radiation are uncertain. We hypothesized that the SASP of preirradiated normal lung tissue would enhance tumor growth. Using a murine experimental metastasis model that allows the assessment of tumor growth in lung, an organ that is well-studied in the context of radiation senescence, we demonstrated that pre-irradiation (2, 4, or 8 weeks before inoculation) resulted in a significant increase in B16 melanoma pulmonary tumor nodule number and size that correlates to the induction of senescence (2, 4, and 8 weeks post-radiation). Delivery of agents that prevent senescence after IR (rapamycin, INK-0128) and delivery of a senolytic agent (ABT-737) with termination of drug therapy at least 5 half-lives before tumor inoculation (8 weeks after irradiation) reduced senescence cells in irradiated lung and were sufficient to prevent radiation enhanced tumor growth. RNA sequencing of pre-irradiated lung tissue from mice treated with rapamycin, INK-0128, or ABT-737 demonstrated a suppression of several aging and SASP related genes with drug treatments relative to radiation and vehicle that are the subject of ongoing study. Together, these data demonstrate the pro-tumorigenic role of the SASP in irradiated tissue and introduces a the dual TORC inhibitor INK-0128 as an effective agent for prevention of radiation-induced normal tissue senescence. These studies

suggest the combination of senescence modifying agents with radiotherapy may lead to a decrease in therapy-induced tumor regrowth.

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Discovery of a new parasite life stage reveals the secrets behind the remarkable success of Leishmania transmission by a vector bite.
Disease vectors transmit pathogens as they blood feed, and most vectors take multiple blood meals during their lifetime. We observed a remarkable effect of a second uninfected blood meal to the Leishmania parasite inside the sand flies. Within 24h after blood feeding by a sand fly carrying a mature parasite infection, the metacyclic promastigotes, previously considered a terminally differentiated infectious Leishmania stage, dedifferentiate to a leptomonad-like stage: we are naming the retroleptomonad promastigote. This new replicative stage shows to be crucial for further amplification of metacyclics resulting in a significant increase in sand fly infectivity. This subsequently translates into 4-fold increase in lesion frequency on animals exposed to sand flies with a second blood meal compared to animals exposed to sand flies with a single blood meal. Also importantly, in the absence of a second blood meal, the majority of Leishmania parasites acquired naturally by feeding on an infected host are lost. This shows how crucial is a second blood meal and the rise of retroleptomonads to ensure vector competence and increase vectorial capacity. RNAseq analysis of midgut-residing retroleptomonads showed a distinct transcript profile validating as a bona fide developmental form. Together, these findings highlight the relevance of multiple blood meals for vector borne pathogens, as our findings have been recently confirmed to have similar effects on mosquitos infected by Plasmodium and viruses. These reveal a novel and fundamental role for multiple blood meals in establishing the pathogen, and most importantly, in perpetually enhancing infectivity of the insect vector. These findings also place readily available blood sources as a critical element in the transmission of vector-borne pathogens, and propagation of vector-borne diseases.

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Big brain, small brain: Drosophila Traip links DNA repair and mitotic functions to suppress neuronal stress response and microcephaly.
Microcephaly is a failure to achieve proper neuron number and brain size during development. Most of the ~30 genes linked to microcephaly function either at the mitotic spindle or in DNA damage repair (DDR). Thus, microcephaly is thought to reflect a loss of neuronal proliferation and/or increased cell death. However, most microcephaly genes have not been well-studied in brain development, and it is

unknown if mutations affecting either mitotic spindles or DDR lead to microcephaly through similar or distinct downstream pathways. We are using the Drosophila brain to study microcephaly gene functions and uncover downstream pathways that mediate microcephaly. In this study, we discovered a combined mitotic and DDR function for the microcephaly gene Traip, and we revealed a critical role for the neuronal stress response kinase wnd downstream of Traip in microcephaly. Traip mutant brains have fewer cells and structural defects. Traip is known to function in DDR; thus, we were surprised to find that Traip mutant neural stem cells (NSCs) were often polyploid, suggesting mitotic failure. We used high resolution live fluorescence microscopy to characterize endogenously tagged Traip transgenes, finding dynamic localization during NSC mitosis where Traip streams on spindles and coalesces at the furrow and midbody. Deleting the nuclear localization signal (NLS) evicts Traip from the nucleus during interphase, thus abrogating canonical DDR functions; however, the Î”NLS transgene rescues Traip mutant phenotypes, suggesting that a mitotic Traip function is sufficient for proper brain development. Our working model is that Traip functions as a mitotic DDR protein to resolve chromosome bridges in late mitosis, and that Traip mutants have persistent bridges leading to mitotic failure, polyploid NSCs and, ultimately, fewer neurons. Using Traip as a model microcephaly gene, we are using whole brain imaging and 3D analysis to screen for suppressors to uncover downstream pathways that play a role in microcephaly. To date, we have found roles for the neuronal stress response MAP3K wnd, Toll signaling, and caspase-dependent cell death in mediating Traip mutant microcephaly phenotypes. We are now testing whether these pathways also mediate the mutant phenotypes of other microcephaly genes, including both DDR genes (hd and MCPH1) and mitotic spindle genes (asp, ana2, and Plk4), and targeting these pathways as potential therapeutic targets to minimize neuron loss in microcephaly.

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Plasma membrane compartmentalization during leader bleb-based migration in melanoma cells under confinement.
Metastatic cancer cells migrating in a confined tissue microenvironment can switch between different modes of migration dependent on the degree of confinement and availability of adhesive ligands. Cells with high contractility are prone to blebbing and when confined in the absence of adhesive ligands, exhibit â€œleader bleb-basedâ€ migration. This mode of migration is characterized by a highly polarized morphology with a long, stable leader bleb (LB) leading the direction of migration, that is separated from the trailing cell body (CB) by a narrow contractile neck. Metastatic cells carrying the BRAFV600E mutation exhibit polarized Erk signaling within the LB that promotes actin retrograde flow to drive fast, directional cell LB-based motility. However, the mechanisms mediating the establishment and maintenance of such extreme cell polarization during LB-based migration are not known. We seek to test the hypothesis that polarization of signaling during LB-based migration is mediated by compartmentalization of lipid and protein organization on the plasma membrane (PM) between LB and CB. To test this, we expressed fluorescent membrane proteins and lipid probes in human metastatic A375 melanoma cells under 3Î¼m confinement with a polydimethylsiloxane (PDMS) or an agarose pad. To address the role of protein topology relative to the membrane in the LB and CB, we analyzed fluorescent fusions of single-pass transmembrane proteins (GFP-GT46 for non-raft, LAT-GFP for raft), those associated with the outer leaflet (GFP-GPI). Localization and photobleaching experiments revealed that transmembrane proteins showed restricted diffusion between LB and CB while proteins on the outer leaflet were free to diffuse. Analysis of an inner leaflet protein (HRas-GFP) as well as a transmembrane proteins associated with cytoskeleton (CD44-GFP) is underway. To address the role of lipid organization in the LB and CB, we analyzed the distribution of phosphoinositides using biosensors (PLCÎ´ PH-GFP for PI[4,5]P2, Akt PH-GFP for PI[3,4,5]P3). This showed that PLCÎ´ PH-GFP labeled the PM evenly, while Akt-GFP was excluded from the blebs. However, our results together reveal a striking degree of PM compartmentalization between LB and CB in melanoma cells migrating under confinement, though the mechanisms behind this still remains elusive. We are currently deciphering the mechanism by which such a compartmentalization is attained and how PM organization regulates bleb dynamics and migration.

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A novel severe late-onset autoinflammatory disease reveals unexpected regulation of ubiquitin signaling at its apex
Posttranslational modification with ubiquitin is critical for regulating a wide range of cellular processes such as proteasomal homeostasis and intracellular signaling. Consequently, dysfunctions in ubiquitylation has been shown to lead to a variety of human diseases. Ubiquitylation requires a three-

step enzymatic cascade, in which the E1 enzyme activates ubiquitin and transfers it to E2 enzymes that cooperate with E3 enzymes to conjugate ubiquitin to specific targets. While much is known about regulation of the ubiquitin system at the level of the E2 and E3 enzymes, ubiquitin activation through the E1 enzyme has traditionally been viewed as a constitutive process. Here, by analyzing whole exomes of undiagnosed disease patients, we discover 23 individuals with severe late-onset autoinflammatory disease, all with variants in the gene encoding the major human E1 enzyme UBA1, at p.Met41. These mutations are mosaic and lineage-restricted to myeloid cells. To interrogate the molecular underpinnings of disease, we utilized cellular models and biochemical approaches. Intriguingly, we find that mutations at p.Met41 result in reduction of UBA1 function and thus loss of cellular ubiquitylation in patient myeloid cells by distinct mechanisms. Canonically, UBA1 is expressed as two protein isoforms through alternative mRNA start codon usage. Met1 start-site translation produces a primarily nuclear protein (UBA1a), while Met41 translation produces a cytoplasmic form (UBA1b). In patient myeloid cells, we find that p.Met41 mutations cause a loss of Uba1b and expression of a non-functional, dominant negative cytoplasmic isoform (UBA1c) generated through downstream start-codon usage at Met67. Surprisingly, we also found reduced Uba1a protein levels in patient myeloid cells. This is due to reduction of an alternatively spliced UBA1 mRNA that preferentially supports translation of UBA1a relative to UBA1b. Taken together, our data shows that UBA1 p.Met41 mutations reduce E1 activity and cellular ubiquitylation by three mechanisms: 1) decreased nuclear UBA1a, 2) loss of cytoplasmic UBA1b, and 3) toxic gain of cytoplasmic UBA1c. This provides the molecular basis for UBA1-related late onset autoinflammatory disease and reveals unexpected dynamic regulation of ubiquitin signaling at the E1 level, in which intricate transcriptional and translational circuits impose spatial control of UBA1 activity during hematopoiesis.

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Cell Biology - General
Peripheral Localization of the NET1 RNA is Required for NET1 Function in Cell Migration
RNA transcripts localize to protrusive regions of cells and this localization is important for cell migration. We have recently shown that RNAs at extending protrusions are actively being translated. However, the molecular consequences of local translation and how it contributes to cell migration is currently unknown. It has been thought that localized transcripts are targeted to specific regions of the cell to increase local protein concentration through their translation. However, this doesnâ€™t appear to be the case for several protrusion-localized transcripts for which the distribution of RNA and protein show little correlation. We aim to understand the role that RNA localization plays in these scenarios. We use, as a model system, the peripherally localized NET1 transcript, which encodes an activator of Rho family GTPases that regulate cell migration. We find that while the NET1 RNA is peripherally localized, the NET1 protein is mostly nuclear. To investigate this, we have developed a method of mislocalizing transcripts in a highly specific manner, which forces the RNA to adopt a more perinuclear localization without affecting total RNA or protein levels. When we mislocalize the NET1 transcript in human breast cancer cells, we find that the level of active NET1 within the cell is reduced, and this is accompanied by a decrease in RhoA activity, focal adhesion size and cell migration speed without any obvious effect on the

overall distribution of the NET1 protein. Strikingly, these observed defects upon NET1 RNA mislocalization are similar in extent to those observed upon almost complete NET1 protein knockdown. These results demonstrate that proper localization of the NET1 transcript at the periphery is a primary determinant of NET1 activity and of its ability to control RhoA-mediate cell migration. Furthermore, we show that, when the NET1 transcript is mislocalized, NET1 has reduced binding with CASK, a protein implicated in the regulation of the actin cytoskeleton, which suggests that localization of the RNA can direct protein-protein interactions. These data describe a novel molecular role of local RNA translation by showing that a particular site of protein synthesis can affect protein activity and interactions resulting in modified cellular behavior.

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Cell Biology - Intracellular Trafficking, Cytoskeleton, and Extracellular Matrix
A New Role for an Old Protein: Multiple Roles of Non-muscle Myosin IIA During Neutrophil Interstitial Migration
Neutrophils are the first responders to different pathophysiological stimuli and their function is dependent on their ability to migrate to the site of action. This complex process involves a multitude of events, including continuous mechanical deformations of the plasma membrane (PM). Migrating neutrophils exhibit coordinated protrusions at the leading edge and retractions at its rear, which are controlled by F-actin polymerization and non-muscle myosin IIA (NMIIA)-based contractions, respectively. To unravel the mechanisms underlying the coordination between PM remodeling and actomyosin cytoskeleton in neutrophils in vivo, we used intravital subcellular microscopy to image their migration during injuries induced in the mouse ear. For comparison, we imaged their behavior in vitro, using a well-established assay. In vivo, neutrophils exhibit a very dynamic PM remodeling characterized by the continuous formation of micron-scale membrane protrusions, which interact with the tissue microenvironment and in particular with the extracellular matrix (ECM). Notably, whereas in vitro NMIIA is localized at the rear of the cell, in vivo NMIIA is also present at the cell leading edge. In this new site, although fully active and contractile, NMIIA does not control membrane retraction, contrary to its documented role in vitro. Besides, NMIIA is recruited to areas of the PM which interact with the ECM, suggesting a possible role in sensing the surrounding microenvironment. We provide evidence that during the early steps of migration, the contractile activity of NMIIA is necessary for the formation of the initial protrusions that ultimately develop into the leading edge. Also, we find that in vivo NMIIA recruitment at the front of the cell is controlled by the Myosin Light Chain Kinase (MLCK), but it is independent of the RhoA/ROCK signaling axis, which operates only at the rear of the cells and it is the main regulator of NMIIA activation in vitro. Super-resolution microscopy indicates that our finding is consistent with substantial differences in F-actin organization at the leading edge between neutrophils migrating in vivo and in vitro, as a result of different modalities of activation of integrin signaling. In summary, this is the first qualitative and quantitative characterization of the actomyosin dynamics in neutrophils in vivo, which unraveled a novel function for NMIIA during migration and as well as a new mechanism regulating its recruitment.

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Cell Biology - Intracellular Trafficking, Cytoskeleton, and Extracellular Matrix
Structure and function of flat clathrin lattices during Epidermal Growth Factor signaling
Clathrin-mediated endocytosis is key to internalize solutes, lipids, and integral proteins from the plasma membrane and is essential for nutrient uptake, lipid homeostasis, and signaling in eukaryotic cells. This trafficking pathway operates with the assembly of clathrin-coated pits which invaginate to form spherical vesicles. Many cells, however, contain a subset of clathrin coats with planar honey-comb like morphology called Flat Clathrin Lattices (FCLs) or plaques. In contrast to clathrin-coated pits, FCLs are poorly studied. FCLs are long-lived on the plasma membrane and can harbor cell adhesion proteins such as Beta5-integrin and the Epidermal Growth Factor Receptor (EGFR). EGF binding to EGFR induces receptor dimerization, transphosphorylation, binding of the scaffold protein Grb2, and activation of kinases including Src. These signaling events play critical roles in cell growth, and their dysregulation is a major driver of tumorigenesis. It has been proposed that reversible phosphorylation can control cycles of endocytic adaptor assembly and disassembly during endocytosis. Yet, how EGFR signaling and the biogenesis of FCLs are connected remains unknown. Here we use quantitative fluorescence and electron microscopic imaging, and biochemical approaches to show that a signaling loop involving EGFR, Src, and Beta5-integrin controls the formation of FCLs at the plasma membrane of human cells. We find that these two receptor systems are linked though Src-mediated phosphorylation of the Beta5-integrin cytoplasmic tail, specifically at Y766, Y774, and Y794. We also observe that EGFR, Src, and Beta5-integrin are spatially organized within FCLs. Specifically, agonist stimulation leads to persistent recruitment of active EGFR, Grb2, and Beta5-integrin into clathrin structures and a corresponding loss of Src. Our ultrastructural analysis shows a four-fold increase in clathrin plaque-covered membrane and plaque size after EGF stimulation. Disruption of the EGFR/Src/Beta5-integrin signaling axis prevents FCLs formation and the persistence of active EGFR and Grb2 within clathrin plaques. These results reveal a mutual regulation of flat clathrin lattices and two different receptors systems to allow dynamic physical interactions and signaling cross-talk at the plasma membrane of human cells. The nanoscale spatial organization of these systems is likely a key regulatory aspect of growth factor receptor signaling in health and disease.

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Cell Biology - Intracellular Trafficking, Cytoskeleton, and Extracellular Matrix
The nanoscale anatomy of exocytic dense-core vesicles in neuroendocrine cells
Exocytosis of cargo-loaded vesicles in neurons, exocrine, and endocrine cells is central for many physiological functions. The key proteins involved in the regulation of exocytosis are known but their nanoscale organization is mostly unknown. Previous attempt to map the anatomy of exocytic vesicle

lacked an accurate representation of vesicle structure. Here, we correlate super-resolution fluorescence (dSTORM) with platinum replica electron microscopy (PREM) to directly visualize proteins associated with single secretory vesicles (dense core vesicles, DCVs) in cultured PC12 neuroendocrine cells in two dimensions (2D). Next, we developed a novel genetically-encoded electron microscopy labeling method for nanoscale visualization of proteins in three dimensions (3D). The decoded molecular morphology of organelles helps bridge the gap that currently exists between the structure, function, and regulation of cellular machinery. We first investigated the spatial organization of Rab-GTPases and their effector proteinsâ€"Rab27a, Rab3a, Rabphilin3a, Granuphilin, and Rim2â€"that are known to support tethering and docking of DCVs to the plasma membrane. We labeled vesicles by expressing the cargo Neuropeptide-Y tagged with the mNeon-green fluorescent protein, along with a DCV protein of interest (POI) tagged with a dark GFP. Next, we unroofed cells and labeled the dark GFP-tagged proteins with Alexa-fluor 647 conjugated nanobody. Labeled cells were imaged with TIRF, dSTORM, and PREM to precisely map the position of proteins on identified vesicles in 2D. To locate these proteinâ€™s axial positions, we generated poly-histidine tagged POIs and surveyed these expressed proteins with NTA-nickel conjugated gold nanoparticles with electron microcopy. For validation of this new labeling method, we imaged well-studied coat proteins that form clathrin-coated vesicles and caveolae. Next, we visualized the 3D locations of the above 5 dense core vesicle-associated proteins using electron tomography. The spatially averaged distribution of targeted gold particles revealed that these proteins coat the entire surface of docked vesicles. This global distribution of these proteins likely aids in the efficient transport, capture, and docking of vesicles in active excitable cells. The nanoscale molecular architecture of DCVs generated from our methods helps uncover how key proteins assemble at the plasma membrane to regulate the docking, priming, and fusion of DCVs in excitable cells.

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Cell Biology - Intracellular Trafficking, Cytoskeleton, and Extracellular Matrix
A role for zinc transporters in the block to polyspermy in mouse eggs
One sperm is necessary for mouse fertilization; two sperm are embryonic lethal. During fertilization, a sperm binds to and penetrates the zona pellucida (ZP), an extracellular matrix that surrounds ovulated eggs. Following fertilization, ZP is proteolytically modified to prevent additional sperm binding. Although complete ZP cleavage takes several minutes, a concomitant release of zinc transiently prevents forward sperm motility and these two independent molecular mechanisms complement one another in preventing polyspermy. In mouse eggs, zinc is stored in cortical granules (CGs) which are vesicles located beneath the egg plasma membrane. Fertilization of ovulated eggs by sperm triggers exocytosis of CGs, releasing zinc as well as ovastacin, a CG-specific protease that cleaves ZP. The mechanism by which zinc accumulates in CGs prior to fertilization remains unknown. In other cell types, members of the zinc transporter family ZnT (ZnT1-ZnT10) have been implicated in zinc trafficking across subcellular organelles, and eight of the ten ZnTs are expressed in mouse oocytes. To investigate the subcellular localization of these transporters, cRNAs encoding each ZnT tagged either with turboGFP (tGFP) or mCherry (mCh) were transcribed in vitro, microinjected and expressed in mouse oocytes. Six ZnTs were located within the endomembrane system, but ZnT2 and ZnT4 co-localized in CGs. When oocytes from a

transgenic mouse expressing ovastacin-mCh were microinjected with either ZnT2- or ZnT4-tGFP cRNAs, ovastacin and ZnT2/ZnT4 were consistently located in different vesicles. Thus, there must be at least two subpopulations of CGs that simultaneously exocytose after fertilization. These results were corroborated by co-expressing either ZnT2-tGFP or ZnT4-tGFP with ovastacin-mCh cRNA in growing oocytes. In addition, we have successfully generated ZnT2KO, ZnT4KO and ZnT2KO/ZnT4KO mouse lines with CRISPR/Cas9. Using ZincBY-1, a zinc fluorescent sensor with nanomolar affinity for labile zinc, we document either a moderate or a substantial decrease of zinc, respectively, in ZnT2KO and ZnT4KO CGs. Current experiments are aimed at determining the effect of the double KO on CG zinc accumulation as well as the effect of absent ZnT2 and ZnT4 on forward sperm motility. Taken together, our results provide novel insights into the post-fertilization block to polyspermy that is essential for successful fertilization and the onset of development.

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Investigating the efficacy and safety of novel and atypical dopamine transporter inhibitors for the treatment of stimulant use disorders
Deaths related to stimulant use has increased dramatically within the US, with cocaine and methamphetamine related deaths in the US doubling over the last five years. Medication-assisted treatments that combine FDA approved medication(s) with behavioral therapy has shown superior outcomes when compared to using only one type of therapy in treating opioid use disorders. Regrettably, stimulant use disorders (SUDs) lack FDA approved pharmacotherapies, resulting an underserved patient population with inadequate treatment options. Our lab has tackled this challenge by synthesizing and studying atypical inhibitors of the dopamine transporter (DAT), the primary molecular target of these illicit stimulants. Through a series of structure activity relationship (SAR) studies based on modafinil, we have discovered bisphenyl atypical DAT inhibitors that have been shown, in pre-clinical models, to mitigate the reinforcing effects of cocaine and methamphetamine, without causing stimulant and reinforcing effects themselves, thus providing potential leads toward medications to treat SUDs. While further studies of these compounds are ongoing, improvements in potency and pharmacokinetics were desirable for discovering pipeline drug candidates. One of the major hurdles medications designed to pass the blood-brain barrier encounter is their ability to also inhibit the human

ether-a-go-go-related gene (hERG) potassium channel, which may lead to lethal cardiotoxicity. Our bisphenyl atypical DAT inhibitors show low micromolar hERG IC50â€™s and while not all hERG inhibitors exhibit cardiotoxicity, it remains a red flag for the FDA. In order to further reduce hERG channel activity while preserving the desired atypical DAT inhibitor profile, a series of novel biphenyl analogues was designed using a combined machine learning and molecular modeling approach to predict reduced hERG channel activity while retaining high DAT affinity. These biphenyl analogues were synthesized and tested in radioligand binding experiments. Several analogues had Ki values in the low nanomolar range (high DAT binding affinities) but also showed significantly lower hERG channel activity, validating the in silico results. Liver microsomal metabolism has been completed and locomotor activity studies are underway to determine if these molecules are behaviorally atypical. Those molecules that do not enhance locomotor activity, as cocaine and methamphetamine do, will be further evaluated in rodent models of SUD.

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Targeting the NRAS 5â€™ UTR with Small Molecules to control NRAS expression

Neuroblastoma RAS (NRAS) is an oncogene that is deregulated and highly mutated in 15% to 20% of Melanomas and Acute Myeloid Leukemias. Constitutively activated NRAS mutations induce the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinaseâ€“AKT signaling pathways, which collectively drive malignant progression. Unfortunately, at the time of this publication attempts to directly target the activated NRAS protein with small-molecule inhibitors have been largely unsuccessful due mostly to its small size and lack of accessible pockets. To date, the most promising strategies to inhibit NRAS signaling include targeting NRAS membrane localization or bypassing NRAS altogether and using inhibitors of downstream signaling molecules. But these approaches have shown mixed results in the clinic. As a result, there is a strong need for an alternative approach in this area, one of which is targeting the NRAS mRNA directly to prevent translation of this oncogenic protein. To this end, the 5â€™ untranslated region (5â€™UTR) of the NRAS mRNA is reported to contain a non-canonical secondary structure G-quadruplex (GQ), that regulates the expression of NRAS. Stabilization of the GQ structure in NRAS by small molecules provides an alternative approach to reduce NRAS expression in cancer cells. However, a major barrier in developing biologically active small molecules that bind to nucleic acids has been the identification of selective interactions. Previous approaches have generally yielded pan-GQ binding molecules, and strategies to generate selective ligands are lacking. Here we use a small molecule microarray (SMM) screen to identify a small molecule that selectively binds to the GQ located in the 5â€™UTR of the NRAS mRNA. Biophysical studies, including thermal melt, fluorescence titration and SPR analysis, demonstrate that the compound binds reversibly to the NRAS GQ structure with nanomolar affinity while showing weak or no measurable affinity for several other GQs. A Luciferase based reporter assay indicated that this compound inhibits the translation of NRAS by stabilizing the NRAS-GQ. Structure probing and sequencing analysis provide further insights into the structure and targetability of the 5â€™ UTR. We demonstrate that the SMM approach can reveal a selective GQ binder for oncogene

inhibition. Efforts toward applying SMMs to other GQ-associated oncogenes are being pursued to discover new selective binding scaffolds.

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Machine Learning versus Artificial Intelligence Methods for Predicting Cardiotoxicity in the â€˜Big Dataâ€™ Era
Cardiotoxicity is a leading concern behind the recall of marketed drugs and failure of clinical candidates. Inhibition of the potassium ion channel, whose alpha subunit is encoded by human Ether-à-go-go-Related Gene, leads to prolonged QT interval in the cardiac action potential. Fatal cases of cardiac arrhythmia have been reported due to drug-induced hERG channel blockade. Several computational methods, including machine learning, have been employed in modeling this endpoint. Reports based on artificial intelligence methods have only recently surfaced in the literature. To this end, we performed a comprehensive comparison of classification models developed using the classical machine learning and modern deep learning methods by employing different molecular descriptors. The training set (~9000 compounds) was constructed by combining hERG bioactivity data from ChEMBL database with data generated in an in-house high-throughput ion flux assay. Prospective validation of the models was performed on ~1700 in-house compounds screened for hERG activity in the same flux assay. We noticed that the deep learning models performed on par with the baseline models (Random Forests and Gradient Boosting). Latent descriptors derived directly from the deep learning architectures provided nearly the same performance as traditional molecular descriptors. We compared our models with other neural network architectures and state-of-the-art models from literature. Our models outperformed these models on the validation data. The study highlights the application of artificial intelligence methods on chemistry big data, readily available from public domain compound repositories, for generation of novel descriptors that are promising for molecular property prediction.

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Chromatin and Epigenetics
Rapid reshuffling of master transcription factors allows exit from plasticity to establish cell-fates
In the pre-implantation mammalian embryo three cell-fates are specified- trophectoderm (TE), epiblast (Epi) and primitive endoderm (PrE), which contribute to the placenta, embryo proper, and yolk-sac, respectively. Epi or PrE fates are determined by the interplay between two master transcription factors (TFs) â€" NANOG and GATA6 and arise from the inner cell mass (ICM) where these two counteracting TFs are initially co-expressed. How NANOG and GATA6 coordinate to maintain plasticity in the ICM and counteract each other to specify distinct cell fates irreversibly is not well understood. Using a Gata6-driven in vitro model of PrE differentiation we define how GATA6 modulates epigenetic mechanisms to promote a PrE fate while suppressing the NANOG-driven Epi fate. We have mapped the changing binding patterns of key PrE-specific TFs (GATA6, GATA4, and SOX17) and Epi TFs (NANOG, SOX2) during differentiation, along with changes in chromatin landscape and transcription, to define how these TFs alter the epigenome and transcriptome. Within four hours of differentiation, we detect widespread changes in chromatin landscape directed by GATA6. PrE-specific genes are upregulated by the combined action of GATA6, GATA4, and SOX17, while Epi genes are downregulated, solely by GATA6, via direct eviction of NANOG and SOX2. Repressive histone marks (H3K9me3 and H3K27me3) play a limited role in achieving repression, and reduction in Epi-specific enhancer activity is the predominant mechanism. We detect very few changes in large-scale 3D-chromatin reorganization, which most often emanate from dramatic redistribution of active and repressive histone marks. Surprisingly, we observe enhanced recruitment of NANOG and SOX2 at GATA6 target sites, despite their progressive transcriptional silencing through differentiation. This could be a mechanism by which the Epi network resists differentiation cues, attempting to maintain plasticity. An alternate mechanism could be GATA6-mediated re-direction of Epi factors to activate PrE genes while reducing their relative levels at Epi genes leading to their repression, effectively promoting a PrE-fate over an Epi-fate. In summary, our study describes how the PrE fate is established and provides a possible mechanism to address the long-standing question of how two master TFs maintain and oppose plasticity in the early embryo.

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Loss of Polycomb Repressive Complex 2 Remodels the Super Enhancer Landscape and Amplifies Master Transcription Factors in Malignant Peripheral Nerve Sheath Tumors
Malignant peripheral nerve sheath tumors (MPNST) are aggressive and highly metastatic soft tissue sarcomas that lack effective treatment. Recurrent loss-of-function mutations in core components of the polycomb repressive complex 2 (PRC2) are prevalent in this devastating disease. Consequently, global loss of the transcriptional repressive marker, trimethylation of histone H3 lysine 27 (H3K27me3), is a feature of human MPNSTs. However, other epigenetic consequences of PRC2 loss remain unclear. Using an inducible expression vector, we restored a functional PRC2 in MPNST cell lines that are PRC2 deficient

(PRC2-null) and profiled the accompanied changes in histone modifications by chromatin immunoprecipitation with massively parallel DNA sequencing (ChIP-seq) and transcriptomics by RNA sequencing (RNAseq). We identified 6135 H3K27me3 peaks that were associated with 2285 genes, which were commonly gained in three PRC2-null MPNST cell lines when a functional PRC2 was restored. Interestingly, over 50% of these genes remained silenced even without H3K27me3, and less than 10% of the highly expressed genes were re-repressed by PRC2 (fold change < 0.7). These 145 genes that were transcriptionally regulated by PRC2 were found to be co-occupied by H3K27me3 and H3K4me3 at their promoters, which is known to occur in bivalent genes. We further investigated the epigenetic changes at the bivalent domains and found that in addition to the gain of H3K27me3, there was loss of its mutually exclusive marker, acetylated H3K27. H3K27ac also is a known marker of super enhancers (SE). Through global SE profiling, we revealed that PRC2 restoration remodeled SE landscape and caused reduction in total SE numbers. Particularly, we identified a group of oncogenic transcription factors (TFs), including forkhead box C1 (FOXC1) and homeobox B8 (HOXB8), which were activated due to the loss of PRC2 and gain of a PRC2-regulated SE. Using single-cell RNAseq on a patient sample from a primary PRC2-null MPNST, we confirmed that these essential SE-driven TFs are valuable biomarkers of malignant cells within the complex tumor microenvironment. In conclusion, we report a novel mechanism underlying the polycomb-regulated transcriptional repression. In MPNSTs, the loss of PRC2 causes activation of a group of essential TFs through remodeling the SE landscape. Our discoveries provide fundamental mechanisms underlying the effective combinatorial treatment of MPNSTs by MEK and bromodomain inhibitors.

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The HSA domain of BRG1 is critical for BRG1-dependent gene expression changes and the interaction of BRG1 with multiple BAF and non-canonical BAF complex members.

The SWI/SNF complex remodels chromatin in an ATP-dependent manner through its ATPase subunit BRG1. Chromatin remodeling alters nucleosome structure to change gene expression, however aberrant remodeling and gene expression can result in cancer. Mutations in certain domains of BRG1, like the HSA domain, have been identified in many cancers. However, although a conserved domain, HSA is still uncharacterized. SW13 carcinoma cells are an ideal model system to study BRG1 function because they do not express BRG1. Previous experiments have shown that SW13 cells expressing wildtype BRG1 (BRG1-WT) express different levels of target genes compared to SW13 cells expressing BRG1 missing the HSA domain (BRG1-HSA). Therefore, we hypothesize that the HSA domain is critical for BRG1 function and elucidating its function will inform SWI/SNF biology in normal and cancer contexts. To test this hypothesis, we generated SW13 tetracycline (tet)-on inducible cell lines for BRG1-WT or BRG1-HSA. For each tet-induced cell line, we performed RNA-seq to identify domain-dependent changes in gene expression at a genome-wide level and compared to them to their respective non-induced cell line as a control. Nearly 1600 genes were differently expressed when BRG1-WT expression was induced, which upon pathway analysis demonstrated an enrichment in a number of cancer pathways. However, most of these genes showed no change in expression when BRG1-HSA was induced. This builds upon previous

data and suggests that the HSA domain is required for most of the differential gene expression. We predict changes in SWI/SNF subunit interactions between BRG1-WT and BRG1-HSA, and to test this we performed Co-IP-MS with BRG1-WT compared to BRG1-HSA. As expected, BRG1-WT binds all expressed members of the core SWI/SNF complex, but BRG1-HSA failed to pulldown 2 members, BCL7C and BAF53a. BRG1-HSA also failed to pulldown 2 members of the non-canonical SWI/SNF complex, which has been recently implicated in cancer and pluripotency, GLTSCR1 and BICRAL. These results are the first to demonstrate that the HSA domain is necessary for traditional BAF function and specific complex formation. To highlight the role that the HSA domain plays in chromatin remodeling and gene expression, we will build upon this data with CUT&RUN profiling and additional interaction studies to identify complex formation dynamics. By elucidating BRG1 function first under normal conditions, we can better understand its role in cancer.

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Identification of a First-in-Class Inhibitor of Histone Lysine Demethylase KDM3B Targeting PAX3-FOXO1-positive Rhabdomyosarcoma and Other Epigenetically-driven Cancers

Rhabdomyosarcoma is the most common soft tissue sarcoma in children and despite aggressive cytotoxic chemotherapeutic regimens, 5-year overall survival for high-risk and recurrent disease is only ~30% and ~17%, respectively. The PAX3-FOXO1 fusion gene activates super enhancer-driven transcription and is the major oncogenic driver in fusion-positive rhabdomyosarcoma (FP-RMS) and represent a unique target in these cancers. To identify precision therapeutics for treatment of FP-RMS for potential clinical trials, we developed a novel cell-based assay which simultaneously monitors PAX3-FOXO1 super enhancer activity and general transcription. We screened 62,643 compounds and identified 64 PAX3-FOXO1 Inhibitors (PFIs) that did not cause general inhibition of transcription or cell death at an early time point (24 hours). Compound PFI-63 was identified as the top hit. RNA-seq and genome-wide CRISPR-Cas9 synthetic lethality studies indicated that PFI-63 was a histone lysine demethylase (KDM) inhibitor. In vitro enzyme inhibition analysis confirmed targeting of KDMs by PFI-63, with highest specificity for KDM3B. Additionally, RNA-seq showed activation of apoptosis and myogenic differentiation pathways while PAX3-FOXO1 target genes were repressed. Western analysis for PARP cleavage confirmed apoptosis and increased expression of MYOG for muscle differentiation. Methylation increase on histone 3 lysines at positions K4, K9, and K27 was confirmed by Westerns. Since poor solubility of PFI-63 in water restricted our ability to perform in vivo validation we performed molecular similarity searches of PFI-63 to identify water soluble analogs. We identified PFI-90, which showed similar RNA-seq and Western findings of increased apoptosis and myogenic differentiation with downregulation of PAX3-FOXO1 targets. Direct enzymatic assays and IC50 measurements demonstrated that PFI-90 exhibited increased cytotoxicity (IC50 of 0.8 uM compared to 2 uM for PFI-63) and superior inhibition of KDMs, again with highest specificity for KDM3B. In summary, we have identified a first-in-class KDM3B semi-selective inhibitor with favorable solubility which has anti-FP-RMS activity in vitro. Structure activity relationship studies are planned and pre-clinical validation by in vivo experiments are

underway. PFI-90 or its analogs represent a potential new therapy for FP-RMS and other epigenetically driven cancers.

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Chromosomes and Nuclear Architecture
Oncogenic LncRNAs drive histone variant mislocalization in cancer cells
Chromosomal instability is a major defining event of cancer progression. The histone H3 variant CENP-A (Centromere protein A) is normally restricted to centromeres where it plays a fundamental role in centromere structure, identity and function. However, in a variety of tumors, we have reported that CENP-A can hijack the H3.3 chaperone pathways to deposit ectopically, thus invading regions such as the chr8q24 locus. Other than the presence of the proto-oncogene MYC, this region is typically a gene desert; however, the existence of a large DNase I hypersensitive site has driven us to examine non-coding transcription from this locus. Intriguingly, several non-coding RNAs are transcribed from the chr8q24 locus, making this region an oasis for non-coding transcription. Long non-coding RNAs (lncRNAs) have less or no protein-coding potential, are over 200 nt in length with diverse cellular functions ranging from genome organization to transcription regulation. Deregulation of lncRNAs is one of the potential hallmark features of cancer progression. Overexpression of chr8q24 locus derived lncRNAs is frequently reported in many cancers and correlates with poor therapeutic outcome and recurrence. Here, we hypothesize that chr8q24 derived oncogenic lncRNAs are unwitting players in altering the local chromatin landscape by recruiting incorrect chaperone-histone variant complexes. We

knocked down the top candidate lncRNAs at chr8q24 locus (PCAT1, PCAT2, CCAT1, CCAT2, and PVT1), to study ectopic CENP-A (eCENP-A) localization in metastatic SW480 colon cancer cells. Interestingly, using coIF-DNA-FISH, we found that disruption of chr8q24 lncRNAs significantly reduced eCENP-A. Releasing the cells from the knockdown treatment significantly rescued the eCENP-A level at this locus. Remarkably, knocking down H3.3 chaperones (HIRA and DAXX) following the lncRNA knockdown prevented the cells from acquiring eCENP-A at the chr8q24 locus. Levels of the CENP-A at centromeres were not affected by the chr8q24 lncRNA perturbation, which confirms that these non-coding transcripts specifically alter the local chromatin from where they were transcribed. Furthermore, we find that the colocalization of kinetochore proteins with eCENP-A at chr8q24 impacts the chromosomal architecture, resulting in an increasing in chromosomal break within this region. These data suggest a novel epigenetic mechanism linking locus and cancer-specific lncRNAs to aberrant chromatin structures in cancer cells.

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Chromosomes and Nuclear Architecture
IDENTIFICATION OF A NOVEL FACTOR THAT ALTERS NUCLEAR ORGANIZATION THROUGH A HIGH CONTENT GENOME-WIDE RNAI IMAGING SCREEN
Keywords: chromatin insulators, high-throughput imaging screen, Nurf301, Oligopaint DNA FISH, chromatin structure Chromatin insulators are DNA-protein complexes that assist the establishment of chromatin 3D organization. By mediating interactions between distant genomic sites, insulators can prevent the spread of repressive chromatin and block the communication between enhancers and promoters to regulate gene expression. Insulator dysfunction can lead to disease and cancer due to the disrupted regulation in oncogenes and tumor suppressor genes, such as myc and Retinoblastoma gene (Rb). In Drosophila, the well-studied gypsy insulator consists of three core integral proteins CP190, Su(Hw), and Mod(mdg4)67.2. Multimerization of insulator complexes forms insulator bodies, of which normal localization is correlated with proper insulator function. To identify novel factors required for insulator body formation, we used a high-throughput imaging approach to visualize insulator bodies after treatment with dsRNA libraries in a modified hematocyte cell line Kc167, which expresses a functional Mod(mdg4)67.2-GFP fusion protein. This strategy identified Nurf301 as a potential novel regulator of gypsy insulator body formation. In vivo luciferase reporter assay suggests Nurf301 promotes gypsy-dependent insulator barrier activity. Co-IP and IP coupled to mass spectrometry results indicate Nurf301 physically interacts with CP190, Su(Hw), and Mod(mdg4)67.2. To reveal the mechanism, we performed ChIP-seq and observed Nurf301 co-localizes with insulator proteins throughout the genome, depletion of Nurf301 results in extensive alteration of gypsy insulator proteinsâ€™ distribution. Since Nurf301 is a nucleosome remodeling factor, we examined published MNase-seq data from Nurf301 null cells and found there is a strong positive correlation between altered nucleosomes and lost gypsy insulator binding sites. To visualize the change of 3D chromosomes organization, we conducted Oligopaint DNA FISH and found that nuclear compaction of gypsy binding sites is specifically changed after depletion of Nurf301. By providing the first report of Nurf301 impacts the gypsy insulator function through chromatin organization, our data provide more insights for future clinical research.

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Identification of regulators of nuclear morphology and chromatin organization
The shape of the human cell nucleus is highly variable amongst cell types and tissues. In addition, nuclear shape changes are associated with diseases such as cancer, and aging. Despite the very fundamental nature of nuclear morphology control, the cellular factors that determine nuclear shape are not well understood. To identify cellular factors that determine nuclear shape, we have performed a high-throughput imaging-based siRNA screen in human immortalized human fibroblast cells and MCF10AT breast epithelial cells using siRNA libraries targeting 867 nuclear proteins including chromatin-associated proteins, epigenetic regulators, nuclear envelope components, and nuclear matrix proteins. We developed a high-throughput image analysis pipeline which quantifies nuclear size and nuclear shape. We identified sets of cell-type specific and proteins required for proper nuclear shape and to maintain nuclear morphology. A number of chromatin modifying enzymes were required for proper nuclear morphology in both fibroblast and breast epithelial cells. Interestingly, most identified factors altered nuclear shape without affecting levels of lamin proteins, know regulators of nuclear morphology. To identify mechanisms regulating nuclear morphology, we characterized lamin-chromatin interactions at the nuclear periphery using biochemical and molecular approaches. We find that lamin A directly binds to histone H3 and is a bona fide â€œhistone readerâ€. These results represent the first systematic exploration of cellular factors involved in nuclear shape determination and identify chromatin related mechanisms as key determinants of nuclear morphology in human cells.

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Sodium Glucose Transporter inhibition increases ketone levels in the human brain
Preclinical studies in models of Alzheimerâ€™s disease and other neurological disorders suggest that

increasing the circulating levels of ketones can improve neuronal health and delay the progression of cognitive impairment. In this proof-of-concept study, we sought to use Magnetic Resonance Spectroscopy (MRS) to demonstrate that empagliflozin, a sodium glucose transporter (sGLT2) inhibitor that is an approved drug for type 2 Diabetes Mellitus, can increase ketone concentration in the human brain. To test this hypothesis, we conducted a 4-week clinical study of 25 mg of empagliflozin daily, with no drug administered on the first visit as a within-subject control. At each of the three visits we used Point-Resolved Spectroscopy (PRESS) to measure the in vivo concentration of the main ketone body, beta-hydroxybutyrate (bHb), in the precuneus of the 17 cognitively normal healthy participants enrolled to date (60.9 +/- 6.8 years old, 11M/6F). The concentration of precuneal bHb increased by approximately 35% within participants taking empagliflozin in a stepwise fashion over the course of three visits (p = .037), controlling for cerebrospinal fluid volume in the voxel of interest. The concentration of other metabolites related to neuronal and glial integrity and neurotransmission were unchanged. This is the first study to demonstrate in vivo changes in human brain ketone concentration in response to the administration of a drug. We found that empagliflozin produced substantial, reliably measurable, and significant elevations in precuneal bHb, without affecting other key brain metabolites. While recruitment continues, these preliminary results suggest that sGLT2 inhibition may be effective in increasing brain ketone concentration. This finding is crucial for designing future clinical trials of sGLT2 inhibitors and other interventions aiming to increase brain ketone levels to stave off cognitive decline in aging and neurodegenerative disorders, such as Alzheimerâ€™s disease.

Nisha Nagarsheth
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Dissecting key determinants that can predict response during adoptive T cell therapy of gene-engineered T cells

Gene-engineered T cell therapy is an emerging cancer treatment strategy, with notable clinical activity in hematologic malignancies. Study of this approach in epithelial cancers, which account for 80-90% of malignancies, has been limited. We initiated a clinical trial of gene-engineered T cells that targets a human papillomavirus (HPV)-16 specific protein, E7, for the treatment of patients with metastatic HPV-16+ cancers. Peripheral blood T cells from the patients were engineered to express a T cell receptor (TCR) that recognizes the E7 antigen in the context of HLA-A*02:01 presented on the tumor cell surface. Patients received a single infusion of these E7 TCR T cells at 1, 10, or 100 billion cells. Six out of twelve patients had objective clinical responses. To discover determinants that predict response to our therapy, flow cytometry, cytotoxicity, and ELISA immune assays were performed to assess the phenotype and fitness of E7 TCR T cells and tumor biopsies were sequenced to identify genetic defects. Sustained, high-level engraftment of E7 TCR T cells in peripheral blood was observed (median 66% of total T cells at 6 weeks) and correlated with cell dose but not with clinical response. Phenotypically, E7 TCR T cells expressed low levels of the inhibitory receptor PD-1 and higher levels of TIM-3 and LAG-3 inhibitory receptors. Also, >90% of infused and engrafted E7 TCR T cells were differentiated effector memory cells. Functionally, the E7 TCR T cells were cytotoxic and produced effector cytokines, including interferon gamma, when cocultured with tumor cell lines ex vivo. E7 TCR T cell expression of inhibitory receptors,

frequency of differentiated effector memory cells, cytotoxic function, and cytokine production did not correlate with patient response. Tumor biopsies were analyzed to identify tumor-intrinsic defects. Treatment-resistant tumors had defects in immune response pathways including damaging mutations in HLA-A*02:01 and beta -2 microglobulin and copy losses of genes in the interferon gamma effector pathway. These pathways are necessary for T cell-mediated recognition and killing of tumor cells. Our clinical trial demonstrates clinical activity for cellular therapy in epithelial cancers. Immune escape through tumor intrinsic defects and not defects in T cell factors determined resistance to therapy. Future trials will examine tumor genomics as a predictive biomarker and be conducted in patients with earlier stages of disease.

Emily Phung
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Respiratory Syncytial Virus Prefusion F protein Vaccine Elicits a Durable Antibody and B Cell Response
Respiratory syncytial virus (RSV) causes substantial morbidity in children and the elderly, with no vaccine available. Multiple vaccination approaches using the RSV post-fusion conformation (post-F) as a subunit vaccine resulted in a modest boost in neutralization and the induction of non-neutralizing antibodies, resulting in no protection. The success of Palivizumab, a monoclonal antibody prophylaxis restricted for use in high-risk infants demonstrates the effectiveness of antibodies in protection from severe disease. Therefore, a vaccine eliciting a substantial and sustainable boost in neutralizing antibodies targeting the pre-fusion conformation (pre-F) of RSV is likely required to provide protection in at-risk populations. A stabilized, pre-F subunit vaccine (DS-Cav1) was evaluated for safety, tolerability, and immunogenicity in a phase I clinical trial. To understand vaccine-induced antibody and B cell responses, we measured RSV F conformation-specific responses by enzyme-linked immunosorbent assay (ELISA) and neutralizing activity to RSV in the sera for up to ten months post-vaccination. In parallel, we tracked the RSV F-specific memory B cell response in the peripheral blood mononuclear cells (PBMC) using fluorescently labeled, tetramerized pre-F and post-F probes and a 17-color flow cytometry panel. We found that vaccination with DS-Cav1 elicited a 9 to 12-fold increase in RSV F-specific antibodies and neutralizing activity in the sera, and RSV-specific antibody was sustained above the week 0 baseline for up to ten months. Similarly, DS-Cav1 vaccination activated pre-F-specific IgG+ and IgA+ memory B cells, with pre-F probe-binding frequencies elevated above the week 0 baseline ten months post-vaccination. Durability was maintained regardless of dose, number of vaccinations, or adjuvant, indicating that a single dose of DS-Cav1 in RSV-experienced individuals may confer protection that extends throughout an entire RSV season. For the first time, a vaccine designed by our knowledge of atomic-level structures and neutralizing epitopes demonstrate the ability to elicit robust and durable neutralizing antibodies in humans, providing a proof-of-concept for structure-based vaccine design.

Anand Singh
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Clinical and Translational Research - General
A novel peptide-based Surface-fill hydrogel nanocomposite attenuates mesothelioma growth
Malignant pleural mesothelioma (MPM) is an aggressive cancer resistant to multimodal therapies. Current therapeutic drug regimens ultimately fail due to a poor understanding of the pathogenic mechanisms. After surgical resection, MPM inevitably recurs from residual microscopic tumor foci. These clinical challenges led us to design a locoregional therapeutic platform delivering microRNA (miRNA), a novel anti-cancer agent, intrapleurally, with the goal of eradicating residual microscopic tumor foci thereby improving the efficacy of multimodal therapy. We engineered a peptide-based hydrogel that can be injected or sprayed directly to coat anatomic surfaces, functioning as a therapeutic depot. Properties of the hydrogel were fine-tuned for biopersistence, therapeutic payload, and cell-specific targeting. In the first step of formulation, miRNA complexed with an amphiphilic cationic peptide forms stable nanoparticles. Next, miRNA-nanoparticles were encapsulated into another peptide hydrogel, formed via self-assembly of the amphiphilic cationic peptide, thus forming a nanoparticle-hydrogel composite. The composite material was nontoxic to MPM cells and demonstrated the efficient release of miRNA-peptide nanoparticles from the large hydrogel matrix in-vitro. Tracking of labeled scrambled miRNA-nanoparticles by confocal microscopy showed nanoparticle uptake by cells was accomplished via clathrin-dependent endocytosis. In mice, the subcutaneous injection of Cy3-miRNA-hydrogel showed sustained miRNA release over three weeks. miRNAs targeting specific molecular vulnerabilities of MPM were used to study the therapeutic efficacy of miRNA-nanoparticle-hydrogel composites. Subcutaneous xenografts treated locally with MPM-specific miRNA-hydrogel composite shrank significantly. Similarly, pleural and peritoneal orthotopic xenografts treated with specific miRNA-hydrogel composite regressed over time. Kaplan-Meier analysis showed that miRNA-hydrogel treatment improved the overall survival of tumor-bearing mice. In order to mimic humans, subcutaneous MPM xenografts in mice were resected, and residual microscopic tumor foci treated locally with MPM-specific miRNA-hydrogel composite inhibited tumor recurrence. Thus, delivery of specific miRNA via a locoregional therapeutic platform in preclinical models of MPM resulted in beneficial anti-cancer effects. This peptide-based material is promising for translational studies of nucleic acid-based locoregional therapy against MPM.

Eleonora Duregon
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Clinical and Translational Research - General
Time-Restricted Feeding and Calorie Restriction Modulate Spontaneous Neoplasm Growth in Female Mice.
In older humans, multiple chronic diseases and increased life expectancy impose a disproportionate socioeconomic burden. Dietary interventions that involve a reduction in daily caloric intake and/or periodic fasting cycles are valuable strategies that can promote healthy aging. Calorie restriction (CR) refers to a reduction of energy intake in the absence of malnutrition and is the most robust intervention

able to preserve health by delaying the onset of disease and increase survival in model organisms. However, the impracticability of chronic CR outweighs the potential long-term benefits in humans. Time-restricted feeding (TRF), i.e. the limitation in the timing of food intake without necessarily reducing caloric intake, can protect against metabolic disorders through the synchronization of the circadian rhythm. We previously showed how single-meal feeding improves health and survival in male mice, regardless of the diet composition. This study compares whether limiting access to ad libitum (AL) food for a few hours per day mimics the beneficial effects of a CR diet. A large cohort of C57BL/6J female mice (n=250) were distributed into five feeding paradigms at midlife: AL, TRF for 8 hours (TRF8), TRF for 4 hours (TRF4), 20 percent CR (CR) and 20 percent CR fed twice a day (CRx2). Pathological analyses at death reveals a shift in fatal neoplasms toward an older age in TRF8 mice. Overall, 84 percent of mice had a neoplasm, with the highest prevalence in the AL group (93 percent) and the lowest in TRF4 (77 percent). Although the mean tumor burden (number of different tumors observed per mouse) among the five groups was similar, AL mice displayed the highest prevalence of high tumor burden values, while the CRx2 mice had the lowest rate of neoplasm. Hematopoietic neoplasms, mainly histiocytic sarcoma and lymphoma, were the most represented malignancies among all groups, although CR mice displayed the highest rate of histiocytic sarcoma (75 percent) and the lowest rate of lymphoma (10 percent). These results indicate that both time- and calorie-restricted feeding regimens can slow down malignant neoplasm progression and extend health span in female mice, even when started in adulthood. By incorporating fasting time into a feeding protocol as a potentially practical strategy to replicate or even augment the beneficial effects attributed to CR, this study may have major implications for translation into human health and clinical applicability.

Dinusha Rajapakse
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Amelotin, an enamel protein is expressed in retinal pigment epithelium (RPE) and associated with hydroxyapatite (HAP) deposit formation in dry age-related macular degeneration (AMD).
AMD is the main cause of irreversible vision loss in aging populations. There are no effective therapies for dry AMD which is the most common form of the disease. Early signs of AMD are drusen which are accumulations of extracellular lipids, proteins, and HAP minerals in the retina. HAP nodules are implicated in the progression of AMD. Drusen deposit growth is associated with precipitation of HAP and binding of proteins to HAP surface, but the proteins involved in the mineralization process is yet to be identified. We have previously reported serum-deprivation of RPE cells in culture mimics some features of AMD and using this model we identified amelotin (AMTN), a protein promoting hydroxyapatite mineralization in tooth enamel. HAP is also formed in our culture model and is blocked by siRNA inhibition of AMTN. Here using human donor eye tissue, we investigated AMTN expression in AMD. 6 normal and 22 AMD pairs of eyes from male and female donors, age range of 65â€“96 years were investigated. Eyes were genotyped for AMD-related polymorphism Y402H of complement factor H. Eyes were cut and sectioned through the macula for microscopy. Immunohistochemistry was performed to identify AMD eyes with lesions and categorize for types of drusen. In situ hybridization probes were used for detection of AMTN mRNA, anti-Amelotin antibody for AMTN protein and Bone-Tag 680RD to

stain HAP. In situ hybridization showed that AMTN is expressed in RPE in eyes with dry AMD, particularly in soft drusen regions containing HAP nodules. AMTN was not found in hard drusen, normal RPE, or donor eyes diagnosed with wet AMD. Immunofluorescence labeling showed AMTN in RPE surrounding soft drusen and isolated drusen with large calcified nodules showed heterogeneous HAP staining, with intense staining of the crusts whereas AMTN was localized in a ring around the HAP structures. Eyes with Y/Y genotype did not contain soft drusen with HAP and had no AMTN signal. In contrast, donor eyes with H/H and H/Y genotypes, had soft drusen with HAP and AMTN. Our findings confirm that AMTN is expressed in diseased human RPE, specifically in dry AMD and may be a key protein in the organization of HAP mineralization in soft drusen implicated in clinical AMD progression. AMTN provides a new possibility for therapeutic intervention. Next, we will perform experiments to identify AMTN specific inhibitors by high throughput screening and test their effects on HAP mineralization on our human AMTN transgenic mice.

Scott Wilkinson
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Clinical and Translational Research - General
Genomic features associated with exceptional pathologic response to intense neoadjuvant androgen deprivation therapy in locally advanced prostate cancer
Background Clinical trials of intense neoadjuvant androgen deprivation therapy (inADT) are based on the premise that earlier introduction of next-generation AR-axis inhibitors may improve outcomes. Although it is still too early to determine a survival benefit, patients whose tumors show significant pathologic responses have lower rates of biochemical recurrence, and nearly every patient with an exceptionally- or completely-responding tumor has remained recurrence-free with > 5-years follow-up. In our recent phase II trial, we evaluated whether patients with locally advanced prostate cancers would benefit from this treatment and whether we could identify molecular features at baseline that correlated with pathologic response. Methods Patients underwent a mpMRI and MR/US guided biopsy prior to receiving inADT. Patients underwent a second mpMRI prior to radical prostatectomy (RP). Pathologic response was measured from H&E slides of RP blocks with residual tumor. Multiple foci of tumor and normal adjacent tissue were identified from biopsy specimens and excised by laser capture microdissection. Whole exome and transcriptome sequencing were performed on 150 DNA and 141 RNA tumor samples from 112 biopsies and analyzed for somatic copy number alterations (SCNA) and mutations or differentially-expressed genes and pathways, respectively. Results Of 39 patients enrolled, 36 underwent RP. There were 15 exceptional responders (ER), defined as residual cancer burden <0.05cc, and 21 incomplete or non-responders (INR). TP53 loss was enriched in INRs (P=0.006), and while deletion of chromosome 6q14-16 was enriched in ERs (P=0.0002), INRs harbored more SCNAs overall (P=0.038). ERs demonstrated increased enrichment for the Androgen Response pathway. The tumor suppressor INPP4B was more greatly expressed in ERs (Q=0.002) and the lncRNA SChLAP1 was overexpressed in INRs (Q=0.004). Conclusion Despite homogeneous clinicopathologic characteristics at baseline, distinct molecular features distinguished a subset of intermediate-to-high risk patients who demonstrated remarkable pathologic responses. By identifying genomic and transcriptomic features at baseline of patients who will respond well to therapy, we will be able to better tailor personalized

targeted therapy. These data highlight the heterogeneity and plasticity of nascent aggressive prostate cancer, which must be considered in the setting of neoadjuvant hormone therapies for optimal clinical management.

Tzu-Ting Huang
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Clinical and Translational Research - General
PI3K/mTOR pathway blockade potentiates CHK1 inhibitor-induced replication stress and genomic instability by increasing CDC45-mediated origin firing and attenuating homologous recombination repair in high grade serous ovarian cancer
High grade serous ovarian cancer (HGSOC), the most common subtype of ovarian cancer, is the most lethal gynecologic malignancy in the U.S. with limited treatment options. HGSOC cells rely heavily on cell cycle checkpoint kinase1 (CHK1)-induced G2/M cell cycle checkpoints for DNA repair, due to universal TP53 mutation causing defective G1/S checkpoints. We previously reported an early clinical activity of a CHK1 inhibitor (CHK1i) prexasertib in recurrent HGSOC patients. To improve the efficacy of CHK1i, we conducted unbiased high-throughput drug combination screens in a panel of HGSOC cells and identified that phosphatidylinositol-3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway inhibitors (PI3K/mTORi) are among the top-ranked drug candidates. The PI3K/mTOR pathway activation is commonly observed (>70%) and associated with increased cell survival and DNA damage response in ovarian cancer. We therefore hypothesized that the combination of CHK1i prexasertib and a dual PI3K/mTOR inhibitor (PI3K/mTORi) LY3023414, would enhance cell death compared to each monotherapy in HGSOC cells. Using clinically attainable concentrations, CHK1i and PI3K/mTORi combination resulted in greater cytotoxicity, DNA damage and chromosome instability compared to monotherapy. PI3K/mTORi also augmented CHK1i-induced replication stress in HGSOC cells, as evidenced by increased pan-nuclear gamma H2AX and phosphorylated RPA double-positive populations, lower replication fork speed, and increased unscheduled new origins. Mechanistically, we identified that combination therapy (i) increases replication initiator CDC45 levels leading to excess new origin firing and (ii) attenuates DNA homologous recombination repair activity, resulting in DNA damage and lethal replication stress. We also noted the CHK1i and PI3K/mTORi combination caused remarkable tumor shrinkage without significant toxicities in vivo. Moreover, we found that ovarian cancer patients with high/high co-expressions of CDC45/RPA1 or CDC45/RPA2 showed worse progression-free survival compared to those with high/low CDC45/RPA1 (15 vs 19 months, $p = 0.003$) or CDC45/RPA2 (19 vs 22 months, $p = 0.007$), suggesting that high/high co-expressions of CDC45/RPA might represent a subgroup of ovarian cancer with high replication stress which may benefit from combination therapy. Together, our data provide preclinical evidence and a clear rationale for CHK1i and PI3K/mTORi-based clinical trials for recurrent HGSOC.

Sarah Davies
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Merkel cell polyomavirus specific T cells are readily generated from healthy donors for adoptive cell therapy
Merkel cell carcinoma (MCC) is a rare and aggressive neuroendocrine skin tumor. Approximately 80% of cases in the US are virus positive MCC (VP-MCC) and are driven by clonal integration of Merkel cell polyomavirus (MCPyV). Use of immune checkpoint inhibitors has greatly improved outcomes for patients with advanced VP-MCC, however less than half of treated patients achieve durable responses. Supplementing a patientâ€™s immune repertoire with tumor-specific T cells can improve tumor rejection in solid tumors, however current cell therapy trials are limited to VP-MCC patients with the most common HLA alleles and adequate lymphocytes counts. To benefit more patients, we sought to develop a platform for generating MCPyV-specific T cells from healthy donors that will be easily translatable to the clinic. MCPyV T antigen (TAg) is an ideal immunotherapy target because it is expressed by all VP-MCC tumors and is required for tumor cell survival. Moreover, because MCPyV infection is ubiquitous, it is likely that HLA-matched healthy donors will possess virus-specific immunity. We expanded MCPyV TAg specific T cells from donor blood using monocyte derived dendritic cells (moDCs) pulsed with synthetic overlapping peptide libraries spanning MCPyV TAg. Pulsed moDCs were co-cultured with autologous CD4+ T cells in the presence of IL-1beta, IL-2, IL-6, IL-7, IL-15, IL-21, IL-23, and TGFbeta for 14 days. MCPyV TAg specific CD4+ T cells were generated from 21/46 (46%) healthy donors without bias for HLA class I or II alleles, demonstrating the broad applicability of this approach. Expanded TAg specific T cells specifically expressed anti-tumor molecules TNFalpha, IFNgamma, granzyme B and IL-2 in response to cognate antigen. TAg-reactive T cells recognized epitopes across all splice variants of TAg and were validated by specific recognition of moDCs natively expressing full-length TAg. Expanded MCPyV TAg specific T cells possessed characteristics favorable for clinical use including polyfunctionality, memory phenotype, and minimal exhaustion marker expression. We have identified a novel production method to readily generate MCPyV TAg specific T cells from healthy donors for potential use as adoptive cell therapy in patients with VP-MCC.

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Discovery of carbohydrate-binding fully human monoclonal antibodies from an unbiased B cell library
Abhorrent surface glycosylation is a hallmark of many tumors and has become an attractive target for the clinical development of carbohydrate-binding antibodies. For example, Unituxin, an FDA approved chimeric monoclonal antibody (mAb), binds to malignancies overexpressing glycolipid GD2 and is currently used in the treatment of pediatric neuroblastoma. Even though carbohydrate-binding antibodies have significant clinical and diagnostic value, there are less than 100 published sequences of human antibodies that bind to carbohydrates. As a result, we do not yet understand how germline B cells are activated to produce high affinity anti-carbohydrate antibodies. Expanding this sequence space

would advance our understanding of how the immune system responds to carbohydrate antigens and provide a framework to produce and design more clinically relevant carbohydrate-binding antibodies. In our current work, we have devised a strategy to discover and characterize novel fully human carbohydrate-binding antibodies. We have profiled a library of hundreds of uncharacterized fully human mAbs donated by ADIMAB, LLC, on our neoglycoprotein microarray to discover novel carbohydrate-binding mAbs. Our glycan microarray is assembled from a diverse range of 738 neoglycoproteins, glycoproteins, and controls. The antibody library originated from a variety of B cell subsets including naïve, IgG memory, IgM memory and plasma cells from healthy human donors. Using a pooled matrix-strategy to conserve microarrays, we have so far discovered 25 fully human antibodies that bind to carbohydrate epitopes present on our array. These novel antibodies bind to a diverse range of carbohydrate antigens, including chitin polysaccharides found on yeast cells, glycolipids GB4 and GD3 found on some tumor cells, and heparosan, which is being considered as an â€œimmune silentâ€ alternative to the polyethylene glycol used in injectable therapeutics. The newly discovered antibodies have been recombinantly expressed and we are working to fully characterize their binding profiles and to explore their potential applications. Additionally, we are working to compile a database of all known anti-carbohydrate antibodies and are in the process of analyzing the data to discover trends or prerequisites for producing a carbohydrate-binding antibody. We hope that this database, fortified with our newly discovered mAbs, will become a useful tool for the design and discovery of carbohydrate-binding mAbs.

Eva Perez Guijarro
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A murine-derived predictive signature correlates melanoma undifferentiation and multipotency with clinical resistance to immunotherapy
Immune checkpoint blockade (ICB), such as anti-PD1/PDL1 or anti-CTLA4, has become the first-line treatment for metastatic melanoma, the deadliest skin cancer. Yet response rates are insufficient while the therapy is associated with severe toxicities. High tumor mutation burden (TMB) and baseline PDL1 expression, increased T-cell infiltrates and treatment-induced inflammatory pathways have shown correlation with clinical benefit, while antigen presentation and Interferon-gamma pathway alterations were detected in some resistant patients. However, the discovery of definitive predictive biomarkers and targets to overcome resistance remain an urgent unmet need. We aimed to identify evolutionary conserved mechanisms of ICB resistance by the analysis of immune-cell and transcriptomic profiles from mouse melanomas, in concert with cross-validation using patient datasets, to build a reliable predictor of clinical benefit. We developed a panel of four genetically engineered mouse models (M1-M4) representing a broad spectrum of molecular and phenotypic subtypes of human melanomas and exhibiting diverse responses to ICB; from fully resistant (M1/M2) to 30-60% response rates in sensitive models (M3/M4). High parametric flow cytometry analysis showed elevated CD4+ and CD8+ T-cells in the sensitive M3 and M4, whereas T-cell dysfunction profiles sustained by macrophages and dendritic cells (DCs) were found in M1 melanomas, which explained its resistance despite high TMB and T-cell infiltrates. In contrast, low-T-cell inflamed melanomas from resistant M2 were supported by the

enrichment of tumor-promoting macrophages but reduced natural killer (NK) cell-conventional type 1 DC axis. Notably, Tumor Immune Dysfunction and Exclusion (TIDE) computational method correlated these profiles with clinical resistance to ICB. Whole-genome comparative analysis of M1/M2 versus M3/M4 transcriptomes revealed a melanocytic plasticity signature (MPS) correlated with patient outcome in response to anti-CTLA4 or anti-PD1. MPS expression was enriched in multipotent melanocytic precursors from the adult hair follicle and embryonic melanoblasts, suggesting that the potency and differentiation status of melanoma can determine ICB benefit. Importantly, the combination of MPS and TIDE significantly improved the prediction of patient survival upon ICB. Our results uncovered both melanoma cell extrinsic and intrinsic determinants of ICB efficacy that could serve as novel targets of immunotherapy.

David Milewski
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Clinical and Translational Research - Therapeutic Oncology and Immunotherapy
Combined Immunogenomic and Immunopeptidomic Analyses Identifies Biologically Relevant Immune Signatures and Novel Immunotherapy Targets in Osteosarcoma
Osteosarcoma (OS) is an aggressive bone cancer with ~850 new cases diagnosed annually in the United States. High dose chemotherapy with surgery has improved the 5-year overall survival to 65%. Unfortunately, relapse is common and no effective second-line therapies have been established. Immunotherapy has emerged as an effective precision medicine modality and involves T cell recognition of tumor associated antigens (TAAs) presented by MHC class I of tumor cells which stimulates cytolytic killing of tumor cells. Currently, little is known about anti-tumor activity of T cells in OS or if T cells can be engineered to specifically target OS tumors. Due to the occasional response of OS patients to immune checkpoint blockade, we hypothesized that OS tumors present immunogenic antigens on MHC class I which are targeted by T cells. Transcriptome analyses on patient OS tumors (n=82) identified a high cytotoxic (CD8+) T cell gene signature associated with increased overall survival, suggesting the presence of an active T cell response in some OS. Comparison of OS tumors, cell lines and normal human tissue controls revealed an abundance of TAAs, including cancer germline antigens (CGA), which have limited expression in adult tissues. PRAME, a CGA expressed in multiple solid tumors and hematological malignancies, was the most frequently overexpressed TAA in OS with normal expression restricted to testis and was validated using immunohistochemistry. To determine how PRAME and other TAAs are processed and presented on MHC class I, we performed large scale immunoprecipitation of MHC Class I complexes, peptide isolation and identification using LC-MS/MS from human OS cell lines (n=3). We identified 3,380 high-affinity MHC class I peptides including peptides derived from PRAME and other TAAs, highlighting the immunogenic nature of OS tumors. Lastly, we tested if OS can be effectively killed through T cell recognition of these antigens. T cells from two healthy donors were transduced with a T cell receptor (TCR) which recognizes an identified PRAME peptide (SLLQHLIGL). In an in vitro co-culture assay, TCR-transduced T cells efficiently killed OS cells and secreted IFNÎ³ with little non-specific reactivity in untransduced T cells. In summary, we report an unanticipated clinically relevant immune infiltration in OS tumors and describe for the first time the immunopeptidome landscape of OS tumors using IP-MS/MS which can be effectively targeted by T cells.

Katherine Masih
Other
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Clinical and Translational Research - Therapeutic Oncology and Immunotherapy
Increased Epigenetic Plasticity and JUN Pathway Activation in Pre-Treatment Pediatric B-cell Acute Lymphoblastic Leukemia Predict Resistance to CD19 CAR T-cell Therapy
Acute lymphoblastic leukemia (ALL) is the most common childhood cancer. The outcome for patients who relapse is poor with a less than 30% survival. CD19 CAR T-cells have shown remarkable response rates between 80-90% in relapsed/refractory disease. Little is known about antigen-independent factors that predict initial resistance to CD19 CAR T-cell therapy. We hypothesized that leukemias that are resistant to CD19 CAR T-cell therapy are distinct from sensitive leukemias and that these differences can be detected prior to therapy. To interrogate differences in the genomes, transcriptomes, intratumoral heterogeneity, and epigenetic landscapes between resistant and sensitive leukemias, we obtained pre-treatment bone marrow aspirates (BMAs) from patients enrolled in a clinical trial at Seattle Childrenâ€™s Hospital. Samples were categorized based on patient response and included 7 resistant and 7 sensitive leukemias as matched controls. We performed whole exome sequencing, bulk RNA-seq, array-based methylation, scRNA-seq, CyTOF, ATAC-seq, and H3K27ac ChIP-seq. Additionally, we established CD19 CAR T resistant and sensitive patient derived xenografts (PDXs) of these leukemias, which are currently being used for in vivo modelling. We identified alterations in known epigenetic modifiers, including CREBBP and EP300, in all resistant leukemias. ATAC-seq revealed relatively more accessible chromatin in resistant patients. The combination of altered epigenetic modifiers and increased accessibility is suggestive of epigenetic plasticity. CyTOF identified a subpopulation with characteristics of both myeloid and lymphoid lineages in one sample that may represent a resistant population of cells selected for under CAR T-cell pressure. Methylation and expression data indicate promoter hypermethylation and lowered expression of JUN pathway genes in sensitive leukemias. Motif analysis of differentially accessible regions in the resistant leukemias showed an enrichment for AP-1 binding proteins, particularly JUN and its partners, which provides a mechanism to avoid caspase-induced apoptosis, the downstream effect of cytotoxic T-cell killing. This study establishes one of the most comprehensive profiling approaches for patient samples. We have shown the association of epigenetic plasticity and JUN pathway upregulation with resistance to CD19 CAR T-cell therapy. We are currently validating these results by modelling our cohortâ€™s therapy resistance in vivo.

Kenneth Ekoru
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Cultural, Social and Behavioral Sciences
Genetic Risk Scores for Cardiometabolic Traits in Sub-Saharan African Populations
There is growing support for the use of genetic risk scores (GRS) in routine clinical settings. Due to the limited diversity of current genomic discovery samples, there are concerns that the predictive power of

GRS will be limited in non-European ancestry populations. Here, we evaluated the predictive utility of GRS for 12 cardiometabolic traits in sub-Saharan Africans (AF; n=5200), African Americans (AA; n=9139), and European Americans (EA; n=9594). GRS were constructed as weighted sums of the number of risk alleles. Predictive utility was assessed using the additional phenotypic variance explained and increase in discriminatory ability over traditional risk factors (age, sex and BMI), with adjustment for ancestry-derived principal components. Across all traits, GRS showed upto a 5-fold and 20-fold greater predictive utility in EA relative to AA and AF, respectively. Predictive utility was most consistent for lipid traits, with percent increase in explained variation attributable to GRS ranging from 10.6% to 127.1% among EA, 26.6% to 65.8% among AA, and 2.4% to 37.5% among AF. These differences were recapitulated in the discriminatory power, whereby the predictive utility of GRS was 4-fold greater in EA relative to AA and up to 44-fold greater in EA relative to AF. Obesity and blood pressure traits showed a similar pattern of greater predictive utility among EA. This work demonstrates the poorer performance of GRS in AF and highlights the need to improve representation of multiethnic populations in genomic studies to ensure equitable clinical translation of GRS.

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Understanding information processing and perceived message believability of simulated Facebook cancer-related posts: Findings from a mixed methods eye-tracking study

While social media (SM) has facilitated the sharing of health information, it has increased the risk for users to be exposed to health-related misinformation. It is critical to understand factors, such as message and user characteristics, associated with how people process and evaluate information on SM. Such endeavors can then inform ways to disseminate accurate health messages and mitigate the spread of misinformation. Using data from an experimental eye-tracking study on simulated cancer Facebook posts, this study examines the association between message conditions and usersâ€™ health literacy on time spent on posts. Participants (n=53) were asked to view simulated Facebook posts about HPV vaccine and sunscreen. Message conditions varied by source (government agency, healthcare organization, or individual), format (narrative vs. non-narrative information), and veracity (evidence-based vs. non-evidence-based). Participants then responded to a survey to assess message believability of posts. A Bayesian hierarchical model was conducted to analyze the role of health literacy on credibility assessment and dwell time across conditions. Areas of interest (AOIs) were created to capture the time spent on an area (e.g. text, source, image) of the post. Dwell time within AOI was defined as â€œtime spent on each AOI divided by total duration of time spent on postâ€. Adequate health literate individuals rated evidence-based posts as being 2.3 times more believable than non-evidence-based posts, 95% HDI [0.74, 3.81]. We further looked into dwell time by conditions controlling for pixel size, participant order, and health topic. For narrative text posts, participants spent more time viewing non-evidence-based compared to evidence-based messages, 95% HDI [7.21, 21.76]. In addition, limited health literate participants spent more time on the source compared to adequate health literate participants, 95% HDI [0.38, 5.99]. When designing SM messages for health promotion, message format and health literacy can affect information processing. Although individualsâ€™ health literacy did not

predict dwell time on the text of a post, participants with low health literacy spent more time scrutinizing the source of the post. This finding may suggest that literacy skills are important to identify accurate information on SM. In an effort to mitigate misinformation, health literacy interventions can help individuals determine accuracy of information on SM.

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Cultural, Social and Behavioral Sciences
Neighborhood deprivation and epigenetic age acceleration
Background: Socioeconomic status (SES) disparities in life expectancy are increasing in the US. Living in a low SES neighborhood is associated with early onset of age-related disease, reduced life expectancy and all-cause mortality. Biologic age captures biologic variation among individuals of the same chronologic age. Prior research suggests living in a low SES neighborhood can accelerate biologic aging through stress- and chemical-related pathways. These studies largely rely on telomere length and allostatic load to measure biologic age. Recently developed epigenetic clocks that utilize blood-based DNA methylation patterns to estimate biologic age may be more sensitive markers than those previously studied. Despite evidence that living in a low SES neighborhood may accelerate biologic age, few studies have assessed this relationship using methylation-based measures of age. Objective: The study objective was to assess the relationship between neighborhood SES and methylation-based biologic age metrics. Methods: The Sister Study is a prospective breast cancer cohort of 50,884 women aged 35-74 years (2003-2009). We used data from a case-cohort sample of non-Hispanic white Sister Study participants for whom we have measured whole blood DNA methylation data (n=2,632). Methylation was measured using an Infinium HumanMethylation450 BeadChip array and results were used to calculate methylation-based age using four previously developed â€˜clocksâ€™ (Hannum, Horvath, PhenoAge, and GrimAge). Metrics were regressed on chronologic age to estimate differences, or â€˜age accelerationâ€™ and transformed into z-scores. Participants were assigned a quantitative relative measure of neighborhood deprivation at the US census-block group level using the â€˜Area Deprivation Indexâ€™ and their primary address at cohort entry. We estimated associations between deprivation and age acceleration using linear regression. Results: Comparing the highest versus lowest quartile of neighborhood deprivation, there was a positive association with age acceleration estimated by Hannum (Beta: 0.20, 95% Confidence Interval (CI): 0.04, 0.37), PhenoAge (Beta: 0.28, 95% CI: 0.13, 0.43), and GrimAge (Beta: 0.41, 95% CI: 0.25, 0.57), but not Horvath clocks. Increasing quartiles of deprivation were associated with GrimAge in a dose-dependent manner (p<0.001). Conclusions: The biological consequences of residing in low SES neighborhoods appear to be quantified by methylation-based markers of aging.

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Cultural, Social and Behavioral Sciences

Sleep inconsistency between weekends and weekdays is associated with changes in brain function during task and rest

Sleep inconsistency is highly prevalent among adults in modern societies and usually related to work-induced sleep restriction and circadian misalignment on weekdays (WD) with subsequent compensatory sleep on weekends (WE). In adolescents, WE-WD sleep inconsistency is associated with impaired attention and higher vulnerability to drug use. Here we investigated changes in brain function associated with WE-WD sleep inconsistency in 56 (30 female) healthy human adults. In our study, WE-WD differences in sleep duration and sleep midpoint were calculated using one-week actigraphy data. All subjects underwent 3Tesla BOLD-fMRI to measure brain activity during a visual attention task (VAT) and in resting-state condition. We found that WE-WD inconsistency of sleep duration and sleep midpoint were uncorrelated with each other (r=.08, p=.58) and influenced attentional performance and brain function differently. Our healthy subjects showed relatively small WE-WD differences (WE-WD: 0.59 hours). Longer WE-WD sleep duration was associated with better attentional performance (3-ball: Î²=.30, t=2.35, p=.023; 4-ball: Î²=.30, t=2.21, p=.032) and greater deactivation of the default mode network (DMN) during VAT (p<.05, cluster-corrected) and greater resting-state functional connectivity (RSFC) between anterior DMN and occipital cortex (p<.01, cluster-corrected). In contrast, inconsistent WE-WD sleep midpoint (WE-WD: 1.11 hours) was associated with worse performance (4-ball: Î²=-.33, t=-2.42, p=.020) and with lower occipital activation during VAT and with lower RSFC within the DMN. Our results document the importance of consistent sleep timing for brain function in particular of the DMN and provide evidence of the benefits of WE catch-up sleep in healthy adults.

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Cultural, Social and Behavioral Sciences

Circadian timing of eating and weight status among adults in the American Time Use Survey

The human body is governed by biological rhythms, which are entrained to the 24-hour day and influence many physiological processes. Recent research examines how the timing of behaviors in relation to these circadian rhythms impact health and well-being. In this project, we use a large, nationally representative dataset to investigate how the timing of eating in relation to the sleep/wake cycle (a proxy for circadian timing) relates to weight status. This project was motivated by experimental studies of time-restricted eating, which suggest that limiting the daily eating interval, eating in the biological morning and limiting eating close to the biological night may promote metabolic health and prevent weight gain. We used the Eating & Health Module of the 2006-08 and 2014-16 American Time Use Survey to examine cross-sectional associations of circadian timing of eating with weight status. Our analytical sample included 38,717 respondents aged 18-64 years with a body mass index (BMI) 18.5 â€“ 50.0 kg/m2. Time-stamped sleep and eating activities based on a single 24-hour time use diary per respondent were used to calculate three parameters: eating interval (time between first and last eating activity); morning fast (time between end of sleep and start of eating interval); and evening fast (time between end of eating interval and start of sleep). We used survey-weighted multinomial logistic regression models to examine associations between each parameter and BMI categories (normal

weight, overweight, obese), controlling for age, sex, education, employment, household size, race/ethnicity, total sleep time, weekend, season, and year. Compared to normal weight individuals, the odds of obesity decreased for each one hour increase in eating interval (OR: 0.97 [95% CI: 0.96 - 0.98], p<0.0001), and increased with each one hour increase in morning fast (OR:1.05 [95% CI: 1.04 - 1.06], p<0.0001) and evening fast (OR: 1.01 [95% CI: 1.00 - 1.02], p<0.05). Contrary to our hypotheses, normal weight individuals reported longer eating intervals and a shorter evening fast than obese individuals. In alignment with our hypotheses, normal weight individuals reported a shorter morning fast compared to obese individuals. Future studies should attempt to tease apart the contributions of diet quality and quantity, circadian timing of eating and social desirability bias as factors influencing the relationship between diet content and timing and health outcomes, including BMI.

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Title removed at the request of the author
Abstract removed at the request of the author

Shaohe Wang
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Developmental Biology
Reconstituting Branching Morphogenesis by Engineering Cell Adhesion
During embryogenesis, many organs undergo branching morphogenesis to form a tree-like hierarchical structure. Stratified epithelia, such as in embryonic pancreas and salivary gland, branch by repeated clefting, where indentations at the surface extend inwards to split one epithelial bud into several. Clefting requires growth factor signaling and involves extensive dynamics of epithelial cells and the extracellular matrix. However, it remains unclear how numerous epithelial buds arise from the interplay of cell and matrix dynamics. Here, we used two-photon microscopy to perform long-term volumetric live imaging at high spatiotemporal resolution using transgenic mouse salivary glands expressing a green nuclear marker together with a red epithelial reporter or a red membrane marker. This imaging strategy has enabled us to follow individual cells within virtually the entire gland for several rounds of clefting. Through quantitative image analysis, we found that the surface epithelial cells form an integral layer with the basement membrane, which together expands uniformly and folds inward to drive clefting that leads to new bud formation. Interestingly, cell divisions of surface epithelial cells always produce one or two interior daughter cells, all of which eventually return to the surface to make a delayed contribution to the surface expansion. We propose that the robust surface return of interior daughter cells is driven by differential adhesion between low- and high-E-cadherin cells, whereas the surface layer integrity is maintained by preferential cell-matrix over cell-cell adhesions of low-E-cadherin cells at the surface.

Importantly, we successfully reconstituted this mode of branching by substantially reducing E-cadherin expression and inducing basement membrane formation in 3D spheroid cultures of engineered cells that normally do not branch. Furthermore, we showed that the reconstituted branching requires integrin-mediated cell-matrix adhesions, and can be promoted by elevating the cell-matrix adhesion strength. Our results demonstrate a fundamental self-organization mechanism based on preferential cell-matrix adhesion greater than cell-cell adhesion that can explain how stratified epithelia undergo branching morphogenesis.

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Developmental Biology

in vivo CRISPR screening for biologically important mir-35 targeting sites in C. elegans

miRNAs play fundamental roles in maintaining appropriate gene expression levels by complementarity between the â€œseedâ€ region at position 2-7 of the 5â€™ end of the miRNA and the 3â€™UTR of its hundreds of targets. Prediction algorithms can provide a long list of candidate genes, and empirical approaches can identify many of the targets bound by a miRNA. However, both approaches are prone to false negatives and false positives. Furthermore, neither of these approaches delineates which of a miRNAâ€™s targets are the most biologically relevant, i.e. those that must be regulated by the miRNA to prevent deleterious phenotypes. Historically, forward suppressor screens in miRNA mutant backgrounds have identified such key targets of a few miRNAs. However, in the case of other miRNAs, such as the essential mir-35-42 family, such suppressor screens were unsuccessful. Therefore, we devised a novel strategy to test the phenotypic impact of disrupting each predicted binding site for mir-35-42 by CRISPR-Cas9 genome editing in C. elegans. This negative selection CRISPR screen not only reduces labor compared to perturbing miRNA binding sites on a site-by-site basis, but it also addresses the high signal to noise ratio when profiling guide RNA depletion in traditional negative selection. More importantly, almost all CRISPR screens reported are conducted at either cellular or tissue level. Our screen is a rare in vivo screen using whole genome-edited animals. This large-scale screen generated 1103 alleles from 570 strains. We analyzed the positional distribution of indels relative to the seed binding region as a metric to infer the phenotypic effects of mutating miRNA binding sites, and this method narrowed down the list from 87 predicted binding sites to six sites selected for validation. The top candidate from the screen, egg laying defective 1 (egl-1) validated in single gene studies: disruption of egl-1â€™s mir-35 binding site resulted in decreased fecundity and embryonic viability. Thus, relieving egl-1 from mir-35-mediated repression partially phenocopies the mir-35-42 mutant phenotypes, and this can be rescued by introducing compensatory mutations into mir-35 that restore egl-1 repression. In summary, this study demonstrates that the application of in vivo whole organismal CRISPR screening has great potential to accelerate the discovery of biologically-important miRNA targets.

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Developmental Biology
Caenorhabditis elegans PIEZO Channel Coordinates Multiple Reproductive Tissues to Regulate Ovulation
Human PIEZO1 and PIEZO2 are newly identified excitatory mechano-sensitive proteins. Dysfunction of PIEZOs caused a variety of human genetic diseases, including dysplasia in the cardiovascular, respiratory, and connective tissues. However, the cellular and molecular mechanisms of PIEZOs in these diseases are less understood. In this study, we took advantage of the reproductive tract of a nematode model C. elegans, which is a facile in vivo system to characterize the functional roles of PIEZOs. The deletion alleles and missense mutants of PEZO-1, the sole ortholog of PIEZOs in C. elegans, caused severe defects in reproduction, including a reduction in brood size, sperm number and ovulation rate. In vivo imaging observations show that oocytes undergo a variety of transit defects as they enter and exit the spermatheca during ovulation. Post ovulation oocytes were frequently damaged during spermathecal contraction. Given that PIEZO is a non-selective ion channel and may regulate spermathecal contractility through Ca2+ signaling pathways, we tested the genetic interactions between pezo-1 mutants and several cytosolic Ca2+ regulators with RNA interference (RNAi). Indeed, pezo-1 interacts genetically with known calcium regulators. Depletion of Ca2+ channels itr-1 and orai-1, and Ca2+ transporting ATPase sca-1, by RNAi substantially enhanced the reproductive deficiencies in pezo-1 mutants. Additionally, the pezo-1 mutants displayed a sperm navigational defect. Sperm that were readily washed out of the spermatheca during ovulation failed to migrate back to the spermatheca, as they normally do in wild type. In hermaphrodites, sperm are guided to the spermatheca by the F-series prostaglandin, and its synthesis requires a precursor from intestinal yolk granules. The observation of a defective yolk granule endocytosis in the pezo-1 mutants suggests that prostaglandin synthesis may be disrupted, which leads to the defective sperm attraction. Using an auxin-inducible degradation system, we depleted PEZO-1 in different reproductive tissues. Reduced brood sizes were observed in each tissue-specific degradation strain, suggesting PEZO-1 may act in different reproductive tissues to coordinate reproduction. To our knowledge, this is the first report of a functional role for PEZO-1 in C. elegans. Lastly, genetic suppressor and chemical antagonist screens will be performed using animals expressing PIEZOs patient-specific alleles to suppress the reproductive defects as a readout.

Md Abul Basar
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Developmental Biology
Human disease mutations reveal an essential function for linkage-specific ubiquitin chain editing during embryogenesis
Posttranslational modification with ubiquitin regulates many aspects of human development. During ubiquitin signaling, cells utilize intricate enzymatic cascades to synthesize topologically different polyubiquitin chain that differentially affect substrate fates. For example, K48-linked polyubiquitin targets protein for proteasomal degradation, whereas K63-linked polyubiquitin modulates protein-protein interaction to regulate diverse cellular functions. Deubiquitylase enzymes (DUBs) regulate

ubiquitin signaling by removing ubiquitin from proteins. DUBs of the ovarian tumor family (OTU DUBs) function through hydrolyzing specific linkage types within polyubiquitin. While some OTU DUBs have been linked to developmental disorders or autoinflammatory diseases, the physiological functions and underlying mechanisms of the OTU DUBs are ill-defined, in particular during embryogenesis. Here, we identify 7 pediatric patients with various neurodevelopmental defects carrying mutations in OTUD5, encoding for a K48/K63-specific OTU DUB. Intriguingly, we find that some OTUD5 variants separate its K48 from its K63 ubiquitin chain cleavage activity. Studying these patient variants in human embryonic stem cell models, we find that the K48-linked ubiquitin chain cleavage activity of OTUD5 is required for neuroectodermal differentiation. Mass-spectrometry analysis of differentiating neuroectodermal cells revealed that OTUD5 interacts with a select group of chromatins remodelers. Cycloheximide chase and proteasomal inhibition assays confirmed that the lack of OTUD5 destabilizes these chromatin remodelers. In addition, ATAC-seq analysis reveals that OTUD5 depletion caused loss of chromatin accessibility at neural enhancers in early stages of neural conversion. From these results, we conclude that the K48-linked deubiquitylation activity of OTUD5 prevents the degradation of a select group of chromatin remodelers to ensure proper neural ectoderm differentiation. Our work identifies an important physiological function of linkage specific ubiquitin chain editing during early embryonic neural fate determination.

Jing Zhang
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NIA
DNA Replication, Damage and Repair
Remodeling of the replisome in response to replication challenge
Proliferation of stem cells drives tissue and organ renewal required for healthy aging. The replication apparatus must duplicate the entire genome, navigating through open chromatin (euchromatin) and more compact regions (heterochromatin). Furthermore, replisomes must overcome numerous obstacles, including chemical blocks in DNA and alternate structures. Failure to overcome these obstructions can result in apoptosis, and/or senescence or genome instability. Replisomes minimally consist of the CMG helicase [CDC45 (C), MCM (M), and GINS (G) proteins], polymerases, and accessory factors. CDC45 and GINS lock the MCM ring around the single strand DNA template for leading strand synthesis. It is known that levels of replisome proteins decline during aging and the cellular response to replication stress is compromised. However, the influence of encounters with blocks on the composition of replisome is poorly understood. Here we report that the replisome can be remodeled upon encounter with a potent block. We developed an approach that enables visualization on DNA fibers of encounters of replisomes with a powerful blocking structure. We discovered that two â€œstressedâ€ replisomes appeared after encounters. Both had lost the GINS proteins which are required for activity of the CMG helicase. One was associated with FANCM, a DNA translocase, mutations in which result in a cancer prone disorder. The other was complexed with the DONSON protein, which is mutant in a microcephalic dwarfism syndrome. DONSON and FANCM are not in a complex with each other but associate with different â€œstressedâ€ replisomes. Remarkably, and quite unexpectedly, the FANCM replisome and the DONSON replisome are distinguished by time and place. DONSON replisomes preferentially associate with early replicating sequences and are in regions characterized by euchromatin histone

marks. FANCM replisomes are more frequent in late S phase, associate with late replicating sequences, and are found in heterochromatin. This distinction is not limited to cells exposed to the blocking agent, but also applies to unstressed cells. CHIP-seq analysis reveals a bias towards early replicating regions for DONSON associated DNA and a bias towards late replicating regions for FANCM associated DNA. Our results reveal a previously undiscovered dynamic in the cellular response to replication stress: that different stressed replisomes are activated in different regions of the genome at different times in S phase.

Suhas Kharat
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DNA Replication, Damage and Repair
5hmC-mediated Stalled Replication Fork Degradation by APE1 Causes Genomic Instability
Poly(ADP-ribose) polymerase inhibitor (PARPi) -induced synthetic lethality of BRCA-deficient cells is being utilized to treat breast and ovarian tumors. However, emergence of resistance to PARPi remains a major concern and understanding resistance mechanisms is of utmost clinical importance. In addition, it provides mechanistic insights into biological processes that are affected by BRCA-deficiency. To identify new regulators of PARPi-resistance in BRCA2-mutant cells, we performed a genome wide siRNA screen in mouse embryonic stem cells (mESCs). We found Ten Eleven Translocation 2 (TET2) loss contributes to resistance to PARPi (Olaparib). Validation of knockdown of TET family of proteins in BRCA2 deficient cells exhibited chemoresistance to not only olaparib but to other PARPi such as veloparib, talazoparib and platinum-based drugs such as cisplatin. TET2 is a metabolic enzyme that oxidizes 5-methylcytosine (5mC) to 5-hydroxymethycytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5CaC) during DNA demethylation. We found TET2 knockdown in BRCA2-deficient cells does not restore homologous recombination. Levels of drug transporters (Abcb1), which have been reported to cause drug resistance in BRCA mutated tumors remained unaltered in TET2 knockdown-BRCA2 deficient cells. Interestingly, we found that TET2 knockdown protects stalled replication forks in BRCA2 deficient cells. Replication fork protection is attributed to the reduction in 5hmC levels on the chromatin and not to changes in the expression of proteins associated with replication fork integrity. Proximity ligation assay revealed that 5hmC is localized on replication fork. Furthermore, we show that increase in 5hmC due TET2 overexpression can induce degradation of stalled replication forks, independent of the BRCA2-status. 5hmC based fork degradation caused genomic instability. We also demonstrate that Base Excision Repair associated apurinic/apyrimidinic endonuclease, APE1, is responsible for degradation of 5hmC, 5fC and 5CaC containing replication fork. Our findings reveal a novel role for 5hmC, an epigenetic mark on the DNA, in maintaining the integrity of stalled RF. Future experiments are focused on increasing 5hmC levels to resensitize chemoresistant BRCA2 deficient cells by enhancing fork degradation.

William Nathan
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NCI-CCR
DNA Replication, Damage and Repair
Mechanistic Studies of Interstrand Crosslink Repair in Non-dividing Cells
The oldest and most commonly used chemotherapy drugs are alkylating agents that work by preferentially damaging and killing rapidly dividing cells. These drugs, including cisplatin and the nitrogen mustards, inflict most of their toxicity through a lesion known as interstrand crosslinks (ICLs). This type of damage works by covalently binding to the two strands of DNA, thereby blocking the unwinding needed for DNA replication and RNA transcription. But slow-dividing, low-grade cancer cells, or the rarely-dividing cancer stem cell, arenâ€™t usually affected by this type of chemotherapy and therefore some patients are untreatable or relapse after chemotherapy. Our aim is to define how the damage from cisplatin, nitrogen mustards, and other crosslinking agents are repaired in these slow or non-dividing cells, with the hope that understanding this repair mechanism will provide an opportunity for new precision medicine. First, we used purified proteins, including XPF/ERCC1, SNM1A, and CSB, and a model ICL-containing DNA substrate to reconstitute replication-independent ICL repair in-vitro. Surprisingly, the biochemical data suggested that this repair of ICLs leads to double-strand break formation. We hypothesized that these double-strand breaks would then be further repaired by the non-homologous end joining (NHEJ) pathway, as this is the double-strand break repair pathway most active in non-replicating cells. To test this, we arrested NHEJ-deficient PreB cells in G1 and then treated them with an extremely efficient experimental ICL agent developed at NCI. ICL treatment in these NHEJ-deficient G1 cells led to an increase in persisting double strand breaks, as determined through staining for markers of double-strand breaks, 53BP1, pKap1, and yH2AX. These breaks were not mappable using the END-seq technique, indicating they occur non-specifically throughout the genome. In NHEJ-proficient G1 cells, no such double-strand break formation downstream of ICLs could be detected. Therefore, we propose a mechanism where in non-dividing cells, ICLs induced by chemotherapy are processed into double-strand breaks which are then resolved through the NHEJ pathway. We speculate that patients with slow-growing tumors defective for NHEJ repair will be highly sensitive to classic ICL-inducing chemotherapies.

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DNA Replication, Damage and Repair
Thanatos-associated protein 1 (THAP1) regulates chemosensitivity of BRCA1-deficient cells
Patients with high-grade serous ovarian carcinoma and germline mutation in BRCA1 exhibit high sensitivity and improved outcome to double-strand DNA break-inducing agents, such as poly(ADP-ribose) polymerase inhibitor (PARPi) and platinum owing to underlying defects in DNA repair via homologous recombination. However, de novo and acquired resistance to these agents is common and pose a considerable, and unsolved, clinical challenge. Here we adopted a systemic approach to identify unexplored factors that could be responsible for resistance to PARPi in BRCA1-defective cells. We used a genome-wide CRISPR-Cas9 knockout library to identify genes in which loss confers resistance to clinical PARPi in BRCA1-dificient cells. In addition to the previously identified genes, we identified THAP1 (Thanatos Associated Protein 1), which did not appear in any previous published screens. We confirmed

that THAP1-depletion in BRCA1-deficient cells were resistant to PARPi, measured by colony formation and chromosome analysis. Moreover, we found that double mutant cells were also resistant to cisplatin. In this context, it is notable that platinum resistant BRCA1-deficient tumors have a significantly lower expression of THAP1 relative to BRCA1 mutant tumors sensitive to platinum (TCGA database). Thus, it is possible that decreased THAP1 expression represents a significant mode of acquired resistance to PARPi or cisplatin in the clinic. Next, we sought to determine molecular mechanism of how THAP1 modulates BRCA1-dependent repair. We found that loss of THAP1 rescued RAD51 foci formation in BRCA1-deficient cells suggesting a role in the 53BP1 pathway. Through the analysis of published THAP1 ChIP-seq data sets and our nascent RNA-seq performed in THAP1-deficient MEFs, we found that THAP1 transcriptionally regulates one of the 53BP1 pathway gene, SHLD1. However, THAP1 does not bind and regulates expression of other 53BP1 effectors (53BP1, RIF1, REV7, DYNLL1 or ATMIN). This suggests that SHLD1 is a direct target of THAP1. We also reconstituted SHLD1 cDNA in THAP1-depleted BRCA1-deficient MEFs and found that it restored sensitivity to PARPi. Thus, THAP1 acts in the DNA damage response through regulating the expression of the 53BP1 effector SHLD1. Altogether these results may lead to therapeutic approaches to overcome and to target specific vulnerabilities in BRCA1-mutant cancers.

Wei Wu
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DNA Replication, Damage and Repair

ATM and PRDM9 regulate SPO11-bound recombination intermediates during meiosis

Recombination between homologous chromosomes during meiosis is initiated by the topoisomerase-like protein SPO11 induced double strand breaks (DSBs). Following MRE11 nuclease removal of SPO11, the DSB is then resected and loaded with DMC1/RAD51 filaments that invade homologous chromosome templates. In most mammals, DSB locations (â€œhotspotsâ€) are determined by the DNA sequence specificity of the methyltransferase PRDM9, and the ATM kinase controls meiotic DSB numbers. But it remains unclear whether PRDM9 and ATM also function in regulating downstream processing events. Here, we demonstrate the first direct detection of meiotic DSBs and resection using END-seq on mouse spermatocytes with little biological material (one mouse). This high sensitivity bypasses the limitations of other hotspot mapping methods that require either impractical quantities of mice (SPO11-oligo seq) or the availability of species-specific, high-quality antibodies (DMC1 SSDS). END-seq captured a strikingly uniform pattern of breakage at all sites that consisted of a strong central peak directly at the site of SPO11 cutting with an accumulation of reads flanking the cut site at a defined distance away. We interpret the central peak to be the direct detection of breaks that covalently bound SPO11 has not yet released from the DSB while adjacent, distal reads reflect minimum and maximum resection endpoints in the population of spermatocytes. In addition, we find that DMC1 limits both the minimum and maximum resection lengths, whereas 53BP1, BRCA1 and EXO1 play surprisingly minimal roles in meiotic resection. Through enzymatic modifications to the END-seq protocol that mimic the in vivo processing of SPO11, we identify a novel structure, the SPO11-bound meiotic recombination intermediate (SPO11-RI) present at the center of all hotspots. We propose that SPO11-RI forms because chromatin bound PRDM9 asymmetrically blocks MRE11 from releasing SPO11. In Atmâ€“/â€“ spermatocytes, the fraction

of SPO11-RI is reduced and trapped SPO11 cleavage complexes accumulate due to defective MRE11 initiation of resection. Thus, in addition to their global roles in governing SPO11 breakage, ATM and PRDM9 are critical local regulators of mammalian SPO11 processing.

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Endocrinology
Role of Phosphorylated Gonadotropin-Regulated Testicular RNA Helicase (GRTH/DDX25) in the Regulation of Germ Cell Specific mRNAs in Chromatoid Bodies during Spermiogenesis
Gonadotropin-regulated testicular RNA helicase (GRTH/DDX25) is a member of the DEAD-box family of RNA helicases which play an essential role in spermatogenesis. There are two species of GRTH, the 56 kDa non-phospho and 61 kDa phospho forms. Our early studies revealed a missense mutation (R242H) of GRTH in the Japanese azoospermic men which resulted in the lack of phospho-GRTH (pGRTH) in in vitro studies. GRTH knock-in (KI) mice with human mutant GRTH gene lack 61 KDa cytoplasmic phospho-species with preservation of non-phospho nuclear form. KI mice are sterile, lack elongated spermatids/spermatozoa with arrest at step 8 of round spermatids (RS) which contain smaller chromatoid bodies (CB). CB is a non-membranous organelle of RS, where mRNAs bound to GRTH transported from nucleus to cytoplasmic sites are temporarily stored, translationally repressed for later transport to polyribosomes for translation at specific stages of spermiogenesis. Owing to the specific function of CBs and importance of pGRTH in spermatid elongation, CBs isolated from the germ cells were analyzed. CBs isolated from KI mice are smaller, highly condensed and lack pGRTH and decreased MVH/Vasa (CB marker protein). RNA-Seq analysis of mRNA isolated from CB revealed 1421 differentially expressed genes (Padj<0.05) with 947 down-regulated and 474 up-regulated genes in KI mice. Expression of genes related to spermatid development, differentiation, chromatin compaction and remodeling (Tnp1/2, Prm1/2, Spem1/2, Tssk 3/6, Grth, Upf2 and Spata3) markedly reduced, and genes involved in RNA transport, storage, regulation and surveillance and transcriptional regulation (Eif1ad and Eif4a2, Ppp1cc, Dctn2, Pabpc1/6, Ybx3, Essrb1, Tent5b and H2al1m) were upregulated in the CB of KI mice and were further validated by qPCR. Notably, mRNAs of Tnp2, Prm2 and Grth which associated with GRTH protein were co-localized with MVH protein in the CB. This indicate the relevance of GRTH as a binder/transport protein of key chromatin remodelers for ensuring their mRNA repression/stability within the CB. In addition, GRTH binding to genes essential for spermatid development and regulation (Tnp1/2, Prm1/2, Grth, Tssk6, Rnf8 and Gcnf) were also found to be markedly decreased in the CB of KI mice. These results demonstrate the importance of pGRTH in the maintenance of biochemical composition/structure of the CB and role in spermatid development, regulation, chromatin compaction and completion of spermatogenesis.

Ashley Xiao
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NICHD
Endocrinology
Neurotrophic factor-Î±1,not BDNF, is critical in preventing stress-induced hippocampal CA3 cell death and cognitive dysfunction in mice
Neurodegenerative disorders such as Alzheimerâ€™s disease have been increasing dramatically in the past few years and affect millions of people in United States. Although the causes of neurodegenerative disorders have been under extensive study, the precise mechanism remains elusive. In our study, neurotrophic factor-Î±1 (NF-Î±1), also known as carboxypeptidase E(CPE) which acts as a prohormone processing enzyme, has been found to exert potent neurotrophic activity in the hippocampal CA3 region, as well as in neuronal cultures. Our studies showed that social combined with physical stress including maternal separation, ear tagging and tail snipping at weaning could lead to complete hippocampal CA3 degeneration and memory deficits in NF-Î±1/CPE-KO mice, despite the presence of normal levels of BDNF and phosphorylated TrkB in the brain. Vice versa, treatment with ANA12,the TrkB inhibitor, did not induce any deficits or degeneration in the hippocampal CA3 after weaning stress paradigm. These observations suggest that NF-Î±1/CPE is critical for the neuroprotective function in the hippocampus during severe stress and independent of BDNF. To further identify the function of non-enzymatic effect of NF-Î±1/CPE , transgenic knock-in mice that express CPE-E342Q (point mutation from E to Q at aa342 to abolish the enzymatic activity of NF-Î±1/CPE) was evaluated. The results showed that the CPE-E342Q mice had intact hippocampal CA3 and exhibited normal learning and memory function after weaning stress, in contrast to NF-Î±1/CPE-KO mice. Cytotoxicity analysis in vitro also confirmed NF-Î±1/CPE-mediated neuroprotective effects against H2O2 and glutamate by activating ERK signaling and increased BCL2. To determine whether NF-Î±1/CPE signal transduction function is mediated via interaction with a membrane receptor, binding studies were conducted on intact HT22 hippocampal cells. Radiolabeled NF-Î±1/CPE bound cell membrane in a saturable manner and with high affinity (Kd=4.37nM). Moreover, inhibitor studies suggest that the receptor does not belong to the tyrosine kinase class. Taken together, our findings show that NF-Î±1/CPE, is a unique trophic factor that is necessary and critical in protecting CA3 pyramidal neurons against severe emotional and physical stress-induced cell death and cognitive dysfunction in mice, independent of its enzymatic activity. Potentially, NF-Î±1/CPE could be a promising therapeutic agent for patients with neurodegenerative disorders.

Shanu Jain
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Endocrinology
Adipocyte Specific Ablation of P2Y14R Improves Glucose Metabolism in Mice with Diet-Induced Obesity
Adipose tissue is one of the major tissues in body regulating glucose and energy homeostasis. White adipose tissue (iWAT) serves as primary site of energy storage, while brown (BAT) and beige AT play a major role in energy expenditure. AT also exerts endocrine effects by secreting various adipokines/ batokines regulating whole body glucose metabolism. Diverse G protein-coupled receptors (GPCRs) are expressed in adipocytes and thus have emerged as potential targets for novel anti-diabetic drugs. P2Y purinergic receptors are a class of GPCRs activated by nucleotides and nucleotide sugars. The purinergic P2Y14 receptor (P2Y14R) is natively activated by UDP-glucose. P2Y14R is expressed in white as well as

brown adipocytes, and receptor mRNA levels was increased in iWAT due to high fat feeding. The potential role of P2Y14R in AT with respect to maintaining whole body glucose homeostasis remains unexplored. To this end, we generated a knockout mouse model lacking P2Y14R selectively in adipocytes (Ad-P2Y14-KO) using Cre/loxP technology. Interestingly, Ad-P2Y14-KO mice consuming a high fat diet gained less weight than the control mice. Body composition revealed that the decrease in body weight gain in Ad-P2Y14-KO was due to a decrease in fat mass. Expression levels of inflammatory markers were reduced in white and brown AT of Ad-P2Y14-KO. Decreased body weight resulted in improved glucose tolerance and insulin sensitivity in Ad-P2Y14-KO. Moreover, fasting blood glucose and fed plasma insulin levels were decreased in Ad-P2Y14-KO. Circulating levels of adipokines such as leptin were decreased in knockout mice, indicating increased sensitivity to leptin in Ad-P2Y14-KO. Mechanistically, lack of P2Y14R signaling increased lipolysis in adipocytes, as plasma free fatty acids levels were elevated in Ad-P2Y14-KO in the fasting state. Liver weight was decreased in Ad-P2Y14-KO indicating a lack of ectopic deposition of fat due to enhanced lipolysis, thereby improving whole body glucose metabolism. Detailed mechanistic studies are underway for understanding the role of P2Y14R in regulating adipocyte biology for therapeutic purposes.

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Endocrinology

The choroid plexus as a novel source of brain insulin and its impact on whole-body metabolism

The choroid plexus (ChP) is a highly vascularized tissue found in the brain ventricles, composed of an apical epithelial cell layer surrounding fenestrated capillaries. Epithelial cells of the ChP (EChP) produce CSF and synthesize neurotrophic factors and other signaling molecules. Through volume transmission, these factors can reach and act on distant brain regions by diffusion and convective flow. We show that insulin is produced in EChP of mice and humans, and its expression and release are regulated by serotonin. Insulin mRNA and immune-reactive protein, including C-peptide, are present in EChP, appearing in much higher levels than any other brain region. Moreover, insulin is produced in primary cultured mouse EChP, and its release is not regulated by glucose. Instead, stimulation of the serotonergic receptor HTR2C leads to activation of IP3-sensitive channels and Ca2+ mobilization from intracellular storages, culminating in insulin secretion. In vivo depletion of brain serotonin by dorsal raphe nucleus ablation negatively affects insulin expression in the ChP. Function of CP-derived insulin was investigated in a mouse model designed with a floxed insulin gene (Ins2). Intracerebroventricular delivery of adeno-associated virus with a serotype 5 capsid (AAV5) containing a gene for the endonuclease Cre induced insulin gene excision exclusively in EChP. Animals lacking CP-derived insulin presented several metabolic abnormalities. In metabolic cages, these animals had a higher respiratory exchange ratio suggesting a preference for carbohydrate utilization for energy production. Even though their fasting glycemia and insulinemia were lower than controls, glucose and insulin tolerance tests revealed no difference between groups. 4 months after the injection, animals lacking CP-derived insulin showed a significant lower body weight. Body composition evaluation by time domain magnetic nuclear resonance revealed significant lower fat content. Open field, elevated plus maze, and fear conditioning behavioral tests revealed no difference between groups. Here, we show for the first time that insulin is

produced by EChP in the brain, and its release is modulated by serotonin. CP-derived insulin has a profound impact on peripheral metabolic parameters, controlling substrate utilization preference, glycemia, and adiposity, Additional work is necessary to unveil the targets and mechanism by which this novel source of brain insulin modulates whole-body metabolism.

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WNK1 Regulates Embryo Implantation In Mice Through The Phosphatase PP2A

Embryo implantation is a prerequisite for establishing pregnancy, and it has been estimated that approximately 30% of female infertility is attributed to impaired implantation. Previous research efforts have identified many important mediators of this process, and yet implantation abnormalities continue to challenge the reproductive health of couples attempting to conceive. This highlights the urgency to identify novel players in this process, which could help combat implantation-failure associated infertility. Differing to the conventional transcriptomic approach, we previously identified with no lysine kinase 1 (WNK1) as a mediator of uterine biology through proteomic profiling. WNK1 was found to be an epidermal growth factor receptor (EGFR) regulated kinase, and WNK1 knockdown in vitro led to impaired decidualization confirming that WNK1 is reproductively functional. To extend these findings in vivo, we crossed Wnk1-floxed mice to the progesterone receptor-Cre mice to drive Wnk1 ablation from the whole uterus (Wnk1d/d). Loss of WNK1 substantially altered uterine morphology, causing endometrial epithelial hyperplasia and adenomyosis. These mice were severely subfertile, with more than 50% reduction in their reproductive capacity. In addition to the abnormal uterine morphology, it was determined that the major cause of subfertility was a delay in implantation, leading to abnormal embryo development and the eventual embryo loss later in pregnancy. Examination of the molecular events during the window of implantation showed that the transcription factor FOXO1, an indispensable implantation mediator was aberrantly localized to the cytoplasm in the Wnk1d/d uteri. Combining analyses from transcriptome, proteome and interactome data uncovered the novel regulatory role of WNK1 in the PP2A-AKT-FOXO1 signaling axis. We show that WNK1 interacts directly with PPP2R1A via mass-spectrometry, the scaffold subunit of the PP2A phosphatase. This interaction is crucial for PP2A activity, as WNK1 inhibition led to reduced PP2A subunits A and C expression in vitro and in vivo. PP2A activity in turn dephosphorylates AKT, and as AKT is a negative regulator of FOXO1, reduced AKT signaling alleviated its inhibitory effect on FOXO1. Ultimately, this permitted the nuclear entry of FOXO1 to transcriptionally regulate implantation-associated genes. Our findings revealed a novel function of WNK1 in mediating implantation in mice through post-translationally modulating AKT and FOXO1.

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Epidemiology/Biostatistics - Demographics and Surveillance

Examining variations in child development outcomes by gestational age at delivery among term births

While preterm delivery is a known developmental risk factor, few studies have examined whether variation in gestational age at delivery among term births has any bearing on childrenâ€™s neurocognitive development. Given the high rate of obstetric intervention during pregnancy in the US, and a recent practice bulletin supporting elective induction at 39 weeks vs. waiting until 40-41 for spontaneous labor, it is critical to determine whether long-term outcomes differ for children delivered at each week of term (37-41 weeks). Children in the Collaborative Perinatal Project (CPP; n=40,057 deliveries between 1959-1966) were assessed for mental and psychomotor development at 8 months using the Bayley Scales of Development, 4 years using the Stanford Binet IQ (SBIQ) domains, and 7 years using the Wechsler Intelligence Scale for Children (WISC) and the Wide Range Achievement Test (WRAT). After fitting adjusted linear mixed models using generalized estimating equations to account for sibling clusters, we found that mean development scores were generally higher for each week of gestation from 37 weeks, with peaks at 40 or 41 weeks and declines in the post-term period (42+). Delivery at 40 weeks was the reference group for all analyses. Children delivered at 39 weeks had lower Bayley mental (-0.77; CI -1.38, -0.16) and psychomotor (-1.30; CI -2.05, -0.55) scores, as well as lower WRAT spelling (-0.57; CI -1.15, -0.01) and reading (-0.97; CI -1.70, -0.25) scores. WISC scores did not vary by gestational age except at 37 weeks for verbal (-1.22; CI -2.14, -0.30), performance (-1.43; CI -2.48, -0.38), and full-scale IQ (-1.78; CI -2.77, -0.79). In Poisson regression, children delivered at 37 and 38 weeks were also at higher risk for having below average scores on all domains across assessment times. Those delivered at 38 weeks had higher risk of being classified as suspect or abnormal on Bayley (RR=1.38; CI 1.20, 1.59) and higher risk of scoring below average on the SBIQ (RR=1.12; CI 1.01, 1.26) and WRAT spelling (RR=1.22; CI 1.08, 1.38) and math (RR=1.20; CI 1.04, 1.38) tests. While small, the improvement in development scores across assessment periods indicates that each week up to 40 or 41 weeks of term gestation is important for cognitive development, suggesting 40-41 weeks may be the ideal delivery window for optimal neurodevelopmental outcomes.

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Epidemiology/Biostatistics - Demographics and Surveillance

Could Use Of Individualized Risk Models Mitigate Health Disparities In Eligibility For Lung Cancer Screening?

Background: The US Preventive Services Task Force (USPSTF) recommends lung screening for ever-smokers aged 55-80yrs with at least 30 pack-years who currently smoke or quit no more than 15yrs ago. However, for the same age and smoking history as whites, African-Americans have higher lung cancer risk, and Hispanics and Asian-Americans have lower lung cancer risk. Incorporating the use of individualized models for lung cancer incidence, lung cancer death, or life-gained into current USPSTF guidelines may reduce racial/ethnic disparities in lung cancer screening. Methods: We used the US-representative 2015 National Health Interview Survey to examine screening eligibility. We consider the following prediction models, when selecting the same number of ever-smokers aged 50-80yrs as are eligible for screening under USPSTF guidelines, combined with those eligible under USPSTF: Lung cancer

risk (Bach, PLCOM2012 and LCRAT models), lung cancer death risk (LCDRAT model), and life gained (LYFS-CT model). We compare the number eligible for screening, proportion of preventable lung cancer deaths (LCDs) prevented (relative sensitivity), number of life-years gained, and screening effectiveness (number needed to screen (NNS) to prevent one LCD), by race/ethnicity. We additionally assessed the calibration of each model in each race/minority by calculating the expected/observed number of cases. Results: Combining model-based with USPSTF eligibility criteria increased the eligible number of African-Americans by 57-118% relative to USPSTF alone, increasing the number of preventable African-American lung cancer deaths by 40-80%. Hispanic/Asian-Americans had lower increases in the number of preventable deaths (10-60% and 10-20%, respectively). Use of models increased the number of life-years gained (whites:20-30%; African-Americans:40-80%; Hispanics:10-50%; Asian-Americans:13-20%). Relative sensitivity was higher and screening more effective in whites and African-Americans than Asian-Americans and Hispanics. However, models estimate risk more accurately for whites than minorities. Conclusions: Combining USPSTF criteria with prediction models selected substantially more African-Americans for screening and would prevent substantially more lung cancer deaths, and gain more life-years, for all racial/ethnic groups. However, all models require more validation and improvement for minorities.

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Epidemiology/Biostatistics - Demographics and Surveillance
Clinical blood tests and cancer surveillance in Li-Fraumeni syndrome
Background: Li-Fraumeni syndrome (LFS) is an autosomal dominant cancer predisposition syndrome, caused primarily by germline pathogenic TP53 variants and associated with a very high lifetime risk of developing multiple primary cancers. Given the high risks and heterogeneity in LFS, cancer screening is essential for an early cancer detection. Current comprehensive cancer screening recommendations in LFS include annual imaging exams and quarterly blood tests, including hormonal biomarkers. While this screening is effective in early cancer detection, the impact of blood tests on the detection of hematological cancers, and adrenocortical carcinoma and germ cell tumors remains unknown. Methods: Our study focused on the interim blood tests, done every fourth and eighth months between annual screening, for early detection of malignancies in 128 participants, children and adults, enrolled in the National Cancer Instituteâ€™s LFS longitudinal cohort study between January 2012 and March 2018. Interim blood test results were paired with cancer diagnosis per intervals of time. Fisherâ€™s exact test (FTE), Generalized Estimating Equations (GEE) methods and clustering on participant identification number (ID) with an autoregressive correlation structure (AR-1) over time were used for statistical analysis. Results: Thirty-two individuals developed 42 cancers during the study period, of which 13 cancers in 12 individuals were diagnosed in the interval between interim screening blood work. One cancer was detected in consequence of a persistent asymptomatic anemia (4 months) shown on the interim blood test, and the diagnosis of colorectal cancer was done after colonoscopy. There was no statistical association between test abnormalities and cancer diagnosis (p-value=0.78; OR = 1.14; 95% CI, 0.29 - 4.03). There was no evidence that presence of any abnormal blood test was associated with a cancer diagnosis within each quarterly interval period (p-value=0.85; 95% CI, -0.12 - 0.48). Additionally,

there was no evidence of association with cancer diagnosis in the subsequent interval period (p-value=0.55; 95% CI, -0.96 - 1.80). Conclusion: Our data suggest quarterly blood work for screening for hematological malignancies may not be of independent benefit in LFS. Longitudinal follow-up is underway to determine the appropriate screening interval in individuals with LFS.

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Epidemiology/Biostatistics - Demographics and Surveillance
Testosterone Therapy in Relation to Cancer Risks among Men in the SEER-Medicare Database
Introduction. In this study, we examined the associations between two forms of testosterone therapy (TT) and prostate cancer risk, stratified by clinical stage or by the presence of primary or secondary hypogonadism. We also evaluated associations between both forms of TT and risks of six additional cancers that are predominant in men. Methods. The SEER-Medicare linkage combines cancer registry data from the Surveillance, Epidemiology, and End Results (SEER) program with Medicare claims of patients aged 65 years and older. We used a nested case-control study design that included 372,696 incident cancer cases, diagnosed between 1992 and 2015. Cancer sites included prostate, bladder, colorectal, esophageal, lung & bronchus, melanoma, and non-Hodgkin lymphoma. We selected 100,000 controls from a 5% random sample of Medicare beneficiaries, frequency-matched to cases on age and year of Medicare enrollment. TT by injection/implantation or topical application and relevant covariates were identified using the healthcare common procedure coding system or national drug codes. We used logistic regression and present odds ratios (OR) and 95% confidence intervals (CI) adjusted for potential confounders, stratified by clinical stage (local/regional vs. distant) or presence of hypogonadism. Results. Both forms of TT were associated with a lower risk of distant stage prostate cancer (injection/implantation OR=0.7, 95%CI:0.7-0.8; topical OR=0.5, 95%CI:0.3-0.7). We observed null associations for TT and risk of local/regional prostate cancer (injection/implantation OR=1.0, 95%CI:0.9-1.0; topical OR=0.9, 95%CI:0.6-1.2). Stratification by stage revealed evidence for positive associations with distant esophageal (topical OR=2.9, 95%CI:1.5-5.7) and local/regional melanoma (topical OR=3.0, 95%CI:1.5-6.3) and an inverse association with colorectal cancer (injection/implantation OR=0.8, 95%CI:0.7-0.9; topical OR=0.5, 95%CI:0.2-0.9). Overall cancer risks did not vary by presence of hypogonadism. Conclusion. Our finding of an inverse association for risk of distant stage prostate cancer may reflect the presence of a distinct etiopathogenic subgroup, when compared with local/regional

disease. Further, TT was also linked to risk of distant stage colorectal and esophageal cancers and increased risk of local/regional melanoma. Thus, TT may play a role in hormonal regulation for these malignancies or may be linked to cancer progression events.

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Epidemiology/Biostatistics - Etiology and Risk
Dyslipidemia in ethnic-diverse pregnant women is associated with placental DNA methylation at loci relevant for cardiometabolic diseases
Aim: Offspring exposed to maternal dyslipidemia during fetal life may be at higher risk of cardiometabolic diseases in later life, such as dyslipidemia, atherosclerosis, hypertension, obesity and type 2 diabetes. This concept of fetal programming of cardiometabolic diseases in later-life is well documented, however the mechanisms are not clearly understood. As a mediator of the maternal-fetal exchange, the placenta can play a role in the programming of health and disease in adulthood. Our aim was to identify placental DNA methylation changes that are associated with early pregnancy maternal dyslipidemia among 262 among racially/ethnically diverse pregnant women from the NICHD Fetal Growth Studies. Materials & Methods: Epigenome-wide analyses using robust adjusted linear regression models were performed to identify placental DNA methylation of CpG sites associated with maternal dyslipidemia status (i.e. high plasma total cholesterol, low HDLc, high LDLc and high triglycerides) in early pregnancy. Genotype-based principal component (PCs) in addition to methylation-based PCs were added to other covariates in the adjustments to account for population structure. We then performed a Bayesian correction for genomic inflation. Genes annotating differentially methylated CpGs were evaluated for gene expression in placenta and tested for enrichment of molecular pathways. Results: At 5% false discovery rate, we found 11 novel significant differentially methylated CpGs associated with high total cholesterol, LDLc and triglycerides, and low HDLc (nominal p-values ranging from 1.21x10-9 to 3.77x10-7). High triglycerides were associated with decreased methylation of cg02785814 (ALX4) and decreased expression of ALX4 in placenta. Genes annotating the differentially methylated CpGs play key roles in lipid metabolism and were enriched in dyslipidemia pathways. Some of these 11 loci are known for their relevance in vascular and structural development of the placenta. Functional annotation found cis-meQTL for genetic loci in ALX4 and EXT2. Conclusion: Among diverse race/ethnic pregnant women, we found corroborating evidence for the role of the ALX4 gene in dyslipidemia due to high triglycerides. These data provide insights into the potential for placental origins of cardiometabolic diseases in later life, and further shed light on the importance of optimizing womenâ€™s cardiometabolic health in early pregnancy or even before pregnancy.

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Epidemiology/Biostatistics - Etiology and Risk

Cigarette smoking and opium use in relation to the oral microbiota in Iran

Background: The human oral microbiota varies between individuals which is likely influenced by environmental exposures and may be associated with oral and certain systemic diseases. Cigarettes and opium contain chemicals and particulate matter that can come in direct contact with oral microbiota and may perturb the microbial ecology of mouth. This study aimed to investigate the association between cigarette and opium use with the oral microbiota. Methods: A total of 558 subjects were included from Tehran, Iran from 2011 to 2015. A questionnaire ascertained demographic information, cigarette and opium use history, and lifestyle characteristics. All participants provided saliva samples and 16S rRNA gene sequencing was performed. Taxonomy was assigned against the Human Oral Microbiome Database. Alpha diversity (i.e., a measure of within individual diversity including amplicon sequence variants [ASVs], Shannon index, Faithâ€™s PD) and beta diversity metrics (i.e., a measure of between individual diversity including Bray-Curtis, weighted, unweighted UniFrac) were computed. MiRKAT and zero-inflated beta regression models were calculated with adjustment for age, sex, BMI and case status. Results: The average number (+/-standard deviation) of ASVs for users of cigarette only (82.1+/-38.6), opium only (76.2+/-40.7) or both cigarette and opium users (77.8+/-42.8) were lower than never cigarette or opium users (95.1+/-44.0). Similar trends were observed for Shannon index and Faithâ€™s PD. The microbial communities differed by cigarette and opium use categories as indicated by the MiRKAT models from the three beta diversity matrices (P<0.05 for all). Nine genera, Corynebacterium, Tannerella, Capnocytophaga, Abiotrophia, Shuttleworthia, Peptostreptococcaceae_.XI..G.1., Lautropia, Kingella and Neisseria, were less likely to be detected in both cigarette and opium users. The relative abundance of the Actinobacteria, Firmicutes, and Proteobacteria phyla was higher in users of both cigarette and opium, and Firmicutes was also higher in users of opium only. The relative abundance of Bacteroidetes and Fusobacteria was lower in users of both cigarette and opium. Conclusion: Cigarette and opium use were related to overall oral microbiota community composition and both the presence and relative abundance of multiple taxa. Further studies are needed to investigate the impact of the changes to the oral microbiota through cigarette and opium use on human health and disease status.

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Epidemiology/Biostatistics - Etiology and Risk

Diabetes-like culture conditions promote breast cancer progression by inhibiting NER (nucleotide excision repair) pathway

Epidemiologic evidence suggests that diabetes negatively affects breast cancer outcomes. Consistent with this, we observed that breast cancer patients with diabetes in our NCI-Maryland cohort experience increased breast cancer-specific mortality, indicating that diabetes may affect tumor biology. Thus, the goal of this study was to understand how diabetes affects breast tumor biology. We performed RNAseq analysis of tumors from 39 diabetes and 57 non-diabetes breast cancer patients. To further corroborate patient data, we treated human breast cancer cell lines with high glucose (25mM) to mimic hyperglycemic condition which is a hallmark of diabetes. We also performed cell proliferation and

migration assays under above hyperglycemic condition. Next, we performed RNA seq analysis for a panel of breast cancer cells (MDAMB175, ZR7530, HCC1500, MDAMB231, MDAMB468) treated with 5 (low) and 25mM (high) glucose. We applied Ingenuity pathway analysis (IPA) and Gene Set Enrichment Analysis (GSEA) with these transcriptomic data to identify specific pathways, cellular molecular functions and genes that are either activated or inhibited under hyperglycemic conditions. Analysis of RNAseq data from breast tumors indicated no significant difference in the proliferation index (based on average expression of 11 cell cycle genes: BIRC5, CCNB1, CDC20, CEP55, MKI67, NDC80, NUF2, PTTG1, RRM2, TYMS, UBE2C) between diabetic and non-diabetic breast tumors. Consistent with this, in cell culture, high glucose treatment did not increase the proliferation of breast cancer cells (MDAMB175, ZR7530, HCC1500, MDAMB231 and MDAMB468). GSEA analysis of tumors indicated the activation of the epithelial to mesenchymal transition (EMT) pathway in patients with diabetes. EMT pathway was also found to be activated in high glucose (HG)-treated breast cancer cells including MDAMB175, ZR7530 and HCC1500. In cell migration assay, hyperglycemia increased migration in a subset of breast cancer cells (MDAMB175 and MDAMB468). IPA analysis of the RNAseq data from breast tumors and high glucose treated cell lines predicted the inhibition of nucleotide excision repair (NER) pathway, a common DNA repair pathway. Taken together, our study indicated that diabetes does not induce a proliferative phenotype in breast cancer, rather induce a metastatic phenotype. Further, diabetes inhibits NER pathways, which may cause genome instability and breast cancer progression, which we currently further investigate.

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Epidemiology/Biostatistics - Etiology and Risk
Moving toward understanding specific pathways of inflammation in pregnancy: prenatal exposure to consumer product chemicals and changes in plasma eicosanoids
Background. Exposure to consumer product chemicals during pregnancy can influence maternal inflammation, making mothers and infants more susceptible to pregnancy-related disorders. Although specific inflammatory pathways can be differentially affected by these chemicals, quantifying distinct pathways is challenging. Eicosanoids, an important class of lipid mediators, influence inflammation through unique pathways and can now be measured in comprehensive lipid panels. Objective. We aimed to determine the association between plasma eicosanoids and urinary biomarkers of three classes of consumer product chemicals among pregnant women. Methods. Our data come from a study of 90 pregnant women nested within a prospective birth cohort study. Maternal plasma and urine were collected at up to three prenatal visits. Plasma was analyzed for 61 eicosanoids, which were grouped into biosynthetic pathways defined by: 1) the fatty acid precursor, including linoleic acid, arachidonic acid, docosahexaenoic acid, or eicosapentaenoic acid; and 2) the enzymatic pathway, including cyclooxygenase, lipoxygenase, or cytochrome P450. Urine was analyzed for three chemical classes, including 12 phthalate metabolites, 12 phenols, and 9 organophosphate flame retardant metabolites (OPFRs). Each eicosanoid-chemical association was examined using repeated measures from generalized additive mixed effects models. Quantile g-computation was used to examine the association between eicosanoids and a simultaneous increase in all chemicals within each chemical class mixture at each

timepoint. Results. Both single-pollutant and mixture analyses showed positive associations between phthalates and phenols with specific eicosanoid pathways. The most consistent associations were between phthalate metabolites and eicosanoids produced by the enzymes lipoxygenase or cyclooxygenase from arachidonic acid. These associations were primarily driven by the metabolites of di-2(ethylhexyl)-phthalate (DEHP). Conclusions. We found higher inflammation-related eicosanoids in maternal plasma was associated with higher exposure to phthalates and phenols, but not OPFRs. These findings can provide insight into specific pathways through which exposure to chemicals in consumer products may be influencing maternal inflammation during pregnancy.

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Epidemiology/Biostatistics - Etiology and Risk
Sedentary behavior (SB), brain-derived neurotrophic factor (BDNF) and brain structure in midlife: A brain Magnetic Resonance Imaging (MRI) sub-study of the Coronary Artery Risk Development in Young Adults (CARDIA)
Background: There is evidence that long sedentary time (ST) is associated with poor brain health but the underlying mechanisms are unclear. Studies have found that BDNF could modify neuronal and synaptic transmission activities and may contribute to brain function. Experimental studies suggest that exercise increases BDNF levels and that low BDNF levels are associated with reduced cognitive function. There are limited data from population-based studies that have examined associations among SB, BDNF, and brain structures. Methods: The CARDIA study is a longitudinal study of persons aged 18-30 years at baseline, who have been followed for 25 years. The study population includes 618 participants (50.3 years old, 51.5% females) from the brain MRI sub-study of the CARDIA study who had plasma BDNF and SB data available at the Year 25 examination. SB was estimated by average ST hours a day from self-reported time spent sitting while watching television, computer use, and riding transportation. Outcome measures included total brain (TB), total grey matter (GM), and total white matter (WM) volume in cubic centimeters (CC). BDNF was log-transformed. ST was categorized into quartiles with the lower 25 percentile (less than 4.3 hours a day) as the reference group. The general linear regression was conducted to examine the following associations, adjusting for age, sex, race, and intracranial volume: Interactions between BDNF and ST on MRI measures; SB and MRI; SB and BDNF; BDNF and MRI; and SB, BDNF, and MRI. Results: Without BDNF adjustment, people sitting for more than 8.5 hours a day had a decreased TB volume of 11.6 CC and decreased GM of 7.5 CC compared to the reference group with p-values of 0.01 and 0.03, respectively. There was no significant difference for WM among different ST quartiles without BDNF adjustment. BDNF interacted with ST quartiles for TB and WM (p-value less than 0.03): Among people sitting for more than 8.5 hours a day, as BDNF increased, TB and WM volumes increased. No interaction effects were detected for GM. Conclusions: BDNF interacted with ST on TB and WM volumes in the study population. The beneficial brain outcomes among people with longer ST indicate potential signaling mechanisms regarding BDNF production in plasma.

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Epidemiology/Biostatistics - Prevention, Prognosis and Response
Statistical approaches using longitudinal biomarkers for disease early detection: A comparison of methodologies
Early detection, i.e., identification of diseases before clinical symptoms emerge, can benefit from tracking longitudinal biomarker changes, which often leads to early treatment and improved prognosis. The Risk of Ovarian Cancer Algorithm (ROCA) is specifically designed for ovarian cancer early detection using longitudinal biomarker cancer antigen 125 (CA125), by incorporating a latent changepoint structure in the longitudinal biomarker trajectory. In contrast, risk prediction quantifies the susceptibility of an individual to develop diseases in the future. Novel statistical methods such as Pattern Mixture Model (PMM) and the Shared Random Effects Model (SREM) have been developed in this context. We investigated the use of risk prediction models, PMM and SREM, for ovarian cancer early detection, with annual screening of CA125. We compared the prediction accuracy and calibration of PMM and SREM with ROCA via analyzing data from the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial and extensive simulation studies. The prediction accuracy was evaluated using the area under time-dependent receiver operating characteristic curve (tAUC) to account for right censored cancer diagnosis time. Calibration was assessed via the calibration plot and the ratio of observed over expected number of diagnosis. The out-of-sample performance was obtained using cross-validation to minimize potential model overfitting. Model robustness to misspecification and different marker screening frequencies were examined in simulation studies. Analysis of the PLCO data showed that PMM had significantly higher tAUC than ROCA (by 1.8-3.4%) and SREM (by 1.6-4.8%) across all cutoff time of interest (0.5-3 years after last screening). An explanation is that with annual screening of CA125, ROCA was not able to estimate the biomarker changepoint structure accurately; while PMM, with CA125 modeled by a smooth function, better captured the abnormality in its trajectory. PMM had better risk stratification than ROCA and SREM, in that it classified more subjects in the high/low-tail probability of having ovarian cancer (PMM high/low: 91.80%/3.30%; ROCA: 87.96%/2.82%; SREM: 54.08%/8.97%). All methods were generally well-calibrated. Simulations showed that PMM and ROCA were more robust to model misspecification than SREM, and that more frequent biomarker screening improved the accuracy of all methods. Future work will extend PMM and ROCA to the survival model framework.

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Epidemiology/Biostatistics - Prevention, Prognosis and Response
mBCRM: a methylation-based risk model for breast cancer
Background: Profiles of genome-wide DNA methylation change with aging and reflect past exposures. Methylation profiles have been used to derive estimators that predict various physiologic metrics and complex traits; these estimators appear to be related to disease risk. For instance, several methylation-based estimators of biological age and immune cell subtypes are markers of future breast cancer incidence. Whether blood DNA methylation can improve clinical risk assessment of breast cancer is an

area of increasing interest. Here, using previously developed methylation-based estimators, we derive a methylation-based breast cancer risk model (mBCRM) to test if blood methylation data can improve breast cancer prediction. Methods: Among a case-cohort sample of 2,774 women enrolled in the Sister Study, we measured whole blood genome-wide methylation profiles using HumanMethylation450 BeadChips when the women were cancer-free. We calculated 41 methylation-based estimators that predict: 14 biological age measures; 11 plasma protein concentrations; 10 immune cell subtypes; and 6 complex traits. Women were randomly assigned into a training set (70%; n=1,941, including 1,108 breast cancer events) and a testing set (30%; n=833, 471 events). In the training set, elastic net regularization was used to select and weight a combination of methylation-based estimators to comprise the mBCRM. The area under the curve (AUC) was calculated to compare prediction of the mBCRM with two existing breast cancer risk models: the Breast Cancer Risk Assessment Tool (BCRAT) and a breast cancer polygenic risk score (PRS). Results: In the testing set, mBCRM was associated with breast cancer incidence (per 1-SD increase, HR: 1.55, 95% CI: 1.28, 1.88, P=6.1×10-6). Although the PRS was the most accurate predictor of breast cancer (AUC: 0.63), it was not significantly better than the mBCRM (AUC: 0.59; P-diff= 0.15). Breast cancer prediction using only the BCRAT was poor (AUC: 0.53). Adding mBCRM to the PRS and BCRAT models significantly improved breast cancer prediction (BCRAT + PRS AUC: 0.64; mBCRM + BCRAT + PRS AUC: 0.67; P-diff=0.01). In a nested case-control study of blood methylation and breast cancer from the EPIC-Italy cohort, mBCRM was externally validated with breast cancer incidence (per 1-SD, OR: 1.33, 95% CI: 1.06, 1.66, P=0.01) and prediction (AUC: 0.59). Conclusions: Incorporating blood methylation data into existing risk models improves breast cancer prediction.

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Epidemiology/Biostatistics - Prevention, Prognosis and Response
Stability of SARS-CoV-2 in human body fluids at different environmental conditions
A novel human betacoronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in late 2019 in Wuhan, China. SARS-CoV-2 is the etiological agent of coronavirus disease 2019 (COVID-19), and currently over 120,000 cases have been reported worldwide in over 100 countries, with over 4,000 deaths. On March 11, 2020, the World Health Organization officially declared a pandemic, marking the first such declaration since H1N1 in 2009. While COVID-19 is generally mild in approximately 80% of cases, severe cases are characterized by pneumonia, respiratory failure, and/or multiorgan dysfunction, and the overall case fatality rate is currently estimated at 1-2%. Transmission is thought to primarily occur through respiratory droplets, although some evidence suggests that a fecal-oral route may also play a role, as gastrointestinal symptoms have been reported in some cases and live virus has been isolated from feces. Furthermore, the duration which SARS-CoV-2 remains viable in human bodily fluids is unknown, although our previous work revealed that SARS-CoV-2 can remain viable on plastic for up to three days at 21-23 °C and 40% RH when diluted in DMEM. As both human bodily fluids and environmental conditions may significantly impact these data, we sought to evaluate the stability of SARS-CoV-2 in blood, sputum, nasal fluid, feces, and urine at three different environmental conditions â€“ hospital (21° C, 40% RH), tropical (27° C, 85% RH), and winter (4° C, 40% RH) â€“ and across twelve different timepoints â€“ 0h, 1h, 4h, 8h, 1d, 2d, 3d, 4d, 5d, 6d, 7d, and 14d.

Aliquots of spiked stocks of each of the five bodily fluids containing 105 TCID50/mL SARS-CoV-2 (isolate 2019-nCoV/USA-WA1/2020) were placed both in sealed tubes and on uncovered polypropylene disks, to evaluate both liquid and surface stability, respectively. Following collection, samples were titrated on Vero E6 cells to assess for viable virus. Given the emergent nature of this outbreak, the data from this experiment are currently pending and analysis is ongoing. Upon completion, this study will provide crucial information for the ongoing public health response effort.

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Prospective Nasopharyngeal Carcinoma Risk Prediction Model for a Population Undergoing Screening
Background: Risk prediction models for nasopharyngeal carcinoma (NPC) in endemic areas have not been established despite knowledge that Epstein-Barr virus (EBV) is the primary cause of this disease and that EBV-related markers are strongly associated with NPC detection. We built a model to predict risk of NPC in a prospective cohort undergoing screening. Methods: Our study was embedded within an ongoing screening trial for NPC in southern China established in 2008 â€“ a prospective cohort of 51,235 participants aged between 30-69 years. We used logistic regression to identify viral and non-viral risk predictors of NPC. We investigated model performance based on discrimination (area under the curve, AUC), adjusting for verification bias. Models were internally validated using five-fold cross-validation. We also estimated absolute risks in our screening area. Results: A total of 151 incident NPCs occurred through 31 December 2016. After adjusting for verification bias, a model including anti-EBV antibody score showed high discrimination (AUC = 0.88, 95% confidence interval [CI] = 0.83 to 0.93). The discrimination was improved by adding screening history (EBV/Screening, AUC=0.93, 95% CI = 0.89 to 0.96, P=4.4×10-6 for difference in AUC) and slightly further improved by adding screening history plus non-EBV risk factors such as sex and family history of NPC (REALNPC, AUC=0.95, 95% CI = 0.92 to 0.97, P=0.007). All models were internally validated. In the screening population, at false-positivities ranging from 10% to 1%, both newly developed models (i.e., EBV/Screening and REALNPC) have similar performances. The 5-year absolute risk estimates of the integrated risk prediction also indicated good separation. Conclusions: Application of EBV-related markers for NPC shows a high prediction ability. As the screening programs mature, screening history should be considered for risk prediction. Modest contributions of non-EBV risk factors for NPC risk prediction were observed. Further study focusing on validation of our risk prediction models is warranted.

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Type 2 diabetes polygenic score in addition to clinical factors for prediction of diabetes incidence in youth

in an Indigenous American population
Background: Type 2 diabetes (T2D)-associated variants, derived from genome-wide association studies (GWAS), have largely been reproducible across populations, but there is limited information on how polygenic scores (PS) based on these variants add to clinical factors for predicting diabetes incidence, particularly in youth from non-European populations. Methods: A prospective cohort study of diabetes was conducted in an Indigenous population native to the Southwestern US. At each exam, a 75-g oral glucose tolerance test was administered for fasting and 2-hour plasma glucose (FPG, 2hPG). Genotypes were available from a GWAS for 2943 participants aged 5-19 years without diabetes at baseline, with imputed genotypes based on whole genome sequence data from 296 study subjects. PS for participants were constructed and weighted using genome-wide significant variants in a T2D GWAS meta-analysis in European-descent populations. Survival analyses were conducted to predict T2D incidence in 2 models: (1) age, sex, modified BMI z score, FPG; (2) age, sex, modified BMI z, FPG, PS. Area under the receiver-operating characteristic curve (AUC) was calculated using a nonparametric method for survival analysis. Decision curve analyses were conducted to evaluate the net benefit of adding the PS to the clinical factors over a range of probability threshold (pt) levels, which define the minimum probability of disease occurrence at which the intervention should be considered. Results: 447 cases of T2D occurred over 30,893 person-years of follow-up (0.15 incidence rate in ~10 years). For the model based on clinical factors alone, cumulative incidence at 10 years was 0.022 in the lowest quartile and 0.24 in the highest quartile. For the model with PS, corresponding values were 0.013 and 0.25. Hazard ratio for the PS, controlled for age, sex, modified BMI z and FPG, was 1.41 per SD (95% CI 1.28-1.56). AUC was 0.75 for the model with clinical factors alone and 0.76 for the model with PS. Over 10 years follow-up and across threshold pt levels from 0 to 0.5: the model incorporating clinical factors and PS had modestly higher net benefit than the model with clinical factors alone, with net benefit of adding the PS higher across moderately high pt values. Conclusion: A weighted T2D PS based on 245 variants was informative in predicting T2D incidence in Indigenous Americans; however, the improvement in prediction and potential net benefit beyond clinical factors alone were modest.

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Targeting the 5â€™ untranslated region of SMN2 as a therapeutic strategy for spinal muscular atrophy
Spinal muscular atrophy (SMA) is a neuromuscular disorder caused by loss of function mutations in the survival motor neuron 1 gene (SMN1). The resulting deficiency of survival motor neuron protein (SMN) causes severe muscle weakness, often within weeks or months of birth. All individuals with SMA have at least one copy of a paralog, SMN2, but a C-to-T transition in this gene results in exon 7 skipping in a majority of transcripts. As a result, less than 20 percent of SMN2 transcripts encode the fully functional SMN protein. Two therapeutics, nusinersen and risdiplam, that promote exon 7 inclusion in the SMN2 transcript have been shown to increase motor function in infants with SMA. While successful, this approach has a ceiling effect that is determined by the abundance of SMN2 transcripts in cells. Increasing the total pool of SMN2 transcripts and increasing the translational efficiency of these transcripts are strategies to overcome the ceiling effect associated with the splice-switching strategy.

We sought to determine whether the 5â€™ untranslated region (5â€™UTR) of SMN2 contains an element that reduces its expression, targeting of which could increase SMN levels. We identified antisense oligonucleotides (ASOs) targeting the 5â€™ end of SMN2 that increase SMN mRNA and protein levels in patient fibroblasts. By pulsing cells with a uridine analog and isolating nascent RNA, we found that the 5â€™UTR ASO does not increase the transcription rate of SMN2. Consistently, by measuring the half-life of SMN transcripts in fibroblasts treated with the 5â€™UTR ASO, we found that the higher steady-state level of SMN mRNA is due to its increased stability. We next tested the 5â€™UTR ASO in combination with a previously developed splice-switching oligonucleotide (i.e., nusinersen). This combination of 5â€™UTR ASO and splice-switching oligonucleotide increases SMN levels more than use of the splice-switching oligonucleotide alone. Finally, we tested the 5â€™UTR ASO conjugated to a cell-penetrating peptide in SMA motor neuron-like cells differentiated from induced pluripotent stem cells. We found that, compared to a non-targeting control ASO, the 5â€™UTR ASO increases SMN levels in this disease-relevant cell type. Our results add to the current understanding of SMN2 regulation and point toward a new therapeutic target for SMA.

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Evaluating the Role of the Basic Helix-Loop-Helix Family Member E40 (Bhlhe40) Transcription factor in Inner Ear Development and Function

The mammalian inner ear is comprised of the auditory sensory organ (the cochlea) and 5 vestibular organs (3 crista and 2 otolithic maculae) responsible for the sense of balance. These organs have specialized sensory hair cells (HCs) arranged into unique mosaic. The complex cellular structures and arrangements of these HCs are crucial for their normal function. How these cells are patterned and specified is an ongoing topic of research. Our laboratory used single-cell RNA sequencing to profile the genes uniquely expressed in different cell types of the developing inner ear. We identified the Basic Helix-Loop-Helix Family Member E40 (Bhlhe40) transcription factor as selectively expressed in the outer hair cells (OHCs) of the cochlea responsible for signal amplification, and in the type I HCs of the utricular macula responsible for precise fast firing in linear acceleration. Based on the selective expression of Bhlhe40 during early post-natal ages, we hypothesized this gene is necessary for normal auditory and vestibular function. To address this possibility, we used a knockout (KO) mouse for the Bhlhe40 gene. First, we used single molecule RNA fluorescent in situ hybridization to validate the expression of Bhlhe40 and its absence in Bhlhe40 KO mice. Second, we used immunohistochemistry to visualize possible changes in the anatomy, morphology, and innervation of the cochlea in Bhlhe40 KO mice. Finally, we examined the possible physiological role for the gene using auditory brainstem response as a measure of hearing sensitivity, distortion product otoacoustic emission as a measure of OHC function, and vestibular sensory-evoked potential as a measure of vestibular function. Our results validate the selective expression of the Bhlhe40 gene to OHCs and type I HCs in neonates. Morphologically, there is a 10% reduction in the number of efferent neuronal fibers that extend to innervate OHCs compared to controls. However, we did not see any physiological differences in auditory and vestibular function of Bhlhe40 KO compared to control littermate mice. Additional work is underway to determine whether

Bhlhe40 KO mice show an increased susceptibility to age related hearing loss or noise-induced hearing loss due to the reduced efferent innervation. Also, we are using quantitative PCR to investigate possible functional compensation by the paralog gene Bhlhe41 that may account for the limited functional effects seen in the Bhlhe40 KO mouse.

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A metabolic shift to glycolysis promotes zebrafish tail regeneration through TGF-B dependent dedifferentiation of notochord cells to form the blastema

Mammals are poor at tissue regeneration, often resulting in permanent damage or complete loss of tissues, organs, and extremities following injury or as a natural consequence of ageing. In contrast, fish maintain a high capacity for regenerating complex tissues after injury throughout their lifetime. Studying these processes should provide insights into the pathways necessary to trigger therapeutic regeneration in humans. We utilize the zebrafish Danio rerio, which are able to regenerate many tissues and organs such as the fin, retina, spinal cord, inner ear, and heart. In particular, the embryonic zebrafish tail serves as an ideal model of appendage regeneration due to its easy manipulation, relatively simple mixture of cell types, and superior imaging properties. Importantly, regeneration of the embryonic zebrafish tail requires development of a blastema, a mass of dedifferentiated cells capable of replacing lost tissue, which is a crucial step in all known examples of appendage regeneration. Using this model, we show that tail amputation triggers an obligate metabolic shift to glycolysis in cells comprising the notochord, which serve as the structural support of the body prior to spine development, during the repositioning of these cells near the amputation site. This metabolic switch is similar to the Warburg effect observed in cancer cells. Inhibition of glycolysis does not affect the health of the embryo but blocks fin regeneration due to failure to form a normal pluripotent blastema. To gain a better understanding of the molecular pathways that are regulated by metabolic signaling, we performed a time series of single cell RNA-sequencing on regenerating tails under normal conditions and in the absence of glycolysis. Strikingly, we detected a transient cell population in the single cell analysis that represents notochord sheath cells undergoing a TGF-B dependent epithelium-to-mesenchyme (EMT) transition to pluripotent blastema cells. We further demonstrate that the metabolic switch to glycolysis is required for TGF-B signaling and blocking either glycolysis or TGF-B receptors results in aberrant blastema formation through the suppression of essential EMT mediators such as snai1. These studies not only provide new insights into tissue regeneration, but also cancer biology by demonstrating that the shift to glycolysis in the Warburg effect is not only utilized by rapidly proliferating cells, but is a cell signaling trigger that induces EMT.

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Gene Expression
GLIS3: A critical player in Polycystic Kidney Disease
GLI-similar 3 (GLIS3) belongs to the GLIS subfamily of Krüppel-like zinc finger transcription factors and plays a critical role in the regulation of several biological processes and is implicated in a number of pathologies. Deficiency in GLIS3 in mice and humans leads to polycystic kidney disease (PKD), a chronic cystic renal disease that eventually leads to kidney failure and affects almost 600,000 people in US. To identify the molecular mechanisms underlying PKD pathogenesis, we studied the changes in gene expression during the development of PKD using whole-body GLIS3-KO and kidney-specific, PAX8-cre Glis3-KO mouse models developed by our group. RNA-Seq analysis of kidneys from 1, 2, and 4-week-old mice demonstrated that during the first month of postnatal kidney development the expression of many genes is significantly induced. However, these genes were not increased in Glis3-KO mice suggesting that loss of Glis3 expression halts postnatal kidney development and cells are maintained in a more immature and proliferative state. The latter explains the observed increase in cell proliferation as measured by EdU incorporation and the formation of renal cysts in the collecting ducts, distal tubules and glomeruli in Glis3-KO mice. KEGG and IPA pathway analysis identified arachidonic acid (AA) metabolism and inflammation among the top pathways and included the major anti-inflammatory AA metabolic genes, Cyp2J5, Cyp2J11 and CYP2C44. LC-MS analysis showed that reduced expression of these genes was accompanied by a decrease in several epoxyeicosatrienoic acid (EA) metabolites with anti-inflammatory properties. This decrease in anti-inflammatory EAs may be in part responsible for the observed increased interstitial inflammation and increased expression of pro-inflammatory genes in Glis3-KO kidneys. ChIP-Seq analysis was performed to determine which genes were directly regulated by GLIS3. Our analysis identified Cyp2J11 as a GLIS3 transcriptional targets. Motif analysis identified a G-rich consensus GLIS3 binding site in the regulatory region of Cyp2J11. Our study identifies a critical function for GLIS3 in postnatal kidney development and the transcriptional regulation of several AA metabolic genes and inflammation, which together play an important role in the progression of PKD in Glis3-deficiency.

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Determining the gene regulatory network for hair cell regeneration in the zebrafish adult inner ear at single-cell resolution
Millions of Americans experience a hearing or balance disorder due to permanent hair cell loss. Hair cells are the mechanosensory cells used in the auditory and vestibular organs of all vertebrates. In zebrafish and other non-mammalian vertebrates, hair cells turn over during homeostasis and regenerate completely after being destroyed or damaged by acoustic or chemical exposure, while in mammals, destroying or damaging hair cells results in permanent impairments to hearing/balance. Transcriptional profiling experiments on adult zebrafish inner ears following injury have identified 2,200 genes implicated in hair cell regeneration. We screened 254 candidate genes and found 10 genes necessary for hair cell regeneration in zebrafish. We hypothesize that appropriate gene regulation by enhancers was

key to how those genes respond during regeneration. Our goal is to identify enhancers involved in repairing a vertebrate inner ear. To identify enhancers in the inner ear and in response to hair cell damage, we developed a transgenic zebrafish to permit conditional and selective ablation of hair cells in the adult inner ear. This model expresses the human diphtheria toxin receptor (hDTR) gene under the control of the myo6b promoter, resulting in hDTR expressed only in hair cells. Cell ablation is achieved by intraperitoneal injection of diphtheria toxin which has a high affinity for the human receptor. On adult zebrafish that undergone hair cell ablation, we investigated the epigenome and transcriptome of single-cells from the inner ear at consecutive time-points following hair cell ablation. Because physical accessibility of genomic DNA is used as a proxy for the â€œactiveâ€ genome, we identified open chromatin locations using single-cell Assay for Transposase Accessible Chromatin with high-throughput sequencing. Machine learning on regeneration induced open chromatin revealed unique cell-specific transcription factor (TF) motif patterns. We correlated enhancer activation with transcription (using scRNA-seq) to identify gene regulatory networks that occur in a regenerating inner ear. We detected a clear pattern of overlapping Sox- and Six-family TFs, suggesting a combinatorial program of TFs determining cell responses and ultimately cell fate. Our ability to correlate cell-type, enhancer activation, and TF expression allowed us to begin to understand the combinatorial â€œcodeâ€ of transcription factors that initiate regeneration and instruct hair cell differentiation.

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High fidelity Cas9 mediates efficient and allele-specific disruption of a mutant human rhodopsin gene in transgenic mice modeling autosomal dominant retinitis pigmentosa

Retinitis pigmentosa (RP) is a group of inherited retinal diseases characterized by progressive death of photoreceptors. Mutations in the rhodopsin (RHO) gene are a common cause of autosomal dominant RP. The RhoT17M mutation, one of over 100 rhodopsin mutant alleles known, is caused by a single bp change (C-T), and it abolishes an N terminal glycosylation site and destabilizes the protein. In this study, we tested if AAV -mediated CRISPR/Cas9 gene delivery could specifically disrupt the T17M mutant allele and rescue the disease phenotype in transgenic mice. A dual-AAV approach was adopted in which the Cas9 cassette was packaged into one vector and the sgRNA cassette together with a reporter gene was packaged into a second vector. Two transgenic mouse lines carrying either the human RhoT17M allele or the RhoP347S allele (as negative control) were used in the study. Mice received subretinal administration of mixed vectors carrying Cas9 and sg RNA targeting the RhoT17M allele in one eye, and the fellow eye received mixed vectors carrying Cas9 and sgRNA against EGFP as a negative control. We compared three Cas9 nucleases, SpCas9, eSpCas9 and HiFiCas9 for on-target and off-target activities. SpCas9 was not able to differentiate between the T17M target and the corresponding WT sequences, as the treatment resulted in markedly higher ERG amplitudes in both the T17M mice and the P347S mice presumably due to inactivation of both transgenes. Sanger sequencing of PCR amplicons spanning the mutant locus confirmed both on-target and off-target events. Treatment with eSpCas9 led to a slight ERG improvement in the T17M mice but not in the P347S control mice. Sanger sequencing revealed only on-target editing events while no off-target events were detected. Significant ERG improvement was

achieved in T17M but not control mice following treatment the HiFiCas9 vector, and Sanger sequencing showed on-target editing events with high indel rate and no off-target editing events. The treated eyes also displayed a much thicker photoreceptor layer and better photoreceptor morphology. CRISPR treatment with HiFiCas9 provides efficient and allele-specific disruption of the T17M human RHO mutant gene. HiFiCas9 exhibited the best on-target activity and specificity suggesting that it could be the preferred Cas9 variant for future treatment of human adRP caused by rhodopsin mutations.

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Studies of OI patient and murine osteoblasts to investigate phenotypic variability of dominant Osteogenesis Imperfecta
Osteogenesis Imperfecta (OI) is a heterogeneous group of bone disorders characterized by bone fractures, growth deficiency and skeletal deformities. Most cases are caused by dominantly inherited mutations in the type I collagen genes, COL1A1 or COL1A2. An important and unexplained feature of OI and many dominant disorders is phenotypic variability, when patients with the same mutation show variable severity of clinical symptoms. Our aim is to find a novel target for OI treatment by understanding modifiers responsible for phenotype variations in OI, utilizing patientsâ€™ osteoblasts (bone forming cells). In the past, OI studies of collagen were limited to dermal fibroblasts due to unavailability of normal osteoblasts controls. However, OI osteoblast transcriptome and function are altered by mutant collagen synthesis. Our access to normal control osteoblasts is enabling the critical first study of OI osteoblasts differentiation and functioning. Osteoblasts cultured from healthy pediatric donorâ€™ surgical discard were subjected to RNA-Seq and mineralization assay to confirm reliability of controls. We focused on COL1A1 mutations Gly352Ser and Gly589Ser, each occurring in two unrelated patients differing in phenotypic severity. Secreted and cell layer collagen of patientsâ€™ osteoblasts showed overmodified chains, indicating the mutations delay collagen folding. Both mild and severe patientsâ€™ osteoblasts deposit significantly less mineral in vitro than controls. Interestingly, osteoblasts of severe patients deposit significantly less mineral in vitro than those of mild patients. RNA-Seq transcriptome profiling showed proteasomal protein degradation, autophagy and vesicle organization to be in top 10 upregulated signaling pathways in comparison to controls, while pathways related to protein translation were downregulated. In parallel, we study the effect of substituting

collagen residue on mouse bone phenotype, comparing our Brtl(Cys) mouse with COL1A1 Gly349Cys and a new model substituting Gly349 with Ser. Both mice have similar small size but Brtl(Ser) are more severe with rib fractures, kyphosis and skeletal undermineralization. Brtl(Ser) calvarial osteoblasts deposit less mineral and collagen fibrils have smaller diameter than wt or Brtl(Cys). This combined human/mouse approach will allow us to explore novel insights into OI osteoblasts compared to controls, effect of single residue variation and positional effects along the collagen helix.

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Neuro+, a genome-wide genotyping platform to study neurological disorders across diverse populations
In a little over 20 years, we have witnessed a revolution in the research area of complex genetic diseases. Despite success, a major limitation is that genomics is failing in diversity. Genome-wide genotyping platforms have been designed to capture genetic variation in European populations and little attention has been paid to designing arrays that can globally cover genetic variation across populations. Here we present the Neuro+ Illumina array, a novel, high-throughput and cost-effective custom-designed content pack to screen for genetic variation in neurological disorders across diverse populations. Neuro+ contains a backbone of 1,914,934 variants (Infinium Global Diversity Array) complemented with a custom content comprised of 95,273 variants implicated in over 60 neurological conditions. Furthermore, it includes > 10,000 tagging variants to facilitate imputation and analyses of 199 neurodegenerative disease-related GWAS loci across populations. Neuro+ can identify rare variants and accurately impute over 15 million common SNPs from the latest release of the Haplotype Reference Consortium, the Genome Asia Pilot Project, the CAPAA African American Panel and TopMed. We envisage this valuable screen tool will standardize genetic studies in neurological disorders across different ancestral groups allowing researchers to perform genetic research and molecular diagnostics underlying brain conditions at a global scale. Neuro+ array will become the reference platform used for the Global Parkinson Disease Genetics Program (GP2) which will generate genetic data for over 150,000 individuals, among other initiatives.

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MUCOSAL-ASSOCIATED INVARIANT T (MAIT) CELLS PROVIDE HELP TO B-CELLS IN VACCINATED AND SUBSEQUENTLY SIV-INFECTED RHESUS MACAQUES
Mucosal-associated invariant T (MAIT) cells, an innate-like T cell subset, serve as a first line of defense against infections. Since the frequency of these cells decreases in HIV infection and they are known to help control opportunistic infections, the study of MAIT cells is of interest in HIV/SIV vaccination and infection. We investigated MAIT cell dynamics and function in rhesus macaque blood and bronchoalveolar lavage (BAL) following mucosal priming with replicating adenovirus type 5 host range mutant (Ad5hr)-SIV recombinants, systemic boosting with SIV envelope protein, and subsequent repeated low-dose intravaginal SIV exposures. We determined the frequency and cytokine production of different subsets of cells by flowcytometry, cytokine production in MAIT cell supernatants by cytokine array, and performed functional assays using the sorted cells. An increased frequency of blood MAIT cells (CD3+CD4-CD8+5-OP-RU+) in response to SIV vaccination was observed, which was maintained 12-weeks post-SIV infection. The BAL MAIT cell population was not as influenced as that in blood and correlation analysis suggested a migration of these cells from the lung to blood. Vaccination increased expression of IFN-Î³, TNF-Î± and Granzyme B, suggesting greater activation of MAIT cells. Following T cell-specific Î±-CD3, Î±-CD28 stimulation MAIT cells showed greater capacity to secrete cytokines and chemokines, such as IL-6, IL-21, MIF, MIP1Î±/1Î², CXCL12/SDF1 and IL-8, that provide help for B-cell activation, migration and regulation, compared to that of CD3+MR1- non-MAIT cells. Secretion of IL-21, MIF, and CXCL12/SDF1 by MAIT cells following vaccination was elevated compared to pre-immunization MAIT cells, suggesting a potential benefit of vaccination. During chronic SIV infection MAIT cell cytokine/chemokine secretion was higher or comparable to pre-immunization levels, suggesting SIV-

infected animals retain the capacity to modulate B-cells. MAIT cell frequencies in blood and BAL correlated or tended to correlate with SIV-specific antibody levels in rectal secretions and with SIV-specific tissue resident memory B-cells, further suggesting their role in providing help to B-cells. Our results suggest MAIT cells can provide help to B-cells irrespective of vaccination and SIV-infection status, although MAIT cells from vaccinated animals showed higher helping capacity. Overall, vaccination enhanced MAIT cell frequency and functionality, which in turn provided B-cell help.

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GAG DETERMINANTS OF SPECIFIC GENOME PACKAGING IN HIV-1 AND HIV-2

To infect a new cell, HIV must package its RNA genome into virus particles. This process is extremely selective, as the HIV genome represents only a small minority (<1%) of all RNAs in the cell. Genome packaging is mediated by interactions between the viral structural protein Gag and the 5â€™ untranslated region of HIV RNA. The nucleocapsid (NC) domain of Gag directly binds viral RNA and is known to be important for packaging. However, NC binds non-viral RNAs at high affinities as well. Consequently, the molecular mechanisms that allow specific genome packaging remain poorly understood. We previously observed a striking difference in RNA packaging between the two types of HIV: HIV-1 and HIV-2. HIV-1 Gag efficiently packaged HIV-2 RNA, but HIV-2 Gag did not package HIV-1 RNA. Thus, these two viruses provide an excellent system to study how Gag:RNA interactions lead to specific genome packaging. To accomplish this, HIV-1-based Gag chimeras were constructed that contained the NC domain of HIV-2 Gag or just the two zinc fingers of HIV-2 NC. We examined RNA packaging by performing fluorescence microscopy of labeled virus particles. We found that the Gag chimeras generated particles at levels comparable to wild-type (WT) HIV-1 Gag. However, the chimeras packaged HIV-1 RNA genomes into significantly fewer particles than WT HIV-1 (60% vs. 95%). Further, unlike WT HIV-1, the chimeras strongly preferred to package HIV-2 RNA over HIV-1 RNA. Importantly, the chimeras were able to replicate in MT4 T cells, but with delayed kinetics compared to WT HIV-1. When re-passaged, the chimeras replicated significantly faster, indicating that the viruses had adapted. Adaptation was found to be due to an S18L mutation in the first zinc finger of HIV-2 NC. This mutation represents a switch from a typical HIV-2 amino acid to a HIV-1 residue at this position and was found to significantly improve viral replication and packaging of HIV-1 RNA. In sum, these findings indicate that the packaging specificity of HIV-2 Gag largely resides within the zinc fingers of NC. The S18 position in the first zinc finger of NC appears to play a key role in genome packaging specificity. These findings provide new insight into the mechanism of HIV genome packaging and may inform efforts to develop antiviral molecules targeting this critical process.

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Quantitative High Throughput Screening to Repurpose Drugs to Treat Myositis
Idiopathic inflammatory myopathies, collectively referred to as myositis, are a group of rare autoimmune diseases characterized by skeletal muscle inflammation, weakness, and often pathologies of the skin, lungs, or vasculature. Current treatment with steroids and immunosuppressive agents are not curative and have serious side-effects. We initiated a drug repurposing program to identify more targeted, less toxic therapeutics to treat myositis. Repurposing is the quickest path for drug development because it involves identifying approved medications currently used to treat other conditions. Although the cause of myositis is not fully understood, muscle from patients have an overabundance of the inflammatory cytokine type 1 interferon (IFN) and a down-stream product major histocompatibility complex (MHC) class I, which targets T cells for cytotoxic attack of host tissue. Genome-wide association studies in myositis have confirmed that alleles of MHC class I are the strongest risk factor, the protein is up-regulated in patient muscles, and forced over-expression in mouse skeletal muscle leads to many features of myositis. We conducted quantitative high throughput screening of >15,000 compound titrations, including all approved drugs, through a series of cell-based assays to identify those that inhibit the IFN-beta stimulated expression of MHC class I in muscle precursor cells (myoblasts). The primary screen utilized CRISPR/Cas9 genome-engineered human myoblasts that contained a pro-luminescent reporter HiBit fused to the C-terminus of endogenous MHC class I. Active compounds were counter-screened for cytotoxicity and validated by MCH class I immunofluorescence, Western blot, and RT-qPCR. All assays were optimized to have Zâ€™-factors >0.5, a statistical measure of assay robustness. Sixty-seven active compounds were identified falling into two major pharmacological mechanisms, Janus kinase (JAK) inhibitors and epigenetic/transcription factor modulators. JAK inhibitors block IFN receptor-associated phosphorylation of STAT transcription factors, the most potent being ruxolitinib, and pilot studies in myositis patients have shown they may be efficacious. Epigenetic modulators included inhibitors of histone deacetylase, DNA topoisomerase II, and transcription factors, like a hypoxia-inducible factor-1 inhibitor echinomycin. Testing in animal models and clinical trials is warranted to translate these therapies to myositis patients.

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Immunology - Autoimmune

TL1A-DR3 signaling promotes autoimmune arthritis by regulating CD4+ T cell metabolic fitness

The level of TNF-like ligand 1A (TL1A), a member of TNF superfamily, is elevated in serum and synovial fluid of rheumatoid arthritis (RA) patients, and is positively correlated with the severity of RA. Death receptor 3 (DR3), encoded by TNFRSF25, is the receptor of TL1A and is mainly expressed by lymphocytes. Mutations and polymorphisms of DR3 in human is linked to RA, suggesting a role of TL1A-DR3 signaling in the pathogenesis of RA. However, the effector cell subsets and how TL1A-DR3 signaling regulates the pathogenesis those cells remains poorly studied. Using newly generated DR3 conditional knock-out mice, we found that T cell intrinsic DR3 expression is required for the development of autoimmune arthritis. This is consistent with single cell RNAseq data from synovial fluids of RA patients, in which CD4+ T cells is the main immune cell subset expressing DR3. To understand the mechanism of TL1A regulating CD4+ T cells function, we performed RNAseq on mouse CD4+ T cells activated in the presence or absence of TL1A. We found that TL1A enhanced glycolysis pathway in CD4+ T cells. To determine whether changes in the expression of glycolytic genes were associated with functional metabolic differences, we performed seahorse glycolysis stress assay and found TL1A treated mouse and human CD4+ T cells exhibited robust glycolysis and glycolytic capacity compared untreated CD4+ T cells. As metabolites is the direct readout of metabolic status, we performed metabolome studies on mouse CD4+ T cells activated in the presence or absence of TL1A, and found higher levels of metabolites in the glycolysis pathway CD4+ T cells activated in the presence TL1A. Moreover, we also found enhanced aminosugar metabolism in TL1A treated CD4+ T cell. Thus, we showed that CD4 intrinsic TL1A-DR3 signaling is required for the pathogenesis of autoimmune arthritis, and TL1A-DR3 signaling endows CD4+ T cells enhanced glycolytic capacity. In the future, we hope to identify effector molecules that translate TL1A-DR3 signaling into metabolism change in CD4+ T cells. These results will shed light on designing novel therapeutic interventions to treat RA.

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Immunology - Autoimmune

Chronic type I interferon disrupts tissue macrophage homeostasis and induces bacterial susceptibility

Overproduction of type I interferon (IFN) is characteristic of systemic autoimmune diseases but its impact on tissue phagocyte function is poorly understood. We hypothesized that chronic elevation of type I IFN, perturbs the metabolic homeostasis of tissue macrophages and macrophage-mediated host defense against pathogens. To test this hypothesis, we used immunity-related GTPase M protein 1 (Irgm1)-deficient mice, which we recently reported to have persistent type I IFN production. Here, we report that in the peritoneum of naive Irgm1-/- mice, embryonic tissue-resident (F4/80hi MHCIIlo) macrophages have reduced expression of the canonical maturation markers F4/80 and Tim4, whereas the monocyte-derived (F4/80loMHCIIhi) macrophage pool was markedly depleted and exhibited decreased MHCII. Reduced MHCII expression was also observed in the monocyte-derived â€˜interstitialâ€™ macrophage population of the lung in Irgm1-/- mice. In addition, tissue macrophages in both locations were found to retain surface expression of the circulating monocyte marker Ly6C, suggesting a systemic deficit in monocyte-to-macrophage differentiation. Similar changes in tissue

macrophage surface markers were observed in Trex1 (Three prime repair exonuclease 1) mutant mice, an independent mouse model featuring chronic, spontaneous type I IFN induction. In vitro studies confirmed that type I IFN compromises differentiation of wildtype monocytes into macrophages. Single cell RNA-seq revealed that Irgm1-/- F4/80hiMHCIIlo peritoneal macrophages have a dramatic shift in gene expression compared to wildtype counterparts, characterized by a marked IFN signature and decreased expression of mitochondrially-encoded genes. These Irgm1-/- macrophages showed decreased mitochondrial mass and membrane potential . Interestingly, we found that type I IFN increases delivery of mitochondria to degradative lysosomes in Irgm1-/- macrophages. Deletion of the type I IFN receptor (Ifnar) in Irgm1-/- mice normalized expression of macrophage maturation markers and restored the mitochondrially active population in the peritoneum. Whereas Irgm1-/- mice had increased susceptibility to intraperitoneal infection with L. monocytogenes, this was rescued in Irgm1-/- Ifnar-/- mice. Taken together, our study suggests that chronic exposure to elevated type I IFN compromises the differentiation and metabolic competence of tissue macrophages, thereby increasing vulnerability to intracellular bacteria.

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Immunology - Autoimmune
Characterization of autoimmune ovarian failure upon removal of the IFN-gamma 3â€™ UTR AU-rich element â€“ a novel mouse model.
Ovarian failure is a physiologic event in menopause, however, when it occurs at less than 40 years of age in women, it is termed premature ovarian failure (POF). POF is idiopathic or due to autoimmunity, infections or enzyme deficiencies. POF affects approximately 1% of women less than 40 years of age and is associated with increased risks of osteoporosis, cardiovascular pathologies and diminished quality of life overall. This therefore necessitates laboratory studies using animal models for development and testing of effective therapies. Existing POF animal models are drug-induced and lack some clinical features. The neonatal thymectomy model however has most clinical features but has a variable POF induction period and is largely dependent on surgery success. A mouse model generated in our laboratory which has a 162 nt AU-rich element (ARE) region substitution with random nucleotides in the 3â€™ untranslated region (3â€™UTR) of the interferon gamma gene results in low but chronic circulating serum levels of interferon gamma. Homozygous mice for the ARE-deletion (ARE-Del-/-) displayed clinical features of systemic lupus erythematosus and primary biliary cholangitis. We hypothesized that these mice may also present autoimmune POF due to their poor reproductive capacity. Using flow cytometry and immunohistochemistry (IHC), we assessed the distribution of immune cells in the ovaries, uteri and spleen (controls). Flow cytometry showed increased CD8+- and CD4+ -T cells, and CD11c+- cells in the ovaries and uteri compared to the wildtype (WT) strain. IHC revealed a dominance of CD8+ T cells in the ovaries and uteri with infiltration of the zona pellucida and granulosa cells of maturing follicles and corpora lutea while sparing the primordial and primary follicles. There were more CD4+-T cells in the homozygous' ovaries and uteri targeting the corpora lutea, granulosa cells and endometrium respectively. Histopathology showed atrophied ovaries and uteri in the homozygous mice while ovarian and uterine weights were also less in the homozygous mice indicative of atrophy. Our study has shown

that the female homozygous ARE-Del mice generated in our laboratory exhibit autoimmune ovarian failure with clinical POF features. Studies are ongoing to establish gonadotropins and sex hormone levels and to determine autoimmune antigens involved. This model holds the potential for the development of specific diagnostics and therapeutics in the management of autoimmune POF.

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Immunology - Autoimmune
Increased expression of lysosomal associated membrane protein (LAMP) 3 in the salivary glands of Sjögrenâ€™s syndrome patients induce apoptosis and contribute to autoantibody production.
Background: Primary Sjögrenâ€™s syndrome (pSS) is one of the most common autoimmune diseases with no approved therapy. In addition to the symptoms of dry mouth, dry eyes, and lymphocytic infiltration in secretory epithelia, a key feature in this disease is the presence of specific autoantibodies, anti-Ro/SSA, anti-La/SSB in 70% of patients. To better understand the underlying changes in salivary gland (SG) gene expression that results in this phenotype, we aggregated publicly available microarray databases to identify overlapping differentially expressed genes and combined this data with analysis of the patient antibody status to identify gene expression changes that were associated with the development of antibody positive disease. This analysis suggested an association with lysosomal associated membrane protein 3 (LAMP3). LAMP3 expression was only previously associated with antigen-presenting cells and some cancers, but its role in pSS was not defined. Methods: The relationship of LAMP3 expression to the development of pSS was studies in mice specifically engineered to express LAMP3 in their salivary glands by retroductal cannulation and infusion of adeno-associated virus vectors encoding LAMP3 (LAMP3-mice). LAMP3 induced changes in cell growth, apoptosis, and autoantigens release were studied in the context of a SG-derived cell by immunofluorescences and biochemical assays. Results: LAMP3-mice developed a progressive SS-like phenotype, with time dependent increase in autoantibodies, and loss of saliva flow. In vitro studies showed that expression of LAMP3 induced lysosomal membrane permeabilization and the release of the lysosomal cathepsins into the cytoplasm of the cell, which resulted in apoptosis via caspases activation. LAMP3 expression also induced the release of extracellular vesicles with autoantigens via an apoptosis-independent pathway. Conclusions: Markers of apoptosis and autoantibody production have long been associated with pSS, but, a fundamental mechanistic understanding associated with this change in state has not been identified. Our study of LAMP3 expression in SG cells signifies a connection between these two observations and suggests a key role for the lysosome and LAMP3 in the development of disease. This finding represents a novel therapeutic strategy in the treatment of pSS.

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Immunology - General
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Justin Malin
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Immunology - General
Stunting of thymic development in conditional Klf6 knockout highlights non-canonical tumor suppressor functionality
The thymus is the central organ for education of immunocompetent and self-tolerant T-cells. The stromal cells of the thymus consist of cortical (cTEC) and medullary (mTEC) thymic epithelial cells, which are responsible for positive and negative selection, respectively, of hematopoietic precursors. While heterogeneity has recently been identified within mTEC, developmental paths remain unclear. Previously, we observed that the expression of the tumor suppressor gene Kruppel Like Factor 6 (Klf6) increases dramatically in both cTEC and mTEC between early embryo and adult. Using a Foxn1cre, we conditionally knocked out Klf6 in thymus. In marked contrast to the stereotypical response to knocking out a tumor suppressor gene, a week after birth, Klf6 knockout (KO) mice had a considerably smaller thymus, and the number of T-cell precursors was down five-fold compared to wildtype. Based on flow cytometry, mTEC counts were reduced more than 25-fold compared to wildtype. These results indicate that gross mTEC development depends on Klf6. At 4 weeks, the thymus and mTEC, in particular, remained stunted, however, the 25-fold drop-off had shrunk to less than 10-fold. When we performed 10X Chromium single cell RNA-Seq on TEC from 4-week old mice we detected organizational shifts in KO mTEC subpopulations, but unexpectedly, no prominent aberration in gene expression. Moreover, based on BRDU incorporation, there were no differences between WT and KO mice in rates of cell proliferation. CD4+ CD8+ thymocyte profiles appeared normal. We hypothesized thymic alterations in Klf6 KO mice had been programmed earlier â€“ during fetal development. Consistent with this, E17.5 mice exhibited stunted thymii, with the mTEC compartment severely stunted. Furthermore, E17.5 mTEC showed significant defects in cell proliferation. Together, these data suggest a reprogramming of the role of the tumor suppressor KLF6 between fetal and early adult developmental stages. Future work will include single cell RNA-Seq and ATAC-Seq in E17.5 mice in order to probe pathways impacted by the loss of KLF6. This work will potentially provide insights into KLF6â€™s regulatory role in thymus and TEC development, while highlighting non-canonical behavior of a tumor suppressor gene during embryonic development.

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Immunology - General

Mapping a functional network of haematopoiesis at single cell resolution by mass cytometry and computational modelling

The haematopoietic system is one of the most thoroughly characterized differentiation hierarchies in mammals. Over the years, a combination of molecular and genetic studies combined with fate-mapping and single cell flow cytometry have provided a detailed picture of the genealogies of the cells of the haematopoietic system. However, the dynamics of interactions between them is still poorly understood. In this study, we used a combination of pulse-chase experiments by mass cytometry combined with a machine learning-based data analysis pipeline, hierarchical agglomerative learning (HAL-x), to probe the functional network of interactions between differentiating immune cells in the mouse bone marrow. We used HAL-x to construct a deep phenotypic map of the mouse bone marrow, identifying more than fifty distinct clusters. Next, in order to understand the dynamic behaviour of the observed clusters of cell types under steady-state conditions, we constructed a correlation matrix between subset frequencies across forty mice. Our results demonstrate significant associations between frequencies of genealogically unrelated immune cell subsets, that serves as an indication of potentially novel dynamic rules of establishing homeostasis in the mouse bone marrow. We decided to test one such association, that of a strong anti-correlation between neutrophils and erythroid lineage progenitor cells, by injecting mice with granulocyte colony stimulating factor (G-CSF). As expected, G-CSF boosted the frequency of Ly-6Glo, CD11bhi immature neutrophils at 24 hours. However, unexpectedly, we also observed a concurrent decrease in the frequency of platelets in the bone marrow. Analysis of pSTAT activation profiles by G-CSF and TPO, the cytokines responsible for differentiation of granulocytes and platelets, respectively, revealed both the above mentioned cell types to be responsive to both cytokines. Moreover, time series analysis of differentiation revealed both cell types to be highly proliferative at steady-state with very rapid half-lives of differentiation. Taken together, these results lead to the hypothesis that functionally, the haematopoietic system behaves more like a network than a hierarchical tree. Moreover, we also propose competition for available cytokines as a potential mechanism to establish homeostasis. Our current efforts are geared towards testing these predictions by using a combination of single cell proteomics, transcriptomics and computational modelling.

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Immunology - General

NEUTROPHILS DRIVE ADIPOSE TISSUE AND MUSCLE WASTING IN CANCER CACHEXIA

Cachexia is a cancer co-morbidity that decreases quality of life and represents an unfavorable prognostic factor. Cachexia manifests primarily with a reduction of white adipose tissue (WAT) and muscle due to metabolic perturbation. Because immune cells control metabolism in WAT and muscle, we hypothesized that the immune cells in the WAT cause the metabolic imbalance that leads to cachexia. Thus, we examined the immune cell profile in WAT of tumor-bearing mice at different time points and we observed an increase of neutrophils and decrease of macrophages prior to wasting. Single-cell RNAseq of WAT and blood cells identified neutrophil clusters that were separated by tissue but not by tumor presence. Pseudotime analysis identified the WAT neutrophils as more mature and activated compared to blood neutrophils with low OXPHOS capacity and high glycolytic capacity. To test whether neutrophils

are responsible for tumor-associated cachexia we depleted neutrophils using an anti-Ly6G antibody. Neutrophil depletion protected tumor-bearing mice against WAT and muscle wasting. Using scRNAseq analysis we also identified two clusters of WAT macrophages with characteristic markers either of resident macrophages or monocyte-derived macrophages. The latter were the only WAT cell type able to secrete IGF-1 and their decrease in the cachectic mice compared to tumor-free mice was paralleled a decrease of WAT IGF-1 protein concentration. In vitro, we observed that IGF-1 was able to induce adipogenesis and lipogenesis in pre-adipocytes. Neutrophil depletion reversed the tumor-induced loss of the IGF-1-producing monocyte-derived macrophages by preventing monocytes differentiation to macrophages and/or by inducing macrophages death. Ongoing experiments suggest a role of neutrophilsâ€™ extracellular traps in affecting monocyte differentiation and survival. These results suggest that the neutrophil/macrophage axis may represent a therapeutic target for treatment of cachexia. Indeed, prevention of cachexia in cancer patients is an important medical need because cachexia greatly reduces patientsâ€™ quality of life quality of life and responsiveness to immunotherapy and chemotherapy.

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Immunology - General
Sustained sensing of commensal-derived products directs polarized immune cell positioning within the liver to optimize host defense
The liver is located at the interface between the intestinal portal vasculature and the general circulation,â€¯employing a complex set of innate and adaptive immune cells to protect from the continuous systemic threat of gut-translocating pathogens. The hepatic vasculature has a highly polarized organization, with both venous and arterial blood entering at the portal triad of each hepatic lobule, then flowing through sinusoids to the central vein (CV). Yet despite this highly asymmetric circulatory organization, the distribution of liver phagocytes or migration of lymphoid elements has largely been described as uniform across the liver lobule. This stands in marked contrast to extensive evidence that in lymphoid organs, cells of the immune system adopt spatially biased positions that provide for more effective host defense than a broadly disseminated pattern. Here we have employed quantitative multiplex imaging, genetic perturbations, transcriptomics, infection-based assays, and mathematical modeling to reassess the functional relationship between liver architecture, immune cell localization, and host defense. We found that both myeloid and lymphoid resident cells are asymmetrically positioned within the liver lobule, concentrating around the entry points for venous blood rich in gut-derived pathogens and molecules (peri-poral region). This positional asymmetry is not a pre-programmed, developmental pattern, but results from sustained MyD88-dependent signaling of liver sinusoidal endothelial cells (LSECs) in response to commensal-derived products. Chemokine gradients, especially of CXCL9, play a major role in establishing this hepatic immune zonation. However, in contrast to the anticipated MyD88-dependent asymmetric production of CXCL9, it was the composition of the peri-cellular matrix to which chemokines bind that was actively regulated by MyD88 signaling in LSECs. Direct experimental testing and modeling showed that a spatially polarized distribution of liver immune cells was more efficient than a uniform distribution in protecting against

systemic dissemination of bacteria. Together, these data reveal that LSECs act as a microbiome sensor. They actively orchestrate immune cell concentration in the sub-regions of the liver most susceptible to blood-borne infection, providing optimal host defense against pathogen spread while limiting disruption of homeostatic organ function.

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Immunology - Infectious Disease

T-cells, IFNÎ³ and Gr1+ cells are necessary for control of acute Crimean-Congo hemorrhagic fever virus infection in mice

Crimean-Congo hemorrhagic fever (CCHF) is a severe illness caused by infection with Crimean-Congo hemorrhagic fever virus (CCHFV). The principal vector and reservoir are ticks of the Hyalomma genus and patients can become infected via tick-bites, handling of infected livestock or during the care of infected patients. CCHF begins as a non-specific febrile illness that can rapidly progress to severe hemorrhagic manifestations and case fatality rates can be as high as 30%. Although clinically apparent CCHF can be fatal, sub-clinical infections are under-appreciated and cumulatively, most people infected with CCHFV will control and recover from the infection. However, the host responses necessary to control the acute infection are unknown and it is unclear if control of acute CCHFV is mediated through cellular or humoral immunity. To investigate the host responses to CCHFV we use a mouse model in which mice are infected with a clinical isolate of CCHFV. Upon infection, mice develop a progressively worsening disease characterized by weight loss, high viral loads, inflammatory cytokine production, severe pathology in the liver and spleen and recruitment of inflammatory immune cells to infected tissues. However, most mice survive the infection and recovery correlated with development of CCHFV-specific B- and T-cell responses. In further studies we have identified that both CD4 and CD8 T-cells are necessary to control the acute infection. Specifically, we found that CD4 T-cells in the liver and spleen produce IFNÎ³ in response to CCHFV and were necessary for the systemic IFNÎ³ response to CCHFV. Furthermore, IFNÎ³ is necessary for control of acute CCHFV infection as neutralization of IFNÎ³ in vivo lead to significantly increased mortality. Additionally, we have identified a critical role for Ly6Chi monocytes and/or neutrophils in control of acute CCHFV infection as depletion of these cell types with an anti-Gr1 antibody lead to uniform lethality in infected mice. Ongoing work is further delineating the role of Ly6Chi monocytes or neutrophils in controlling CCHFV and evaluating the hypothesis that T-cells control acute CCHFV through recruitment and IFNÎ³-mediated activation of Ly6Chi monocytes and neutrophils in infected tissues. Lastly, ongoing work is evaluating the hypothesis that CTL activity of CD8 T-cells is a further necessary T-cell effector function for control of acute CCHFV infection.

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Immunology - Infectious Disease

Characterization of a GPR35 nonsense mutation in a previously healthy patient with non-HIV cryptococcal meningoencephalitis.

Cryptococcus is a facultative intracellular fungal pathogen that causes disease in diverse hosts. Globally, cryptococcal meningoencephalitis (CM) is responsible for ~11% of AIDS-related deaths and carries a mortality rate upwards of 30-50% in the HIV-uninfected individuals, including solid organ transplant recipients, those immunosuppressed for cancers and autoimmune diseases, and the previously healthy with no apparent underlying predispositions at a rate of ~1:500,000 in the United States. While T-cell defects related to immune suppression in HIV is a well-known risk factor for CM, little is known about the immune defects underlying disease in the previously healthy. In a cohort of 94 previously healthy patients with CM, whole exome sequencing was performed identifying rare alleles predicted to have deleterious functional consequences (CADD >30). Pathway analysis identified G-protein coupled receptor (GPCR) signaling (p-value=1.9E-2) and cell adhesion (p-value =2.0E-3) deficits as enriched in this population. One such GPCR mutant allele consisted of a heterozygous STOPGAIN mutation in the orphan receptor GPR35 (GPR35C275X). GPR35 has been previously associated with inflammatory conditions, including inflammatory bowel disease, type 2 diabetes, and coronary artery disease. In the present studies the mutant allele was associated with increased surface expression on human innate immune cells and prolonged Ca2+ signaling following ligand stimulation, indicating a defect in receptor internalization and desensitization similar to the effects of C-terminal truncations of the chemokine receptor CXCR4 in the autoimmune disease WHIM. Healthy donor monocytes were found to highly express GPR35 in response to cryptococcal antigen, TLR4-receptor stimulation with lipopolysaccharide (LPS), and cell stress. GPR35 stimulation of human monocytes with the synthetic agonist, zaprinast, suggested a synergistic effect on IL-1ï¿½, G-CSF, and TNF-ï¿¡ production, and an antagonistic effect on production of anti-inflammatory cytokines. Differentiation of CM patient-derived inducible pluripotent stem cells (iPSC) into macrophages indicates that the GPR35C275X patient allele exhibits reduced LPS-induced cytokine potentiation but does not alter M1/M2 polarization of macrophages. Ongoing experiments utilizing GPR35C275X knock-in and GPR35 knock-out mice currently being generated in our lab will further determine the role of GPR35 in innate immunity and susceptibility to CM.

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Immunology - Infectious Disease

Pre-birth Tissue-Specific Immune Programming

Environmental exposures, especially during the developmental window from conception to early life, can have long-term impacts on individual health. In particular, infections during pregnancy by severe, vertically transmittable pathogens can lead to numerous adverse outcomes for offspring. Unclear is to what extent transient, non-transmittable infections affect the well-being of offspring. To address this question, we used an attenuated strain of the gut pathogen Yersinia Pseudotuberculosis (YptbYopM) as a model. Tracking of YptbYopM after oral infection of timed-pregnant mice showed that infection by this pathogen is highly transient and incapable of vertical transmission to offspring. Strikingly, offspring of transiently infected dams harbored profound alterations in gut immunity, including substantially higher

numbers of T helper 17 cells (Th17) in both the small and large intestine but not at other barrier tissues throughout adulthood. Cross-fostering and serum transfer experiments demonstrated that soluble factors from maternal circulation were sufficient to prenatally imprint offspring intestinal immunity. High-throughput screening revealed this in utero imprinting is mediated by the cytokine interleukin-6 (IL-6). Using mice with a cell-specific deletion in the IL-6 receptor, transient infection-induced maternal IL-6 was found to act directly on fetal intestinal epithelial cells and as a result epigenetically enhance their expression of factors (e.g. serum amyloid A) that are associated with epithelial-immune cells communication. Consequently, offspring delivered from transiently infected dams displayed enhanced Th17-mediated protective immunity against intestinal infections including, Salmonella typhimurium and Citrobacter rodentium. In contrast but in line with heightened activation of epithelial cells, offspring from transiently infected dams were more susceptible to experimentally induced intestinal inflammation. Our results thus propose transient maternal infections can prenatally imprint a defined IL-6-dependent program on intestinal epithelial-immune cell crosstalk that promote protective immunity but in defined settings exacerbates susceptibility to inflammatory disorders.

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Immunology - Infectious Disease
The transcription factor BACH1 promotes tissue damage and host susceptibility in Mycobacterium tuberculosis infection by reducing expression of Gpx4, a major negative regulator of ferroptosis
Ferroptosis is a type of regulated necrotic cell death induced by the accumulation of intracellular free iron and toxic lipid peroxides. We have recently described a role for ferroptosis in Mycobacterium tuberculosis (Mtb)-induced macrophage necrosis based on its biochemical requirements and its blockade both in vitro and in vivo by a lipid peroxidation inhibitor (fer-1). To help validate these findings and further delineate the mechanism involved in this process we have analyzed Mtb-induced cellular necrosis and host resistance in mice genetically deficient in or overexpressing glutathione peroxidase (Gpx4), an essential regulator of ferroptotic cell death. To do so we generated conditional-knockout mice for Gpx4 by using creERT2, CD45cre and LysMcre systems to target whole body, hematopoietic compartment and myeloid lineage cells, respectively. Mice were infected mice with a virulent Mtb strain H37Rv by aerosol inoculation. Disease progression was evaluated by CFU burden, lipid peroxide levels and induction of pulmonary necrosis as assessed by intravenous injection of Sytox dye which stains DNA

in necrotic cells. After aerosol low dose Mtb infection these conditional-knockout mice showed both increased lung necrosis and substantially elevated pulmonary and splenic bacterial burdens. The severe disease outcome observed in Gpx4-deficient mice contributed to early animal death. In the opposite direction, transgenic mice overexpressing Gpx4 when infected at high dose with Mtb, an in vivo model of Mtb-induced tissue necrosis, were found to display decreased bacterial burdens as well as reduced pulmonary necrosis. Interestingly, genetic deletion of BACH1, a transcription factor known to repress antioxidant genes including Gpx4, increased the levels of intracellular glutathione as well as enhanced expression of Gpx4 in Mtb-infected macrophage in vitro and in lungs of Mtb-infected mice. Moreover, Bach1-deficient mice infected at high dose with Mtb displayed a significant reduction in bacterial loads as well as lung necrosis along with lowered levels of lipid peroxides. In addition, Bach1-/- mice showed increased resistance to high dose Mtb infection as well as enhanced survival. Together, these findings provide genetic evidence further delineating the role of ferroptosis as a mechanism of host cell death and tissue necrosis in Mtb infection and implicate both Gpx4 and Bach1 as potential targets for host-direct therapy of TB.

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Immunology - Innate and Cell-mediated Host Defenses
TLR5 protects against pulmonary fibrosis by preventing dysbiosis
Idiopathic pulmonary fibrosis (IPF) is a fatal and incurable lung disease with a mean survival of 3-5 years from diagnosis. Recently, host defense genes have been implicated in the pathogenesis of IPF; increased lung bacterial burden has been detected in IPF patients and has been associated with disease progression and severity. Toll-like receptor 5 (TLR5) is an innate pattern-recognition receptor for bacterial flagellin found on leukocytes and various epithelial cells, including intestinal and lung epithelia. Up to 10% of the population carry a dominant-negative minor allele, a single nucleotide polymorphism (rs5744168) for the TLR5 gene, that abolishes or severely impairs its signaling. In the present study, we have identified rs5744168 minor allele carriers to be susceptible to idiopathic IPF based on a cohort of 1133 patients and 2903 control subjects. Furthermore, we have demonstrated that lung-healthy rs5744168 minor allele carriers are susceptible to lung injury in response to ozone exposure and smoking, suggesting that TLR5 may protect the lung against injuries, an upstream event preceding the development of pulmonary fibrosis. We found Tlr5-deficient mice to be susceptible to lung fibrosis compared to control littermates after bleomycin lung injury. Excitingly, we found that a Tlr5 agonist drug, which is in clinical trials for human use for the treatment of liver cancer, protected mice against pulmonary fibrosis. Mechanistically, RNA sequencing revealed the upregulation of antimicrobial genes in Tlr5 agonist-treated mice, suggesting that TLR5 may protect against microbiota-induced lung injury. Indeed, Tlr5-deficient mice displayed increased lung dysbiosis and injury after bleomycin treatment compared to control littermates. Antibiotic treatment rescued both lung injury and fibrosis in Tlr5-deficient mice, suggesting that the protective effect of TLR5 is dependent on the presence of microbiota. In conclusion, our present study proposes several novel concepts: 1) TLR5 is the first human genetic risk factor for IPF that is upstream of microbiota changes; 2) TLR5 activation may not only treat established disease, but also prevent the progression of pre-clinical fibrotic lung disease; and 3) TLR5 agonism

represents a novel therapeutic approach to treat IPF through the modulation of the lung microbiota and may have wider implications for a variety of diseases associated with dysbiosis and infections.

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Immunology - Innate and Cell-mediated Host Defenses
Neurological Decline During Tauopathy is Associated with Microglial Dysfunction and CD8+ T cell Engagement
Alzheimerâ€™s disease (AD) and other tauopathies are some of the most common forms of dementia and affect more than 50 million people worldwide. Commercial therapeutics treat symptoms but do little to mitigate disease pathology and progression because the underlying cause of disease pathogenesis remains elusive. Major risk factors for the development of AD include mutations in several genes such as ApoE and TREM2. Importantly, these genes are linked to the homeostasis and function of microglia, which are a central nervous system (CNS) resident macrophage population responsible for surveilling the parenchyma and clearing cellular waste. We therefore sought novel insights into how these cells shape development of a primary tauopathy. We were also interested more broadly in the question of whether peripheral immune cells respond to a tauopathy and if so whether these cells are influenced by microglia. To study a primary tauopathy, we used a well-established model in which mice express the 383 amino acid isoform of human tau with the P301S mutation under control of the murine Thy1 promoter. In humans the P301S mutation is associated with early onset frontotemporal dementia, and homozygous mice expressing this mutant Tau protein develop a severe neurodegenerative disease at 5-6 months of age. We used high-dimensional flow cytometry to profile the CNS immune landscape in P301S mice during the onset and development of tauopathy. These studies uncovered a subset of disease-associated microglia and infiltrating CD8+ T cells that emerged as P301S mice developed symptoms. Interestingly, immunohistochemical studies revealed that the CD8+ T cells appeared to directly engage and form clusters around activated microglia in the spinal cord gray matter, suggesting local antigen presentation. A subset of these CD8+ T cells also established CNS residency, as evidenced by their expression of CD103 (a marker for tissue resident memory T cells). To evaluate the role of microglia in T cell interactions and disease progression, we depleted these cells using a CSF-1R inhibitor. This treatment reduced CD8 T cell clusters in the CNS but also significantly enhanced tau pathology and neuronal dysfunction, leading to increased mortality. We propose that healthy microglia play a crucial role in limiting the spread of pathogenic tau. This function appears to deteriorate with time, triggering local antigen presentation, engagement by CD8+ T cells and significant neurological decline.

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Immunology - Innate and Cell-mediated Host Defenses

Identification of a 9kb homozygous deletion in SAMHD1 in an Ashkenazi Jewish patient with Aicardiâ€“Goutières syndrome

We present a 33-year-old man who was investigated for a long standing illness that included neurological impairments and systemic inflammation. Lesional skin biopsy showed evidence for panniculitis with areas of lipoatrophy. He developed progressive severe contractures and is wheelchair-bound. Brain MRI revealed bilateral basal ganglia lacunar infarcts with peri-ventricular white matter changes. Parents are of Ashkenazi Jewish (AJ) ancestry and there was no family history of similar illness or consanguinity. Collectivly, his clinical findings indicated a disorder in the spectrum of primary interferonopathies including Aicardi-Goutières syndrome (AGS). AGS manifests as early-onset encephalopathy and often results in severe intellectual and physical disability and is caused by mutations in genes encoding proteins that are critical for nucleic acid metabolism and sensing. Exom sequencing was performed but no pathogenic SNV variants were identified in AGS-associated genes and no damaging variants were found in other promising candidate genes. A variety of copy number variation (CNV) calling algorithms (XHMM, CoNIFER, and CNVnator) were applied but did not identify any pathogenic deletions or duplications. To increase read coverage of relevant coding regions, targeted NGS of 230 genes associated with autoinflammtory diseases was performed on the patient and his parents. Downstream analysis using â€˜Atlas-CNVâ€™ revealed a homozygous deletion encompassing exon 1 of SAMHD1 in the patient. PCR amplification using primers flanking the predicted deletion generate a 500bp in the patient and his carrier parents, but not in healthy controls. Subsequent Sanger sequencing of the amplification product confirms a 9kb homozygous deletion in the patient. Quantitative RT-PCR analysis demonstrated a complete loss of SAMHD1 expression in the patient and a 50% reduction in both parents. The identified 9kb deletion is found in gnomAD SV v2.1 with a minor allele frequency of 9.2-5 and previous studies in the AJ population estimated the frequency of this deletion at approx. 0.8%. In conclusion, our study shows that phycisians and genetic testing providers should be aware of this rare SAMHD1 deletion in patients presenting with neurological features and inflammation. This deletion could be easily missed if conventional Sanger sequencing or NGS based methods are applied. Identifying this mutation early in life can point to an appropriate treatment with targeted biological therapy.

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Immunology - Lymphocyte Development and Activation
Regulation of T-bet induction during innate and adaptive lymphocyte development
Type 1 T helper (Th1) cells, group 1 innate lymphoid cells (ILC1s) and conventional natural killer (cNK) cells play critical roles in type 1 immune responses. However, while Th1 cell differentiation occurs upon the engagement of T cell receptor- and co-stimulation-mediated signaling in a specific cytokine environment, cNK cells and ILC1s are pre-developed and ready to immediately respond to pathogen invasion. Although the master transcription factor T-bet (encoded by Tbx21 gene) is responsible for all those cell subset development, the differences and similarities of T-bet induction during Th1, ILC1 and cNK cell development is unclear. In this study, by analyzing epigenetic imprints on the enhancers of the Tbx21 gene locus, and through deleting or mutating cell subset-specific enhancer(s), we compared the mechanisms of T-bet induction in above cell subsets. We found that the STAT binding site in the Th1-specific Tbx21 enhancer is essential for T-bet expression during Th1 cell differentiation both in vitro and upon Toxoplasma gondii infection, but dispensable for the development of cNK cells and ILC1s. On the other hand, deleting the core-region of the cNK cell-specific Tbx21 enhancer only diminished the T-bet expression in cNK cells, and resulted in an immature phenotype of those cells, but without affecting ILC1 and Th1 cell development. Unlike IL-12-STAT4-mediated T-bet expression in Th1 cells, we found that IL-18-RUNX3 mainly controlled the induction of T-bet in cNK cells through the specific enhancer. While the mechanism of T-bet induction in ILC1s is still under study, our current results demonstrated that innate and adaptive lymphocytes may utilize distinct mechanisms to induce T-bet expression for their development.

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Immunology - Lymphocyte Development and Activation
Dysregulated TGF-Î² Signaling Leads to Cell-Intrinsic Changes in T cell Development that Promote Allergic Inflammation
Variants in genes in the Transforming Growth Factor Beta (TGFb) signaling pathway are associated with allergic diseases, suggesting that this cytokine may be a central player in the allergic disease. The role of TGFb in preserving T cell mediated self-tolerance is well-appreciated, but how disruption in this pathway promotes allergic inflammation is not clear. TGFb signals through its heterotetrametric receptor TGFÎ²RI and II, leading to phosphorylation and subsequent nuclear translocation of Smad proteins 2 and 3. We previously demonstrated that patients with Loeys-Dietz Syndrome (LDS), an autosomal dominant disorder caused by mutations in TGFBR1 and TGFBR2, are highly predisposed to food allergy, asthma, allergic rhinitis and eczema. Both LDS patients and mice harboring a knock-in allele (Tgfbr1mut) of an LDS mutation known to cause severe disease in humans exhibit increased levels of total and food-specific IgE, consistent with their allergic tendencies. These serologic changes were associated with increased numbers of T follicular helper (Tfh) cells, which promote memory B cell and plasma cell formation, and decreased T follicular regulatory (Tfr) cells, which suppress humoral immune responses, in LDS patients and mice. Using a mixed bone marrow chimera model, we demonstrated that these alterations in T cell development are cell-intrinsic. Furthermore, OVA specific OTII cells harboring the Tgfbr1mut were more likely to accumulate in the peyerâ€™s patch, less likely to upregulate Foxp3, and more likely to differentiate into Tfh cells in response to orally ingested OVA. Tgfbr1mut OTII cells also produced more IL-4 and less IL-10 in response to OVA. These findings suggest that TGF-Î² signaling plays an important role in T cell development and function that, when disrupted, promotes allergic inflammation.

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Single cell RNAseq analysis reveals profound abnormalities in the distribution and diversity of thymic epithelial cells in Rag1 mutant mice
The thymus contains a heterogeneous population of stromal cells which orchestrate T cell development and selection. Recent works highlighted the complexity of thymic stromal cells, and medullary thymic epithelial cells (mTECs) in particular. However, the developmental origin, hierarchy, and function of these subpopulations remain ill-defined. Moreover, the characterization of cortical TECs (cTECs) has been largely restricted to the adult thymus. Impaired lymphostromal cross-talk in the thymus of subjects with combined immunodeficiency is associated with abnormalities of thymic architecture and TEC maturation. Here, we compared TEC distribution and gene expression in wild-type (WT) and in mice carrying Rag1 hypomorphic mutations observed in patients with immune deficiency and immune dysregulation. Single cell RNA-seq analysis of TECs isolated from adult Rag1 mutant mice revealed an excess of cTECs, which segregated in different clusters, confirming our flow cytometry and

histopathology results. The mTEC compartment, albeit reduced, showed a similar distribution of previously described subsets (mTEC I-IV), suggesting perturbation of mTEC development rather than mTEC differentiation into functional subsets. To address whether such abnormalities of cTEC and mTEC abundance and subset distribution in Rag1 mutant mice may reflect defects in TEC development, we extended scRNA-seq analysis to TECs from WT mice of neonatal age. A similar pattern was observed in TECs of adult Rag1 mutant mice and WT mice of neonatal age, indicating altered TEC development when Rag1 activity is perturbed. This defect correlates with a decreased number of double-positive thymocytes, suggesting that impaired lymphostromal cross-talk in the thymus of Rag1 mutant mice (and patients with similar defects) is associated with abnormalities of TEC composition which may contribute to altered immune tolerance that is often observed in these conditions. We have further refined the complexity of TECs and shown that impaired development of T cells in combined immune deficiency has profound effects on the composition and maturation of both cTECs and mTECs.

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A novel and human-specific CD46-KLF/SP interaction mediates gene expression required for successful Th1 induction
Th1 cells are critical for the host defense against intracellular pathogens and viruses. Signals mediated by autocrine activation of the human-specific complement receptor CD46 during T cell receptor (TCR) stimulation are vital to Th1 induction in human CD4+ T cells, but how exactly CD46 at a molecular level mediates this role is currently undefined. CD46 is expressed in different isoforms on human CD4+ T cells that can bear either one of two distinct cytoplasmic domains: CYT-1 or CYT-2. Upon TCR-driven autocrine CD46 stimulation, the intracellular domains of CD46 are cleaved by gamma-secretase and translocate to the nucleus where particularly CYT-1 drives the expression of genes coding for nutrient-influx-channels (e.g. glucose transporter GLUT1 and the amino acid transporter LAT1) and mTORC1 activity that mediate metabolic adaptations fundamentally required for cell growth, expansion, and Th1 effector functions. The lack of a DNA binding domain in CD46-CYT-1 precludes it from acting directly as a transcription factor (TF) and we hence hypothesized that CYT-1 regulates gene expression via direct interaction with specific TF activator and/or repressor complexes. Indeed, CUT&RUN experiments performed using our novel antibody raised against cleaved CYT-1 identified key members of the KLF/SP TFs gene family as potential interacting partners of CD46-CYT-1. Subsequent ELISA and Microscale Thermophoresis (MST) experiments with recombinantly-produced purified proteins and synthetic CYT-1 or scrambled peptides confirmed specific, strong, dose-dependent CYT-1/KLF/SP TFs interactions. Functional studies revealed that CYT-1 fostered KLF/SP TFs binding to appropriate DNA motifs. Genome-wide comparison of the KLF/SP TFs and CYT1 bound genes in T cells revealed their enrichment in crucial basic cell-physiological pathways. Moreover, the CUT&RUN data in conjunction with ATAC-seq analyses indicated that this novel CYT-1/KLF/SP axis may control general chromatin remodeling â€“ a notion we are currently exploring. These data define a novel and critical human-specific pathway of gene regulation and further underpin the vital role of intracellular/autocrine complement in the regulation of normal cellular activity.

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Immunology - Tumor Immunology
Identification of novel ligands that alter the function of type II NKT cells
Natural killer T (NKT) cells are distinct innate lymphocytes which recognize lipid antigens in the context of the major histocompatibility complex-like molecule CD1d. When activated, these cells can rapidly produce a variety of cytokines and modulate innate and adaptive immune responses. There are two main subsets of NKT cells, termed type I and type II NKT cells. Type I NKT cells are characterized primarily on the basis of their invariant TCRÎ± expression and reactivity to the glycolipid Î±-galactosyl ceramide (Î±GalCer), whereas type II NKT cells express a different and more diverse TCR repertoire than type I NKT cells. Currently, the most widely studied antigen for type II NKT cells is sulfatide. Sulfatide-reactive type II NKT cells are reported to have immunosuppressive function in autoimmune diseases and cancer. We previously showed a role for sulfatide-reactive type II NKT cells in suppressing tumor immunosurveillance in a murine model of lung metastasis. The activation of type II NKT cells in this model by the injection of sulfatide increased the development of lung metastasis. Sulfatide (C24:1) is composed of a galactose head carrying a sulfate group and beta-linked to a ceramide portion formed by a 24-carbon fatty acid chain with 1 double bond and a sphingosine chain with 1 double bond. The structure of the ceramide influences the interaction of the TCR with the lipid-CD1d complex and determines the reactivity to the lipid. We synthesized diverse sulfatide-analogues varying in the number of double bonds and hydroxyl groups in the ceramide. In vitro, analogues having a phytosphingosine sphingoid base activated both type II and type I NKT cell hybridomas. In contrast, the analogue C24:2 with two double bonds and a sphingosine base activated only the type II NKT cell hybridoma. In vivo, the C24:2 analogue significantly reduced the development of lung metastasis, showing distinct and opposite effects from sulfatide. Splenocytes from wild type mice stimulated with C24:2 produced larger amounts of both Th1 and Th2 cytokines than C24:1, and the IFNg/IL-13 ratio was much higher. In contrast, IFNg knockout mice were not protected by C24:2, suggesting that C24:2 promotes tumor immunity by an IFNg-dependent mechanism. The identification of new ligands inducing altered functional activity of type II NKT cells could be of great interest for developing new anti-tumor immunotherapies.

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Microbiota regulates intratumoral dendritic cells via a type I interferon - natural killer cell axis
Microbiota can affect tumor development and response to therapy, however, the underlying mechanisms are unclear. Tumor infiltrating dendritic cells (tiDCs) are a rare population of antigen presenting cells needed for anti-tumor immunity. Here, we addressed the role of microbiota in regulating tiDCs. As a reductionist approach, we used mice with or without microbiota [germ-free (GF)

or given broad-spectrum antibiotics] and found a decrease in tiDCs in mice without microbiota consistent across 5 syngeneic tumor models. Amongst several proinflammatory cytokines, we found a reduction of type I IFNs in GF mice at both the protein and mRNA level. Furthermore, type I IFN receptor knock-out mice displayed impaired response to therapy and reduced tiDCs, similar to mice lacking microbiota. Type I IFNs are well-known regulators of NK cells, which have been recently shown to recruit cDCs into the tumor via production of the chemokine XCL1. Remarkably, we found reduced NK cell frequencies in each tumor model for which tiDCs were decreased in the absence of microbiota. Through scRNAseq and transcript-specific RNA flow cytometry, we found that NK cells are the major source of Xcl1 in the tumor microenvironment (TME) and that GF NK cells produce lower amounts and are overall less activated. Administration of microbiota derived c-di-amp, a known stimulator of type I IFN, to GF mice increased intratumoral levels of Ifna, Ifnb1, and Xcl1 resulting in the rescue of DC infiltration and control of tumor growth. To determine the translational relevance of our findings, we analyzed whole tumor RNAseq data from melanoma patients treated with checkpoint inhibitors. We found that the signatures decreased in mice devoid of microbiota were similarly reduced in non-responder (NR) patient tumors: DC, NK cells, XCL1, and type I IFN. We further confirmed the role of microbiota via fecal microbiota transplants (FMTs) giving feces from responder (R) and NR patients to GF mice prior to tumor implantation. We showed that mice receiving R FMT had more tiDCs and NK cells compared to mice given NR feces. Overall, our data provides a novel mechanism by which microbiota-derived c-di-amp can alter the production of type I IFN in the TME leading to NK cell activation, increased DC recruitment, and better anti-tumor response. Through leveraging our findings of microbial influence, we hope to extend our studies to use targeted approaches to manipulate microbiota and improve response to cancer therapy.

Maja Buszko
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Immunology - Tumor Immunology
Characterization of novel CD25 antibodies that deplete human regulatory T cells in humanized mouse model.
Regulatory T cells (Tregs) are key players in the suppression of immune responses and in the maintenance of immune homeostasis. Due to their central role in pathogenesis of multiple conditions, Tregs are an attractive target for immunomodulatory drug development. Tregs exhibit an activated phenotype and share the expression of many activation antigens with activated conventional T cells (Tconv). Our goal in this project was to generate and characterize monoclonal antibodies (mAbs) that would specifically react with human Tregs. mAbs were raised in mice by immunization with purified, in vitro expanded human Tregs. In contrast to widely available anti-CD25 mAbs that equally bound both activated Tregs and activated Tconv, we identified several mAbs that bound CD25 on expanded Tregs, but failed to bind to CD25 on expanded Tconv. To determine whether a similar selective binding could be demonstrated in vivo, we used the xeno-GVHD model in which NOD-SCID-gc-/- (NSG) mice are engrafted with human peripheral blood mononuclear cells (PBMCs). Several of the mAbs bound to CD25 expressed on Tregs, but not to CD25 expressed on activated Tconv. To test the effect of in vivo administration of the Treg specific mAbs, one of the clones, 2B010, was selected and injected

intravenously (i.v) 2 weeks after PBMCs engraftment. Injection of 2B010 resulted in markedly reduced Tregs frequencies and numbers, much less depletion of CD25+ activated Tconv, and no reduction of CD25+CD8+ T cells. Further studies will address the effect of these mAbs at reversing the Treg-mediated protection of xeno-GVHD and enhancing tumor immunity in reconstituted NSG recipients bearing transplanted tumors.

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Systematic Analysis of Symmetry in Membrane Proteins
Membrane proteins are encoded by around one third of a given genome and play key roles in transmission of information and chemicals such as neurotransmitters into the cell. Available membrane protein structures have revealed an abundance of symmetry and pseudo-symmetry, which arose not only by the formation of multi-subunit assemblies, but also by repetition of internal structural elements. In many cases, these symmetry relationships play a crucial role in defining the functional properties of the proteins. For example, to allow substrates to diffuse down their concentration gradient, many ion channels create pores in the membrane from units arranged around a central axis. In this context, a systematic study of symmetry should provide a framework for a broader understanding of the mechanistic principles and evolutionary development of membrane proteins. However, available symmetry detection methods have not been tested systematically on this class of proteins because of the lack of an appropriate benchmark set. Therefore, we collected membrane protein structures with

unique architectures and manually curated their symmetries and pseudo-symmetries to create the MemSTATS dataset. Using MemSTATS, we compared the performance of four widely used symmetry detection algorithms and pinpointed areas for improvement. To address the identified shortcomings, we then developed a robust symmetry detection methodology called MSSD, which takes into consideration the restrictions that the lipid bilayer places on protein structures. MSSD detected symmetries with significantly higher accuracy and lower false positive rate compared to any other tested method. Consequently, we used MSSD to analyze all available membrane protein structures and presented the resultant symmetries in a freely available online database called EncoMPASS (encompass.ninds.nih.gov). Using EncoMPASS, we then proceeded to quantify both the extent and diversity of symmetry relationships in known structures of membrane proteins. Ongoing work is addressing diverse questions relating to evolution by analyzing symmetries across species, to the driving forces for oligomerization and fusion by investigating symmetric interfaces, and to function and mechanism by using symmetry to identify and verify ion binding sites.

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Predicting risk of late age-related macular degeneration using deep learning
Age-related macular degeneration (AMD) is the leading cause of legal blindness in all developed countries. Based on clinical features, the disease can be classified into early, intermediate, and late stages. In some people, AMD advances to the late stage slowly; in others, the disease progresses faster and may quickly lead to a loss of vision in one or both eyes. There is, therefore, a critical need to make accurate time-based predictions of progression to late AMD. In this project, I propose a deep learning framework to automatically identify AMD severity from color fundus images of both eyes. Different from previous methods, our algorithm takes advantage of deep learning and extensive domain knowledge in clinical ophthalmology. It also mimics the human grading process by first detecting individual risk factors in each eye and then combining values from both eyes to assign an AMD score to each patient. Thus, our model closely matches the clinical decision-making process, which allows an ophthalmologist to inspect and visualize an interpretable result. Evaluation on a 12-year multi-center prospective cohort study of the clinical course shows that our model performed better on the ability to predict AMD severity on a patient-level than retinal specialists (accuracy 0.67 vs 0.60). In addition, our model can be combined with the survival analysis to predict the risk of progression to late stage of AMD over a wide time interval (1-12 years). When trained on â€œbig dataâ€ from 82 US retinal specialty clinics, which represents the largest longitudinal AMD dataset ever used in deep learning, and validated against an independent test dataset, the model achieved high prognostic accuracy (five-year C-statistic 86.4) that substantially exceeded that of retinal specialists using the two existing clinical standards. These results highlight the potential utility of using deep learning methodologies to assist and enhance clinical decision-making in patients with AMD. Taken together, our proposed framework helps clinicians optimize treatments, lifestyle interventions, and follow-up/reimaging regimens. It also sheds the light on the implementation of screening and early detection programs in the diagnosis, prevention, and cure of AMD.

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Non-genetic cell-to-cell heterogeneity maps the variability of immune responses against tumors
Significant progress has been made in harnessing the immune system to attack tumor cells. Long-term cancer remission has been achieved for some patients by therapies involving the use of tumor infiltrating lymphocytes, or antibodies blocking proteins that downregulate the immune response. Nevertheless, the majority of cancer patients (usually ~70%) will only have partial or no response to these therapies. Most studies trying to understand the variability in the responses to these therapies have focused on differences in the genetic composition of the patient or their tumors, and on differences in environmental factors. Here, we explore a third possible contributing factor, namely that cell-to-cell variability naturally observed in genetically identical cells could account for the stochasticity in the final outcome of the aforementioned cancer immunotherapies. As a starting point, we mixed B16 melanoma cells expressing ovalbumin peptide (OVA) and OVA-specific CD8+ T cells from OT-1 mice at different ratios, having dozens of technical replicates for each ratio. We used flow cytometry to measure the concentration of different cytokines, the expression levels of cell surface markers, and the number of cells at different timepoints. We found that there was a range of conditions that lead to high variability between replicates, with T-cell derived IFN-g in the supernatant varying more than three orders of magnitude and correlating with the ability of T cells to control B16 tumor cell growth. In the process of mapping the different potential sources for this variability we found that the response of tumor cells to IFN-g is highly heterogenous. Additional results suggest that this cell-to-cell heterogeneity, coupled to a positive feedback loop between IFN-g and antigen presentation by tumor cells, can lead to the drastic replicate-to-replicate variability observed in our experiments. In summary, we have demonstrated how digital-ness in immunotherapeutic outcomes can be traced to the non-genetic cell-to-cell heterogeneity of T cell activation and tumor response to inflammation. We will show how quantitative modeling of these feedback loops highlight key dynamic hallmarks of a productive immune response against tumors. We will also discuss how we further test our quantitative insights with microfluidic models and in vivo mouse models, towards delineating key dynamic features that switch cancer treatment towards better outcomes.

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Small RNA-mediated regulation of carbon and nitrogen metabolism in Escherichia coli
The ability to rapidly respond to changes in their environments is critical to the survival of bacteria. One mechanism of regulating bacterial gene expression during stress conditions is through small RNAs (sRNAs). These are 50 to 300 nucleotide transcripts that function by base pairing to their mRNA targets to alter translation or stability. sRNAs play a role in regulating gene expression during a wide range of stresses including iron starvation, oxidative stress, and membrane stress. We recently discovered a new sRNA, GlnZ, which is derived from the 3â€™ UTR of the Escherichia coli glutamine synthetase gene glnA. Examination of GlnZ levels in different nitrogen sources by northern analysis showed that GlnZ levels are highest during stationary phase and upon nitrogen starvation. These experiments revealed a loss of GlnZ production in a strain lacking ntrC, the nitrogen stress response regulator, indicating that NtrC transcriptionally regulates GlnZ levels. We also observed decreased GlnZ levels in cells grown in glutamine as the sole nitrogen source. This regulation is post-transcriptional since it is still observed when GlnZ is expressed from a heterologous promoter. Assays of RNase mutant strains showed that the down-regulation is dependent on RNase III, as GlnZ levels remain high in glutamine in cells lacking RNase III. Interestingly, this glutamine-dependent RNase III degradation is also seen with other sRNAs that are not involved in nitrogen metabolism, suggesting a more general effect of glutamine on RNase III activity. A global RNA-seq approach termed RNA interaction by ligation and sequencing (RIL-seq) identified aceE, sucA, and glnP, which encode proteins involved in glycolysis, the TCA cycle, and glutamine transport respectively, as potential base pairing targets of the GlnZ sRNA. Western analysis and lacZ fusions revealed that GlnZ overexpression leads to decreased levels of AceE, SucA, and GlnP. Additionally, deletion of glnZ led to a growth defect for cells recovering from nitrogen starvation, demonstrating a clear role for GlnZ in responding to nitrogen deprivation. Together, these data reveal a role for GlnZ in regulating carbon and nitrogen metabolism in response to changes in nitrogen availability.

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Microbiology and Antimicrobials
PE/PPE mediate nutrient transport across the outer membrane of Mycobacterium tuberculosis
Mycobacterium tuberculosis (Mtb) infection remains a leading cause of death worldwide, resulting in 1.5 million deaths in 2018. Mtb has a highly impermeable outer-membrane composed of a series of unusual lipids including phthiocerol dimycocerosate (PDIM). Proteins that may serve as porins to transport nutrients across the outer-membrane in Mtb have long been sought but with very limited success. Approximately 10 % of the Mtb genome is made up of proline-glutamate (pe) and proline-proline-glutamate (ppe) families of genes, but their functions are poorly characterized. In this study, we discovered that a member of PPE family protein, PPE51, mediated nutrient transport across the outer-membrane of Mtb. The ppe51 deletion mutant was unable to grow on glucose or glycerol, also showing a strong defect in the uptake of 14C-glucose and 14C-glycerol. Both growth and uptake defects of the mutant were complemented by expression of wild-type ppe51 or a porin gene mspA from Mycobacterium smegmatis (Msmeg). Interestingly, loss of the outer-membrane lipid PDIM, which compromised the impermeability of outer-membrane, also rescue the growth of mutant. The outer-membrane localization of PPE51 was proved by a cell surface protein biotinylation assay and flow cytometry analyses which is based on surface detection of proteins in whole cells using antibodies. By screening the transposon library and chemical cross-linking, we identified the PPE51 partner protein, PE19. And the deletion mutant of pe19 displayed same phenotypes with the âˆ†ppe51 mutant. These results indicated that PE19/PPE51 serve as porin to mediate glucose and glycerol across the outer-membrane. We next asked whether this porin-like function was specific for the PE19/PPE51 pair or if other PE/PPE pairs had similar functions. We further identified PE20/PPE31 and PE32/PPE65 mediating magnesium and phosphate across the outer-membrane respectively. The growth defects of mutants in corresponding substrates limiting media are also PDIM-dependent. All slow-growing mycobacteria, including Mtb, have an expanded repertoire of pe/ppe, but lack any detectable homolog of the porin

seen in the fast growing mycobacteria (like Msmeg) that have few pe/ppe pairs. PDIM, like the PE/PPE proteins, is also restricted in distribution to the slow-growing mycobacteria. Our results indicated that PDIM determined the low permeability of outer-membrane in slow-growing mycobacteria, and the PE/PPE proteins act as solute-specific channels.

Tiffany Zarrella
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Microbiology and Antimicrobials
Investigating the polymicrobial interaction between Pseudomonas aeruginosa and Staphylococcus aureus
In nature, microorganisms are typically found in multispecies communities, however most species are studied in isolation. In cystic fibrosis, a disease which is plagued by persistent polymicrobial respiratory infections, the interactions of the bacterial pathogens Pseudomonas aeruginosa and Staphylococcus aureus affect disease severity. Elucidating the molecular bases for these interactions could identify potential targets to modulate the multispecies infectious community for treatment. It is common for bacteria to sense other microbes and respond antagonistically within complex communities. Since P. aeruginosa frequently resides in multispecies environments and has the capacity to secrete a plethora of antimicrobials, we hypothesized that P. aeruginosa senses and responds to the presence of S. aureus. We found that P. aeruginosa senses molecules released by S. aureus, and responds by producing antimicrobials that are active against S. aureus, independent of the previously described bacterial cell wall sensing by P. aeruginosa. To identify the specific S. aureus exoproducts being sensed, we designed an array of promoter-reporter constructs based on transcriptional changes in P. aeruginosa after S. aureus exposure. Of the promoters tested, seven had significantly higher expression of the fluorescent reporter mScarlet upon exposure to S. aureus secreted products, and showed dose-dependent responses. We then utilized these promoter-reporter strains to screen an arrayed S. aureus transposon mutant library and identify mutants that were deficient in inducing the reporters. Interestingly, supernatant from S. aureus mutants lacking genes required for the biosynthesis or export of the metallophore staphylopine (StP) failed to induce reporter expression in one class of promoters. StP binds Zn, as well as other heavy metals, and Zn addition repressed this promoter, suggesting that the metal-binding activity of StP induces reporter expression. Induction of additional promoter-reporters was not affected by the StP mutants or [Zn], which suggests that P. aeruginosa senses S. aureus via additional pathways as well, and we are currently working to identify these. Our findings thus yield new insight into interbacterial communication and the underlying molecules. Future work will focus on the molecular mechanisms governing antimicrobial production pathways in P. aeruginosa that could be novel targets for the development of therapeutics for chronic bacterial infections.

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Molecular Biology - Prokaryotic and Eukaryotic
Overlapping and competing roles of the two RNA-binding proteins, ProQ and Hfq
Base-pairing regulatory RNAs modulate gene expression at the post-transcriptional level where they affect the translation and/or stability of their target RNAs. In bacteria, the major class of base-pairing regulatory RNAs are small RNAs (sRNAs), which affect every aspect of bacterial physiology, including pathogenicity. In many cases, the base-pairing between two RNAs is facilitated by an RNA-binding protein (RBP) that serves as an RNA chaperone, such as the conserved Hfq protein in bacteria. However, recent studies have shown another family of RBPs, the FinO-domain proteins, also bind sRNAs. To examine the global contribution of the FinO-domain ProQ protein in Escherichia coli, we took advantage of a high throughput sequencing methodology, RIL-seq (RNA Interaction by Ligation and sequencing), which enables transcriptome-wide identification of RNA pairs bound to an RBP. We used RIL-seq to examine the RNA populations and RNA-RNA interactomes for both Hfq and ProQ. First, Hfq and ProQ were found to bind different sets of RNAs, with Hfq binding mRNAs and sRNAs equally, and ProQ primarily binding mRNAs. This hints ProQ and Hfq might have different roles. Surprisingly, a comparison of the RNA-RNA interactomes revealed that a significant fraction of the RNA-RNA pairs captured on ProQ were also present on Hfq, suggesting the two proteins may have overlapping and/or competing roles. One such overlapping pair is between RybB, a previously-characterized sRNA known to act under cell envelope stress, and a novel sRNA RbsZ. We found that RbsZ regulates RybB, leading to RybB degradation. This in turn disrupts the ability of RybB to regulate its own targets. This regulation was mediated by Hfq and blocked by ProQ. Examination of changes in the E. coli transcriptome upon pulse overexpression of ProQ showed that the protective effect of ProQ was true for many RNAs. This suggested that E. coli ProQ has a global role in protecting or stabilizing RNA transcripts. Finally, ProQ was reported to be critical in osmoprotection and ProQ RIL-seq performed under high salt conditions revealed a shift in some of the top RNA-RNA pairs found on ProQ. This implies that the interplay between Hfq and ProQ might differ under osmotic stress. This study illustrates the need to further understand the complex network of RBPs regulating overlapping or unique subsets of RNAs, which are critical for bacterial prosperity in a host or in the environment in rapidly changing environmental conditions.

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Molecular Biology - Prokaryotic and Eukaryotic
An E3 ubiquitin ligase as a novel target for anti-malaria therapy
Malaria is one of the deadliest infectious diseases worldwide. Despite more than a century of efforts to eradicate malaria, the disease remains a major and growing threat to the public health due to multi drug resistance (MDR). Emergence of MDR signifying to discover novel targets/drugs to combat malaria parasites and one such targets which is currently most explored is â€œparasiteâ€™s ubiquitin systemâ€. Protein ubiquitylation is an important post-translational regulation, which has been shown to be necessary for life cycle progression and survival of human malaria parasites- Plasmodium falciparum (Pf). E3 ubiquitin ligases (E3s), a key player in protein ubiquitination and proteasomal

degradation, are novel potential drug targets. E3s are very diverse and are major determinants that provide specificity for the substrate/protein recognition to be ubiquitinated. The hypothesis behind this study is that targeting the E3 ligase functions of ubiquitin system would selectively kill the malaria parasite. We generated transgenic P. falciparum parasite lines with specific E3 ligases tagged with pSli-HA-glmS sequence that enables us to knockdown (KD) the expression of E3 ubiquitin ligase (E3-3). Our drug assay results evidently indicated that proper expression of this protein (E3-3) is essential for the growth of parasites and KD of this protein causes parasites vulnerable to antimalarial drugs. KD of an E3 ligase (E3-3) reduces ubiquitination of many parasite proteins. Changes in specific protein expression after E3-3 KD were identified using 2D gel electrophoresis and tandem mass spectrometry (MS-MS), many of the proteins match the known protein targets of artemisinin- a currently most potent drug to fight malaria. E3-3 KD renders parasites more sensitive to artemisinin derivatives and changes protein expression of P. falciparum multidrug resistant protein 1 (PfMDR1) and P. falciparum chloroquine resistance transporter (PfCRT). Therefore, the P. falciparum E3-3 ligase could play a significant role in parasite modulating the expression of key drug transporters and/or targets. Thus, E3 ligase could be potential drug target for treating malaria infections.

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Molecular Biology - Prokaryotic and Eukaryotic
The Role of HEAT Domain Mutations in mTOR Signaling, Complex Formation, and Cancer
Background: Mechanistic target of Rapamycin (mTOR) is a critical nutrient sensor and regulator of cell metabolism. Previously, we demonstrated that a single point mutation in the HEAT domain (R628C) of mTOR resulted in plasmacytoma formation. Bioinformatic analyses revealed that cancers preferentially select for missense mutations where R mutates to C, H, or W. While kinase activating mutations on mTOR have been discovered, little is known about HEAT domain mutations. Elucidating the HEAT domain mutations of mTOR, where key partners are suggested to bind, is critical to our understanding of mTOR complex formation and signaling. mTOR forms three major complexes, mTOR complex 1 (mTORC1, Raptor/S6K1), mTORC2 (Rictor/Akt), and mTORC3 (mEAK-7/S6K2). Thus, we hypothesize that mutations spanning the HEAT domains of mTOR result in differential mTOR complex formation.
Methods: To address effects of R to C mutations on mTOR signaling, we generated transgenic mice harboring the R628C mTOR mutation, and seventeen plasmids containing cancer-associated mTOR R ïƒ C mutations: 173, 281, 311, 398, 526, 553, 604, 619, 628, 672, 731, 755, 881, 886, 1080, 1090, 1301 (from cancer patients via the TCGA cBioPortal). We cultured E14.5d MEFs from male and female (WT and KI R628C, littermate paired) mice. Second, we transfected H1299 human cell lines with FLAG-tagged mTOR WT or mutant constructs. Cells were starved in DMEM lacking amino acids (DMEM-AAs), and reintroduced DMEM+AAs and 10% FBS for 1 hour, with or without rapamycin (mTORC1 inhibitor).
Results: We determined that mutations in the HEAT domains result in aberrant mTOR complex formation and have identified specific regions within the HEAT domains which are responsible for forming different complexes. R628C KI MEF cells resulted in decreased sensitivity to rapamycin treatment. Rïƒ C mutations in H1299 human cancer cells resulted in complex selectivity, where certain mutants favored mTORC1, 2, or 3 and some resulted in no complexes. Thus, our work demonstrates that

mutations in the HEAT domain of mTOR significantly affect mTOR signaling due to preferential complex formation. Conclusion: These results were surprising because mTOR complex selectivity due to HEAT domain mutations have not been demonstrated. Further study of these HEAT domain mutations will improve our understanding of mTOR complex signaling and may help to develop personalized therapies for the specific cancers associated with these mutations.

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Aberrant post-translational modifications (PTM) characterize the hepatic proteome of methylmalonic acidemia (MMA)
Organic acidemias (OAs), such as methylmalonic acidemia (MMA), are a group of inborn errors of metabolism that typically arise from defects in the catabolism of amino- and fatty acids. OAs are difficult to treat and have multisystemic manifestations, leading to increased morbidity and mortality. Accretion of acyl-CoA species is postulated to cause intracellular toxicity. Here, we explore an alternative pathophysiological consequence of impaired acyl-CoA metabolism: the accumulation of aberrant posttranslational modifications (PTMs) that modify enzymes in critical intracellular pathways, especially during periods of increased stress. Using a mouse model that recapitulates the hepatic mitochondriopathy of MMA (Mmut-/-;TgINS-MCK-Mmut) and donated human liver tissues from MMA patients and controls, we surveyed PTMs in hepatic extracts with acyl-lysine antibodies through Western blot analysis. We discovered widespread hyperacylation in the MMA mice compared to controls. Next, we prepared anti-PTM antibody columns, purified hepatic extracts from MMA and control mice, and performed mass spectrometry to characterize the PTM proteome. Hyperacylation of enzymes involved in glutathione, urea, arginine, lysine, tryptophan, valine, isoleucine, methionine, threonine, and fatty acid metabolism were detected in the MMA mice but not controls, and further validated via immunoprecipitation analysis and Western blotting. Immunoprecipitation experiments using MMA human and murine hepatic extracts confirmed hyperacylation of urea cycle enzyme Carbamoyl Phosphate Synthetase 1 (CPS1) compared to respective controls. Tandem mass spectrometry analysis indicated 15 sites of hyperacylation including sites associated with CPS1 inactivation and hyperammonemia. We also found increased acylation of Optic Atrophy 1 (OPA1), a mitochondrial enzyme which maintains mitochondrial cristae. Immunoprecipitation analysis both short and long OPA1 isoforms were modified when purified from the kidneys of MMA mice compared to controls which could inhibit OPA1 function leading to loss of cristae. Overall, hyperacylation of key enzymes in pathways known to be dysregulated in MMA likely contributes to altered metabolism and identifies a new set of targets for therapeutic intervention. Through in vitro analysis we have identified the deacylase capable of removing MMA related PTMs and are developing an AAV therapy targeted at removing hyperacylation and restoring metabolic homeostasis in MMA mice.

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MiR-936 regulates the number and strength of excitatory synapses by inhibiting TMOD2
MicroRNAs (miRNAs) are short, non-coding RNAs of âˆ¼22 nucleotides that regulate gene expression at the translation level and play vital roles in the function, assembly and plasticity of synapses. miR-936 is a primate-specific miRNA expressed in the brain and increased in the dorsolateral prefrontal cortex (DLPFC) of people with schizophrenia (SCZ). The significance of miR-936 overexpression to schizophrenia is unknown. The goal of this study is to investigate the function of miR-936 and the consequence of miR-936 overexpression. We first used postmortem human brains to examine miR-936 expression pattern in DLPFC. miR-936 is enriched in cortical layer 2/3 and expressed in glutamatergic and GABAergic neurons but not in glial cells. The DLPFC of SCZ samples have elevated miR-936 in layer 3 and 4. To investigate the function of miR-936 in neurons, we over-expressed miR-936 in glutamatergic neurons differentiated from human induced pluripotent stem cells (iNs), and co-cultured them with iNs expressing channelrhodopsin to elicit synaptic transmission via optical stimulation. Overexpression of miR-936 in iNs led to a decrease in the number of excitatory synapses. Electrophysiological recordings of iNs overexpressing miR-936 showed that both the amplitude and the frequency of miniature excitatory postsynaptic currents (mEPSCs) were reduced. Optical stimulation-evoked excitatory synaptic responses in miR-936 overexpressing neurons had a lower ratio of AMPA and NMDA receptor-mediated currents (AMPA/NMDA ratio, reflecting synaptic strength) and intact paired-pulse ratio (an indicator of presynaptic release probability). Blocking endogenous miR-936 with a miR-936 sponge led to increases in excitatory synapses, the frequency and amplitude of mEPSCs, and AMPA/NMDA ratio. We further identified TMOD2 (an actin binding protein) as a target gene of miR-936 that is suppressed by miR-936 overexpression to alter synapses. These results indicate that miR-936 restricts the number of synapses and the strength of synaptic transmission by inhibiting TMOD2 expression. These findings suggest that in the DLPFC of individuals with SCZ, miR-936 upregulation can lead to a reduction of glutamatergic synapses and weakening of excitatory synaptic transmission, which contribute to the hypofrontality of SCZ.

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An integrin receptor mediates Tau fibril-dependent neurotoxic activation of primary astrocytes
Tau is a microtubule-stabilizing protein that is prone to misfolding. Abnormal accumulation of Tau and propagation of Tau pathology are observed in Alzheimer's disease (AD). The progression of unfolded Tau has suggested an unfolded protein transfer mechanism along neuronal connections. However, the presence of Tau pathology in astrocyte cannot be explained by it. Previous studies showed that astrocytes readily take up Tau. The internalization of Tau into astrocytes presumably require a specific receptor(s), the identity of which is currently unknown. On the other hand, while neurons would seem to be the main players in AD, astrocytes also contribute to it given their close physical proximity to neurons and their crucial roles in maintaining homeostasis and regulating neuroinflammation. Two different states of reactive astrocytes, termed as "A1" and "A2" respectively, have been identified. A2 astrocytes are protective, whereas A1 astrocytes are neurotoxic. In addition, Astrocytes can respond to unfolded proteins released from neurons, implying the existence of neuron-glia crosstalk in brain. Here we identified a cell receptor, integrinÎ±VÎ²1, as Tau receptor in astrocyte by employing proximity labeling based proteomic mapping. Knockdown of integrinÎ±VÎ²1 in primary astrocyte strikingly reduce the uptake of Tau fibrils. Moreover, the binding of Tau fibrils can strongly induce the integrin signaling in an integrinÎ±VÎ²1 dependent manner, indicating that integrinÎ±VÎ²1 not only mediates the entry of Tau, but also transduces signaling from the cell surface. Additionally, we find that Tau fibrils and monomers differentially induce inflammation in astrocyte as indicated by differential up-regulation of pro-inflammation cytokines, chemokines, and nitric oxide synthase (iNOS). More importantly, gene expression studies show that Tau fibrils can convert astrocyte into A1 status, which further induces the death of neurons. Knockdown of integrinÎ±VÎ²1 and its downstream adaptor Talin can abolish these phenotypes. Inhibition of the focal adhesion kinase (FAK), an established mediator of integrin signaling, has a similar effect. Taken together, we have identified integrinÎ±VÎ²1 as a Tau receptor in astrocyte, which mediates Tau entry. Additionally, we found Tau fibrils can invoke inflammation response and convert astrocyte into neurotoxic A1 status to kill neuron, and our results strongly suggest that the integrin-talin-FAK pathway plays a key role in the process.

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Glutamate and GABA co-release in the lateral habenula
Neurons that utilize either glutamate or GABA as signaling molecules are widely distributed in the brain and have been studied for decades. However, we recently demonstrated that lateral habenula (LHb) contains axon terminals that co-release glutamate and GABA from distinct pool of vesicles located in the same axon terminal. Glutamate-GABA co-releasing axon terminals are originated from neurons that co-express the vesicular glutamate transporter 2 (VGLuT2) and the vesicular GABA transporter (VGaT) (VGluT2+ VGaT+ neurons) located in both the ventral tegmental area (VTA) and the entopeduncular nucleus (EPN). In this study, we determined GABA and glutamate co-releasing properties from VTA and EPN inputs to the LHb. To selectively activate VGluT2+ VGaT+ inputs from VTA or EPN, we crossed

VGluT2::Cre mice with VGaT::FlpO mice and injected intersectional viral vectors that express channel-rhodopsin in the VTA or EPN. 6 to 8 weeks later, we prepared LHb slices to optogenetically activate VGluT2+ VGaT+ inputs from VTA or EPN and determined their co-releasing properties by patch clamp electrophysiology. We found that LHb activation of VGluT2+ VGaT+ inputs, from VTA or EPN, produced monosynaptic excitatory postsynaptic currents (EPSCs) blocked by CNQX, and monosynaptic inhibitory postsynaptic currents (IPSCs) blocked by Bicuculline. By current-clamp recordings, we found that LHb activation of VGluT2+ VGaT+ inputs from VTA produced inhibition of LHb neurons due to higher GABA currents. In contrast, LHb activation of dual VGluT2+ VGaT+ inputs from EPN produced activation of LHb neurons due to higher glutamate currents. By different frequency stimulation of VGluT2+ VGaT+ inputs from VTA or EPN, we found that glutamate was rapidly depleted from the presynaptic terminals while GABA release was maintained. These findings indicate that glutamate and GABA have different release probabilities, supporting that glutamate and GABA are packed in independent synaptic vesicles. Finally, we determined if LHb activation of glutamate or GABA metabotropic receptors modulates co-release of glutamate and GABA from dual VGluT2+ VGaT+ inputs. We found that LHb activation of metabotropic glutamate receptors increases GABAergic transmission from VTA VGluT2+ VGaT+ inputs while LHb activation of GABA B receptors decreases glutamatergic transmission from EPN VGluT2+ VGaT+ inputs, indicating that metabotropic receptors have modulatory effects on LHb co-release of glutamate and GABA.

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CONTROLLED ASTROGLIOGENESIS IN CHEMICALLY DEFINED (SERUM-FREE) CONDITIONS, BYPASSES NEUROGENESIS AND ENABLES AUTOMATED, HIGH-THROUGHPUT GENERATION OF ASTROCYTES FROM HUMAN PLURIPOTENT STEM CELLS
Astrocytes play important roles in normal brain development, function, and various pathological conditions. Derivation of human astrocytes from induced pluripotent stem cells (iPSCs) is an attractive approach for disease modeling and drug discovery; however, present protocols are variable, inefficient, and last up to 6 months. Here, we developed a highly efficient, chemically defined and controlled astrocyte differentiation protocol that requires neither serum nor genetic manipulation. By identifying and simultaneously manipulating several critical pathways, we obtained astrocytes from iPSCs with over 90% efficiency in less than 30 days. These cells displayed astrocyte morphologies and expressed typical markers, whereas genes indicative of other cell types (e.g. neurons, oligodendrocytes) were absent. Compared to the popular dual-SMAD inhibition (dSMADi) approach for neural induction, our method resulted in more efficient generation of symmetrically dividing BLBP+ radial glial cells within 7 days. By day 14, BLBP+ cells differentiated into S100B+ astroglia while TUJ1+ neuroblasts were absent, followed by NFIA expression (day 21). At day 30, strong induction of CD44 and glutamate transporter SLC1A2 was observed. Cell maturation over two passages then robustly induced expression of GFAP and HEPACAM (day 50). iPSC-derived astrocytes were capable of taking up the neurotransmitter glutamate, displayed calcium transients, stored glycogen, promoted neuronal survival, neurite outgrowth and synaptic activity when co-cultured with neurons. We also utilized iPSC-astrocytes for disease modeling (e.g.

Alexander disease, Zika virus infection), high-throughput drug screening and cell grafting experiments in mice. Lastly, the protocol was automated using a robotic cell culture system, which now enables the standardized production of billions of well-characterized human astrocytes.

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Pin1 isomerization of Kv4.2 regulates seizure intensity in mice
Epilepsy is a common disorder affecting an estimated 1% of the US population. This condition is characterized by anomalous excitation of neural networks that manifests in recurrent seizures. While the etiology of epilepsy is multifaceted, Kv4.2-containing voltage-gated K+ ion channel dysfunction is often associated. Kv4.2 has shown to be functionally downregulated in response to seizure; however, the molecular mechanisms underlying this modulation are uncertain. To address this, we performed a proteomic screen to detect proteins in complex with Kv4.2. The screen revealed Pin1, a peptidyl-prolyl isomerase that binds to target proteins upon their phosphorylation and regulates post-translational modifications. Using co-IP and co-immunostaining in neurons, we confirmed their interaction. To identify Pin1 binding sites on Kv4.2 and the phosphorylation signaling involved in Pin1 recruitment, we used a synthetic peptide pulldown assay with peptides containing three putative Pin1 binding motifs: pT602, pT607, and pS616. The pulldown assay revealed that Pin1 binds strongly to T607. Further, co-expression of different kinases with Kv4.2 in HEK293 cells revealed p38 MAPK as primarily responsible for phosphorylating T607. We then assessed if the p38-Pin1-Kv4.2 interaction is modulated by seizure. GST-Pin1 pulldown from brain lysates revealed PTZ-induced seizure enhanced p38-Pin1-Kv4.2 interaction. To further probe the role of this interaction in vivo Crispr-cas9 was used to generate novel T607A knockin mice(Kv4.2TA) that abolished dynamic Pin1 binding to Kv4.2. To assess the role of this cascade on neuronal physiology, we used patch clamp electrophysiology in pyramidal cells of hippocampal slices. Kv4.2TA cells displayed a reduction in AP firing relative to WT in response to somatic current injections. This reduced excitability is traced to increased Kv4.2-mediated current as we found an increase in current in Kv4.2TA cells in outside-out somatic patches. Finally, we probed seizure intensity in WT vs Kv4.2TA mice. In response to IP kainic acid injection, Kv4.2TA mice exhibited reduced seizure intensity over an hour-long period relative to WT mice. The reduced cellular and network excitability in Kv4.2TA mice could be recapitulated in WT by pharmacological block of p38 kinase and Pin1. Therefore, we have identified a novel signaling cascade that can be a target for therapeutic intervention to mitigate seizure intensity in epilepsy by reducing Kv4.2 downregulation.

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Kdm4ab: A novel epigenetic regulator of vertebrate neural development
Epigenetic regulators such as DNA and histone modifying enzymes add cell-type-specific epigenetic marks that help to establish tissue-specific gene expression patterns and cell fates during development. Although large-scale recessive mutant screens have identified genes directing epigenetic regulation in invertebrates, similar screens to identify developmental epigenetic regulators have not been carried out in vertebrates. We have developed the first transgenic epigenetic reporter in a vertebrate, a transgenic zebrafish line that reliably reports changes in tissue-specific epigenetic silencing and activation. The transgene uses a CpG island â€œepigenetic silencing cassetteâ€ fused to a ubiquitous promoter driving destabilized green fluorescent protein (GFPd2). Using this epigenetic reporter line, we are performing a large-scale chemical (ENU) mutagenesis screen to identify tissue-specific epigenetic regulators. Thus far, we have isolated more than twenty mutants that display defects in epigenetic silencing in a variety of different organs and tissues, including liver, intestine, heart, blood, and pharynx, to name just a few. We are using RNA-seq based mapping methods to identify the defective genes in these mutants. We mapped one mutant specifically defective in epigenetic gene activation in the brain to a previously uncharacterized histone-modifying gene, kdm4ab. We confirmed that kdm4ab is the defective gene by performing morpholino knockdown and by generating additional kdm4ab CRISPR/Cas9 alleles. We are currently investigating the role of the kdm4ab and closely related kdm4aa genes in neural development and brain-specific gene regulation programs, using gene expression and epigenetic profiling, detailed phenotypic analysis, and experimental embryology. These studies are shedding important new light on the tissue-specific epigenetic mechanisms that direct neural developmental programs.

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Integration of single cell transcriptomes and chromosome accessibility to detect regulatory elements critical to interneuron development
GABAergic interneurons are a heterogenous cell population and their dysfunction is associated with numerous brain disorders. Interneurons are born in the ganglionic eminences (MGE, LGE and CGE), with distinct subtypes arising from different GE domains. Despite substantial evidence suggesting that initial interneuron subtypes are specified within these neurogenic niches, only a handful of fate-determining genes have been identified. One possible explanation is that progenitors contain epigenetic signatures

directing cells towards a particular interneuron fate that are not yet apparent in the transcriptome. Ample evidence indicates that epigenetic mechanisms induce heritable changes in gene expression and are critical in mediating cell fate and differentiation in a variety of tissues. Yet we lack a comprehensive understanding of how epigenetic states influence interneuron cell fate in normal development and disease models. To explore the relationship between interneuron cell fate and epigenetic mechanisms in neural progenitors, we performed single cell RNA (scRNA-Seq) and single cell assay for transposase-accessible chromatin (scATAC-Seq) sequencing to determine the transcriptome and chromatin accessibility in the embryonic MGE, LGE, CGE and cortex. This allows us to screen for candidate enhancers and other non-coding regions regulating expression of specific interneuron subtypes. We detected distinct genes and corresponding chromatin profiles that are restricted to specific progenitor zones and interneuron lineages. By examining ATAC peaks in lineage-specific genes across cell types, we detected putative enhancers associated with specific GEs and potentially distinct interneuron subtypes. To expand on these initial observations, we generated a conditional knockout of Enhancer of zeste homolog 2 (Ezh2) in MGE progenitors. Ezh2 regulates histone methylation and acts as a transcriptional repressor; thus loss of Ezh2 likely disrupts chromatin architecture and gene expression. These Ezh2 cKO mice displayed a significant decrease in PV+ interneurons in multiple brain regions. We are currently performing scRNAseq and scATACseq on the MGE of Ezh2 cKO to assess how the transcriptome and chromatin landscape is altered in these mice. These insights increase our understanding of how epigenetic modifications regulate interneuron fate and could open new avenues for understanding how disease-associated genes or genomic loci could perturb interneuron fate and maturation.

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Shared and unique dimensions of psychopathology associated with multiple, broadly distributed brain configurations

Brain imaging research has not yet delivered reliable biomarkers for psychiatry. One challenge is the high comorbidity of mental disorders, which might be addressed by analytic approaches designed to identify highly associated patterns in both symptom dimensions and imaging data. This is particularly relevant to youth, where the seeds of chronic psychopathology may manifest as co-occurring symptoms of anxiety, irritability and disruptive behavior. Our primary aim was to discover shared and specific dimensions of anxiety, irritability, and disruptive behavior that are grounded in intrinsic brain connectivity. The second aim was to test the robustness of these latent dimensions in an independent sample. This was a cross-sectional functional magnetic resonance imaging study. The discovery sample (N=183) and the independent replication sample (N=326) comprised healthy volunteers and individuals diagnosed with an anxiety disorder, disruptive mood dysregulation disorder and/or attention-deficit/hyperactivity disorder. Psychiatric symptoms were rated by both the participant and the parents using standardized symptom questionnaires. Participants also provided 10 minutes of resting-state functional magnetic resonance imaging data. Functional connectivity was assessed using partial correlations between 216 network nodes including subcortical structures. Principal component analysis was used for dimensionality reduction prior to identification of maximally correlated latent dimensions

of functional connectivity and clinical symptoms. Results are considered significant at 0.05, corrected for multiple testing. In the discovery sample, we identified four latent clinical variates â€” two transdiagnostic capturing inhibitory difficulties and negative affectivity, and two specific for either disruptive behavior or anxiety â€” that were associated with unique but widespread connectivity patterns (all r > .86). The transdiagnostic variates consistently manifested across varying input-to-participant ratios and replicated clinically in the second, independent sample indicating robustness against overfitting and sample-specific covariation. These findings emphasize the relevance of shared features of mental disorders and suggest that multiple, broadly distributed brain configurations instead of consistent, universal patterns of brain connectivity underlie similar clinical phenotypes, which will require a diverse set of approaches to identify targets for novel interventions.

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The Parkinson' s disease kinase LRRK2 promotes tubulation and vesicular sorting from membrane damaged lysosomes

Mutations in the leucine rich repeat kinase 2 (â€‹LRRK2â€‹) gene are a cause of familial Parkinsonâ€™s disease (PD) and variants in the LRRK2 locus are associated with the sporadic form of the disease. LRRK2 pathogenic mutations are gain of function mutations that produce a toxic hyperactive protein. Nonetheless, the biological functions of LRRK2 remain incompletely understood. Additional human genetic data nominate the lysosome as a crucial organelle for PD, and although cells carrying LRRK2 pathogenic mutations display lysosomal defects, the specific role of LRRK2 in the lysosome remains unclear. Here, we observed that LRRK2 is recruited to lysosomes that have a partially ruptured membrane in mouse primary astrocytes. Our data suggest that LRRK2 recruitment to membrane damaged lysosomes is parallel and independent to lysophagy. The proximity labeling method APEX2, coupled with quantitative mass spectrometry, revealed the motor adaptor protein JIP4 as a LRRK2 interactor at the lysosomal membrane. Using confocal microscopy, combined with pharmacological and genetic tools, we observed that LRRK2 is able to recruit JIP4 to permeabilized lysosomes in a kinase-dependent manner. Indeed, the LRRK2 pathogenic mutation located in the kinase domain (G2019S) increases JIP4 recruitment to the lysosomal membrane. LRRK2 recruitment of JIP4 occurs through two small GTPases, RAB10 and RAB35. Both RAB proteins are phosphorylated by LRRK2 (in their Thr73 and ThrT72 sites respectively) at the lysosomal membrane which preserves the membrane localization of both RABs and allows them to recruit JIP4. Using super-resolution live cell imaging microscopy and focus ion beam electron microscopy (FIB-SEM), we observed that once at the lysosomal membrane, JIP4 promotes the formation of LAMP1-negative/LRRK2-negative lysosomal tubules that release membranous content from ruptured lysosomes. Released vesicular structures are able to interact with other healthy lysosomes. Thus, we described a new process that uses lysosomal tubulation to release vesicular structures from permeabilized lysosomes, being the first evidence of lysosomal sorting in mammalian cells. LRRK2 orchestrates this process that we name LYTL (LYsosomal Tubulation/sorting driven by LRRK2) that, given the central role of the lysosome in PD, is likely to be disease relevant.

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APOE gene methylation is associated with cognitive performance in middle-aged urban adults
DNA methylation (DNAm) is the addition of a methyl group to the 5â€™ carbon of the cytosine nucleotide adjacent to a guanidine (CpG site). It influences transcriptional activity and is related to the development and progression of many diseases, such as the pathophysiology of brain aging and cognition decline. Apolipoprotein E (APOE) allele e4 is a known risk for cognition decline and Alzheimer's disease (AD). However, there are few studies that examine whether altered DNAm in the APOE genomic area affects cognitive outcomes, especially in racially diverse middle-aged adults. Our aim is to identify DNAm sites in the APOE genomic area that are associated with cognitive performance in a diverse community-dwelling human cohort. We assessed the DNAm level of CpG sites in APOE and proximal genes including nectin cell adhesion molecule 2 (NECTIN2) and apolipoprotein C1 (APOC1) in 464 participants averaging 46 years old using Illumina Methylation EPIC BeadChip, a powerful test for high density DNAm markers. Cognition was measured using neuropsychological tests that assessed global mental status, memory, visuoconstruction and spatial ability. Regression analysis was used to determine the association of methylation levels with cognition performance, adjusting for socio-demographic characteristics (age, sex, race, poverty status and education), known risks for poorer cognitive performance (APOE genotype, smoking, diabetes mellitus, hypertension, and cardiovascular disease status) and immune cell composition. We identified two CpG sites associated with cognition measurements in the fully adjusted regression models after correction for multiple testing. We discovered that the methylation level of cg00397545 in the promotor region of APOC1 is inversely associated with Card Rotation Test, which measures two-dimensional spatial ability (P=0.000177). This CpG site has been reported to be correlated with AD-related biomarkers in AD patients. Importantly, we identified elevated methylation level of a novel CpG site cg10178308 within the promotor region of NECTIN2 which is significantly associated with lower Benton Visual Retention Test score (P=0.000084). This test captures immediate visual recall. Finally, using machine learning, we predicted the cognition test scores in a testing data set with moderate accuracy. Our results indicate that DNAm in the APOE genomic area influence cognitive performance and may be considered as an early indicator of cognitive decline.

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Methylphenidate alters the balance between the default mode network and the executive network during working memory performance under threat
Background Anxiety disorders are among the most prevalent and debilitating mental conditions.

Unfortunately, treatment remains suboptimal in many cases. Improving treatment requires a better understanding of the factors that modulate anxiety. Interactions between anxiety and cognition are well-documented. While deleterious effects of anxiety on cognitive function are well-established, certain cognitive processes, such as performing a difficult task, can reduce anxiety. The neural mechanisms underlying such anxiolytic effects remain poorly understood. This study uses a pharmacological manipulation of cognitive function to examine the neural interactions between cognitive and anxiety-related networks. Specifically, three factors were orthogonally manipulated: cognitive function via methylphenidate, anxiety via experimentally induced anxiety with threat of shock, and cognitive performance difficulty via n-back tasks. Methods Single oral dose of 20 mg methylphenidate (MPH) (cognitive enhancer) or placebo (PLA) was administered 90 minutes prior to testing. In this double-blind between-group design study, 50 healthy adults (HA, 25/drug-group) performed a working memory (WM, 1-back and 3-back tasks) in the MRI scanner, under threat (potential electrical shocks) or safety (no shocks) conditions. This event-related study was analyzed using AFNI afni_proc.py and 3dMVM scripts, examining drug influence on the interaction between threat and WM load. Results During safety, the placebo and methylphenidate groups did not differ in their neural responses to WM, showing similar activation of the central executive network (e.g., DLPFC). However, methylphenidate (vs. placebo) influenced the effect of threat on cognitive processes. Methylphenidate prevented the deactivation of the DMN which was prominent during placebo under threat, but at the same time magnified the engagement of the CEN compared to placebo under threat. Conclusion These preliminary results suggest that methylphenidate alters the balance between the DMN (internalizing processes) and CEN (cognitive function) when challenged by anxiety. This may have critical implications for understanding mechanisms underlying interactions between cognition and anxiety. Work is underway to clarify the behavioral correlates (cognitive performance and anxiety ratings) of these neural findings.

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Automated Utricular Hair Cell Counting by Digital Image Analysis Based on Nuclear Staining
Aminoglycoside antibiotics are widely used to treat life-threatening bacterial infections, and cisplatin is a platinum-based chemotherapy drug that is highly effective in treating a variety of cancers. Both of these treatments have ototoxic side effects that lead to permanent loss of sensory hair cells in the inner ear resulting in hearing loss and/or balance disturbances. Our lab utilizes ex vivo preparations of adult mouse utricles as a model system to study the mechanisms underlying ototoxic drug-induced hair cell death. The utricle model system is the best-characterized in vitro preparation for studies of mature mammalian hair cells. However, efficient and reliable quantification of cultured hair cells has been a persistent challenge with this model system. Specifically, the commonly used hair cell marker myosin 7a results in a diffuse cytoplasmic stain that is not conducive to automated quantification and must be quantified by hand, a labor-intensive task. More importantly, myosin 7a immunolabeling is retained in dead hair cells, making it impossible to accurately quantify the percentage of surviving vs. dead hair cells after ototoxic drug treatment. Here we have developed a method that allows automated quantification

of surviving hair cells in utricle preparations. To achieve this, we stained adult utricle cultures for Pou4f3, a hair-cell specific transcription factor. Importantly, staining for this marker results in highly specific and discrete nuclear signal. In addition, by staining for apoptotic cells in parallel with Pou4f3, we determined that Pou4f3 is only expressed in living hair cells, making it a better marker than myosin7a. We then utilize the binary function within ImageJ to define the nuclear Pou4f3 signal and automate the quantification. Furthermore, we have written a macro that automates the entire process from image loading to a final quantified image that can be immediately evaluated for accuracy. The user is then able to manually correct any mis-quantification via an image overlay indicating the counted nuclei. Overall, this method significantly reduces the time spent quantifying hair cells in ex vivo utricle cultures while simultaneously increasing the accuracy of quantification and the identification of surviving hair cells.

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Neuronal and astrocytic extracellular vesicles in blood reflect brain pathology
Background: Extracellular vesicles (EVs) are membranous nanoparticles that are continuously secreted by all cells, including brain neurons and astrocytes, and circulate in all biofluids. The cargo of circulating EVs depends on the physiological or pathological state of their cell of origin raising the possibility of using them for â€œliquid biopsyâ€. Multiple studies have shown that neuron- and astrocyte-derived EVs (NDEVs and ADEVs) in plasma provide biomarkers for Alzheimerâ€™s disease (AD), including p181-Tau, total Tau, synaptic proteins, and complement. Nevertheless, the relationship between NDEV and ADEV cargo and brain pathology in AD has been hypothesized, but never demonstrated. Methods: We analyzed NDEVs and ADEVs from ante-mortem plasma samples from patients with definitive AD who reached autopsy (40 with AD pathology only, 14 with AD plus Lewy body pathology, 26 with AD plus vascular dementia) and 22 cognitively normal sex and age matched controls. We immunoprecipitated NDEVs targeting neuronal marker L1CAM and ADEVs targeting astrocytic marker GLAST. We measured p181-Tau, total Tau, synaptic proteins in NDEVs and complement in ADEVs. We measured three common EV markers, CD63, CD81 and CD9, to normalize measurements for differential EV yield. Results: Normalized NDEV Tau and pTau-181 levels were higher in AD ($p<0.05$) and mixed pathology groups compared to controls ($p<0.01$). Furthermore, the pre-synaptic marker synaptophysin was higher, whereas the post-synaptic marker synaptopodin was lower in AD and mixed pathology groups compared to controls ($p<0.05$). There was a strong positive correlation between synaptic proteins and Tau ($r>0.64$; $p<0.0001$) and p181-Tau ($r>0.51$; $p<0.0001$). Normalized ADEV Complement C5 and C5a levels were higher in mixed pathology groups compared to controls ($p<0.01$). Ongoing analyses will determine relationships between EV biomarkers, pathologic scores for various pathologies (CERAD, Braak stage) and ante-mortem cognitive scores, as well as identify differences between pure AD and mixed pathology dementia. Results: Demonstrating pathologic correlates offers the strongest possible evidence for the convergent validity of biomarkers. The present study shows for the first time that NDEV and ADEV biomarkers satisfy this criterion and may be used to detect in vivo brain pathologic changes for AD and related dementias.

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Neuroscience - General
BETA-CARYOPHYLLENE: A NOVEL THERAPEUTIC APPROACH FOR COCAINE USE DISORDER
Cocaine use disorder (CUD) continues to be a serious health problem worldwide. Despite intense research, there is still no FDA-approved medication for it. Recent efforts to discover potential effective therapeutics have focused on the endocannabinoid system because of its identification as a neurobiological substrate underlying drug addiction. In the last decade, there has been growing interest in beta-caryophyllene (BCP), a volatile phytocannabinoid present in high proportions in cannabis and large numbers of spice and food plants including black pepper, thyme and cloves. This dietary additive, approved by the FDA, has been shown to produce promising therapeutic effects for multiple neuropsychiatric disorders. Surprisingly, the therapeutic potential of BCP in the treatment of drug abuse and addiction has not been explored. Here, using gold standard animal models of drug abuse, we systematically evaluated the potential therapeutic utility of BCP against cocaine-related behaviors in rodents. We also assessed the mechanisms of action by which BCP might produce these effects. In a series of experiments, we found that BCP attenuated cocaine-enhanced electrical brain-stimulation reward and optogenetic intracranial self-stimulation driven by activation of dopamine neurons in DAT-cre mice, both outcomes indicating reduced cocaine reward efficacy and, by extension, reduced cocaine abuse liability. Intriguingly, when administered systemically or orally, BCP attenuated cocaine self-administration in rats, again demonstrating its ability to reduce cocaine abuse. The reduction in cocaine self-administration was blocked by GW6471 and GW9662, and partially by AM630 (antagonists to peroxisome proliferator-activated receptors: PPAR alpha and gamma and to CB2 receptor), suggesting the involvement of PPAR alpha and gamma and CB2 receptors. BCP also reduced drug-primed reinstatement of cocaine seeking and cocaine conditioned place preference, indicative of its preventative effects against relapse. When BCP was substituted for cocaine, rats ceased responding, suggesting BCP itself has low liability abuse. Our findings suggest that BCP has exceptional promise as a therapeutic candidate in the treatment of CUD. Importantly, given its good oral bioavailability and the advantage of being an already FDA-approved non-toxic dietary additive, BCP is a valuable candidate for drug repurposing programs in translational medicine.

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Sharp-wave discharges and reduced inhibitory neuron density are signatures of poor cognitive outcome in aging
Memory loss associated with old age can have devastating effects on quality of life, independent living, and oneâ€™s identity. Preventing or reducing these consequences, however, necessitates a better understanding of the neural processes that underlie â€œsuccessfulâ€ versus â€œunsuccessfulâ€ cognitive aging. Certain brain wave oscillations, which can be measured by electroencephalogram (EEG), support long-term memory formation and are known to change with age. However, the impact of these oscillatory changes on memory formation in advanced age is unclear. Here we used a multi-scale experimental approach, bridging behavior, brain waves, and neural circuity. More specifically, we utilized a well-characterized rodent model of normal cognitive aging to examine the relationship between age-related changes in brain waves and the hippocampal â€œgatekeepingâ€ capacity of parvalbumin+ (PV+) inhibitory neurons. In a pilot study of nine aged rats (24 months), animals were tested in the Morris water mazeâ€”the gold standard for assessing spatial memory. Seven adult rats (9 months) served as controls. Similar to aged humans, we observed considerable inter-individual variability in memory capacity among aged individuals. We then recorded EEGs for 24 hr using multichannel electrodes. Unexpectedly, we observed a considerable amount of â€œseizure-likeâ€ activity among aged rats compared to adults. The function and impact of these â€œsharp-wave discharges (SWDs)â€ remain a major debate in the field. Our data showed that aged rats with memory impairment had significantly more bouts of SWDs than adult rats (p=0.005) and aged rats with intact memory (p=0.024). These results indicate that increased SWD activity is tightly coupled to cognitive outcome in aging. The density of PV+ neurons in the dentate gyrus, a region important for spatial memory, was also coupled to age-related cognitive status, where density was significantly decreased in aged-impaired rats (p= 0.005) but not age-unimpaired rats (0.99) compared to adult rats. Together these data identify two signatures of poor cognitive outcomes in aging, namely more SWD activity and reduced inhibitory drive. Follow-up studies will assess the degree of synchronization between SWDs and PV activity by simultaneously recording EEGs and PV activity via fiber photometry. The current results spark the exciting prospect that these signatures can serve as therapeutic targets in order to improve memory among aged individuals with memory loss.

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Role of orbitofrontal and piriform cortex in relapse to fentanyl seeking after food choice-induced voluntary abstinence
There are few preclinical studies of fentanyl relapse, and these studies have used experimenter-imposed

(forced) abstinence procedures. In humans, however, abstinence is often voluntary, with drug available in the drug environment but forgone in favor of non-drug alternative rewards. We recently developed a rat model of relapse after food choice-induced voluntary abstinence. Here we used the model to study the role of orbitofrontal cortex (OFC), previously implicated in heroin relapse after forced abstinence, in relapse to fentanyl seeking after voluntary abstinence. We trained male and female rats to self-administer palatable food pellets for 6 days (6-h/day) and intravenous fentanyl (2.5 µg/kg/infusion) for 12 days (6-h/day). We assessed relapse to fentanyl seeking after 13-14 voluntary abstinence days, achieved through a discrete choice procedure between fentanyl infusions and palatable food (20 trials/day). In both sexes, relapse after food choice-induced abstinence was associated with increased expression of the activity marker Fos in OFC. Pharmacological inactivation with muscimol+baclofen (50+50 ng/side) of OFC decreased relapse to fentanyl seeking. We then determined projection-specific activation of OFC afferents during the relapse test by using Fos plus the retrograde tracer cholera toxin B (injected into OFC). Relapse to fentanyl seeking was associated with increased Fos expression in piriform cortex (Pir) neurons projecting to OFC, but not in projections from basolateral amygdala and thalamus. Pharmacological inactivation of Pir with muscimol+baclofen decreased relapse to fentanyl seeking after voluntary abstinence. Next, we used an anatomical disconnection procedure to determine whether projections between Pir and OFC are critical for relapse to fentanyl seeking. Unilateral muscimol+baclofen injections into Pir in one hemisphere plus unilateral muscimol+baclofen injections into OFC in the contralateral but not ipsilateral hemisphere decreased relapse. Our results identify Pir-OFC projections as a new motivation-related pathway critical to relapse to opioid seeking after voluntary abstinence.

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Increased dynorphin-kappa opioid receptor function in heroin withdrawal-induced hyperalgesia
Opioid use disorder is a major worldwide public health concern and opioid overdose deaths have reached epidemic proportions in the US. Opioids are potent analgesics, but repeated exposure to opioids lead to opioid-induced hyperalgesia (OIH) during withdrawal, which is hypothesized to be an important factor in drug seeking and relapse. Heroin users in acute withdrawal and ex-users in protracted abstinence show an increased sensitivity to pain. Our hypothesis is that the dynorphin-kappa opioid receptor (dyn-KOR) stress system is sensitized in opioid dependence and functionally involved in OIH. Therefore, we investigated the potential of the brain penetrating KOR antagonists 5'-guanidinonaltrindole (GNTI, 30 mg/kg, SC) and nor-binaltorphimine (nor-BNI, 30 mg/kg, SC) to reverse OIH in rats and mice. Animals of both sexes were included to test for potential sex differences in the role of dyn-KOR in behavior. Rats and mice were first tested on the hot plate to determine their sensitivity to the analgesic effects of heroin (0-1 mg/kg, subcutaneous). Female rats were less sensitive to the analgesic effects of heroin compared with males, whereas male and female mice were equally sensitive to heroin-induced analgesia. Thus, higher doses of heroin were used for female rats to produce hyperalgesia. Opioid-induced hyperalgesia was induced by daily injections of heroin in rats (2 mg/kg for males and 6 mg/kg for females, once a day, subcutaneous) and mice (escalating doses: 5-40 mg/kg,

twice a day, subcutaneous). The animals were tested with an electronic von Frey device 6 h (rats) or 24 h (mice) into heroin withdrawal. We found that treatment with KOR antagonists reversed OIH in rats of both sexes, an effect that lasted longer in females. Female mice, despite exhibiting similar heroin-induced analgesia as male mice, did not develop a significant OIH. Mice lacking the pro-dynorphin gene had higher baseline (i.e., without any treatment) paw withdrawal thresholds and were resistant to OIH. These findings indicate sex and species differences in OIH and the functional role of KOR in OIH. Our results suggest the dyn-KOR as a potential target for understanding pain produced by opioid withdrawal in individuals who were exposed to chronic opioids.

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Orbitofrontal cortex activity is necessary for encoding valueless sensory associations
The orbitofrontal cortex (OFC) is considered one of the least understood parts of the brain. Tasks used to assess OFC function have nearly exclusively been value-guided behavior where the value of one option is evaluated against another. What all of these had in common was that direct experience with rewards was involved. Thus OFC was thought to contribute to behavior only when direct changes in, or computations of, value occurred. We now know this is not the case. In sensory preconditioning, subjects are taught one valueless cue predicts another (A-B) and later learn cue B predicts a biologically meaningful stimulus (food). Thereafter subjects will respond to A as if it predicts food, even though cue A itself was never directly paired with food. The value of A is inferred on-the-fly. OFC was shown to be necessary for this inference of value, and neuronal recordings showed that this was correlated with the ability of OFC neurons to encode valueless A-B pairings. OFC is involved in representing structure among environmental features, and value (as well as valueless sensory associations) are just features of this structure. While OFC shows correlates of valueless sensory associations, it is not known whether this activity is necessary for later inference of value. It is possible these correlates are simply downstream of sensory processing in other brain regions. If this is the case, then OFC interference during A-B pairing would not affect later value inference. If this is not the case and OFC is necessary for valueless learning, then inference of value would be impaired. I used optogenetics in rats to directly address this. I infused rats with Halorhodopsin (HALO), a light gated ion channel for neuronal inhibition, or yellow fluorescent protein (YFP), a control protein, and implanted fiber optics in OFC. YFP served as the control group that received equal surgical, infusion, light stimulation, and conditioning procedures. Groups only differed in whether OFC activity was inhibited during A-B pairing. HALO animals were not able to infer the value of A. The YFP control group, which had intact OFC function during A-B pairing, was able to appropriately infer the value of A, which itself was never rewarded. OFC activity is necessary for the formation of valueless sensory associations. These findings demonstrate that OFC serves a broader role in learning than previously thought, contributing to processing and encoding of valueless sensory associative information.

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Neuroscience - Neural Circuits
Assessing integration of afferent inputs to medial olivocochlear neurons in vitro using a novel slice preparation, the wedge slice
Medial olivocochlear efferent neurons (MOCs), which reside in the auditory brainstem, provide feedback to the cochlea to modulate cochlear responses to sound. Though the properties of MOC synapses onto outer hair cells in the cochlea are well documented, the intrinsic and synaptic physiology of MOCs themselves have not been thoroughly explored. Our lab recently showed that the MOCs receive inhibitory input from ipsilateral neurons of the medial nucleus of the trapezoid body (MNTB). This result indicates that excitation (from T-stellate neurons in the contralateral cochlear nucleus [CN]) and inhibition (from the MNTB which is innervated by globular bushy cells in the contralateral CN) to MOCs are both driven by the contralateral ear. To understand how MOCs integrate these multiple sources of sound-evoked input, we developed a novel slice preparation which preserves presynaptic circuitry while facilitating patch-clamp electrophysiology. Our preparation, a wedge-shaped slice, contains an intact auditory nerve root and CN on the thicker side (1.2mm) and then tapers to the thinner side (0.3mm) where MOCs can be accessed for whole-cell patch-clamp recordings for detailed study of synaptic inputs. With this preparation we can stimulate the auditory nerve directly, preserving the approximate timing of inputs as they enter the auditory brainstem and allowing for intrinsic CN circuit activation and synaptic plasticity to occur at synapses upstream of MOCs. Our data suggest that both excitatory and inhibitory inputs to MOCs can be activated via auditory nerve stimulation, with excitation and inhibition arriving with distinct latencies. To examine the impact of the inhibitory input on MOC firing properties, both sets of afferent inputs were activated using train stimulation (20x, 100Hz) while recording in current clamp. After quantifying spiking in the control condition, GABA and glycine receptor blockers were added to the bath and the protocols were repeated. Blocking inhibition increased spike probability and decreased the latency to first spike indicating spike suppressing inhibitory effects. These experiments allow us to determine the combined influence of excitation and inhibition on the MOCs, and how each affects MOC output and in turn, cochlear modulation. Additionally, this preparation can be lent to other systems to improve connectivity of long-range inputs and enhance circuit analyses during in vitro slice physiology and imaging.

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Representations for grasp-relevant parts of objects in the human intra-parietal sulcus
Much of our daily activities are comprised of real-time processing of object shapes to plan an interactive movement. A comprehensive understanding of visual object processing, thus, requires studying vision in the context of goal-directed movements. Despite its importance, most studies of object processing have used passive viewing of images or simple tasks stripped of any interaction to investigate the neural underpinning of visual object processing. This shortcoming is mainly due to the technical challenges of

taking real objects to the MRI scanner and allowing participants to interact with the objects while remaining stable inside the scanner. Here, we designed a set up to allow participants to view and grasp 3d-printed objects inside an MRI scanner. The objects were put on a table positioned over the body, and participants viewed the object through a mirror while wearing a custom helmet to minimize their head movements. We examined the role of occipito-temporal and parietal object selective regions in processing the shape of objects to plan a proper grasp movement. Objects were one of four mug-shaped items composed of a handle part (either curved or straight) and a body part (either round or rectangular. Participants were instructed to grasp the handle. In a slow event-related design, the visual presentation of the object and the grasping movement were carefully separated from each other. We focused on two shape-selective regions of interest, one in the lateral occipital cortex (LOC), and one in the inferior intra-parietal sulcus (inferior IPS). A pattern classification analysis was performed on the visual responses to discriminate either the two objects with the same body and different handles (handle classification) or the two objects with the same handle and different bodies (body classification). LOC showed similar classification accuracies for the body and handle, but inferior IPS showed significantly higher pattern classification for the handle than the body. These results suggest that regions in the occipito-temporal cortex process all incoming visual input about the objects, while regions in the intraparietal sulcus extract only the visual information relevant for proper interaction with objects.

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Modulation of risk assessment behaviors by the anterior hypothalamus
During the past decade, neuronal circuits in the ventromedial hypothalamus (VMH) have been implicated in the regulation of aggression. Moreover, recent studies showed that neurons in the VMH project to the anterior hypothalamic area (AHA) and activation of these axonal projections in the AHA evokes defensive behaviors in mice. It is widely accepted that maladaptive regulation of fight-or-flight stress responses is a contributing factor to the development of many psychosomatic disorders. Therefore, we sought to determine how this VMH-AHA pathway encodes for defensive behaviors and the specific neuronal types within the AHA underlying these behaviors. For this, we expressed channelrhodopsin-2 (ChR2; n = 6 mice) or YFP control (n = 4 mice) in VMH neurons and used optogenetics to manipulate VMH axonal projections in the AHA. First, we observed that photostimulation of VMH-AHA circuitry increased blood glucose levels and decreased fasting-induced refeeding compared to control mice. Using a real-time place avoidance task, we found that VMH-AHA photostimulation induced avoidance for the stimulation-paired chamber and increased compulsive grooming in the unpaired side of the arena. Moreover, VMH-AHA photostimulation during a cliff avoidance task promoted risk taking as mice increased head dipping and falls, further supporting that VMH-AHA circuitry promotes risk taking behaviors. Recently, a population of parvalbumin (PV)-expressing neurons in the AHA was identified and linked to the regulation of sympathetic function. We used ChR2-assisted circuit mapping in brain slices and found that VMH neurons are synaptically connected to AHA-PV cells. Additionally, electrophysiological characterization of AHA-PV neurons (n = 30) revealed that they are fast spiking, which may be beneficial in escape situations requiring rapid

output. To determine whether these PV neurons are essential for risky behavior, we used caspase-mediated ablation of these neurons (n = 7 mice) and observed significantly reduced risk-taking behavior compared to control mice (n = 6 mice) in elevated plus maze and light/dark tests. Together, our findings show that gain of VMH-AHA function and deletion of AHA-PV neurons regulate risk assessment in opposite directions. This indicates that the anterior hypothalamus is necessary and sufficient for orchestrating risk-taking behaviors.

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High fat diets tune hypothalamic and mesolimbic feeding circuits to calorically dense foods
Dieting is notoriously difficult, particularly with increasingly convenient availability of calorically dense foods, such as those high in fat. Despite an ever-expanding number of dietary guidelines, sustained internal drives to consume energy rich foods over lower calorie alternatives often surmount efforts to control body weight. The neural mechanisms promoting adverse dietary choices remain poorly understood. However, two neural feeding circuit nodes influenced by diet include Agouti-related peptide (AgRP) neurons of the hypothalamus and the mesolimbic dopamine (DA) system, which encode homeostatic need and hedonic value, respectively. Here, we used a devaluation paradigm in mice coupled with cutting-edge in vivo fiber photometry to perform longitudinal recordings of AgRP neuron activity and DA release during standard diet (SD) presentation following a history of high fat diet (HFD) access. Behaviorally, we found HFD exposure dramatically and persistently reduces SD consumption in a manner independent of sex, body weight changes, or functional leptin and melanocortin-4 signaling. Fiber photometry recordings revealed one week of HFD robustly suppressed AgRP neuron responses to SD presentation during periods of hunger, which correlated with reduced SD intake through 8 weeks of HFD access. Although AgRP neuron responses to SD were blunted by one week of HFD exposure, effects of the gut hunger hormone ghrelin, or various gastrointestinal satiety signals, remained intact at this time point. These findings suggest early altered responses to SD are independent of functional capability of peripheral signaling mechanisms on AgRP neurons. Conversely, one week of HFD consumption sufficiently reduced AgRP neuron responses to intragastric infusion of calories, highlighting potentially shifted metabolic activity. Within the mesolimbic DA system, HFD blunted DA release in the nucleus accumbens during SD pellet approach and retrieval beginning one week post HFD consumption. To mimic strict dieting, mice were withdrawn from HFD for two weeks. Even following HFD abstinence, AgRP neuron responses to SD remained weakened and large responses to HFD were intact. Together, our results uncover diet-induced neural circuit mechanisms that underlie the challenges associated with dieting and making healthy eating decisions.

Christopher Kim
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Training recurrent spiking networks under biologically plausible synaptic constraints
Recent advances in deep learning ushered a large number of artificial neural network (ANN) models that can capture the complex and intricate features of neuronal activities. However, ANNs suffer from interpretability and biological plausibility as scientists open the â€œblack boxâ€ to understand the underlying mechanism that enables ANNâ€™s superior performance. To overcome these issues and gain insights into the computational capability of biological neural networks, we investigated if the balanced network, which is an important class of spiking network model that has firm theoretical and biological basis, can be trained to learn complex tasks. Our goal was to train spiking network models in the balanced state that (1) used neurons with spiking mechanism, (2) obeyed biologically plausible constraints on synaptic weights, and (3) exhibited network dynamics observed in cortical circuits. Based on our previous work that showed spiking networks can learn to generate arbitrarily complex activity patterns, we imposed further constraints on the synaptic weights in order to learn connectivity structure that respected Daleâ€™s law (i.e., excitatory and inhibitory connections) and maintained strong synaptic strength during learning. We found that such synaptic constraints naturally gave rise to large trial-to-trial variability in the spiking activity after a successful training, and the network exhibited distinct features of inhibition-stabilized dynamics observed in cortical circuits. Furthermore, we found that individual neurons in the balanced network can learn to generate complex activity patterns by adding only sqrt(K) plastic synapses while the existing K synaptic connections remained unchanged. Our finding demonstrates that the conventional view on the balanced state that it can only perform linear computations holds only at the level of population firing rate. Individual neurons in the balanced state has the capacity to perform nonlinear computations with minimal changes in the synaptic connections as long as the firing rates of individual neurons are constant on average. In the future, our model will be trained with more sophisticated learning algorithms developed for spiking networks to further inquire about the computational capabilities of biologically plausible networks.

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â€œDopamine Neuron Challenge Testâ€ for early detection of Parkinsonâ€™s disease

Parkinsonâ€™s disease (PD) is the second most common neurodegenerative disease and the most common movement disorder, affecting 1% of the population over the age of 60. The hallmark pathological feature of PD is the progressive loss of dopamine (DA)-producing neurons in the substantia nigra pars compacta (SNc), resulting in a cohort of motor deficits that worsen over time. Since these motor symptoms typically do not manifest until more than 60% of SNc DA neurons have been lost, early detection of PD is difficult. The goal of this study is to develop a sensitive and practical laboratory test that can be used for detecting PD at an early stage. We hypothesize that at the resting state, when DA neurons fire at relatively low frequencies, the initial loss of SNc DA neurons during early PD will not result in a detectable decrease in extracellular DA levels because the remaining DA neurons can compensate by the increasing their baseline firing rate and unit DA release per neuron. However, if we use pharmacological agents to challenge DA neurons to induce a surge of DA release from all DA neurons, a difference in total DA release between PD patients and healthy controls may be revealed because the magnitude of the DA surge is mainly determined by the total number of DA neurons when the unit DA release per neuron has been normalized to the same high level by the challenging agents. We further hypothesized that the difference in the magnitude of the drug-induced DA release between PD patients and control will translate to different elevations in the levels of DA metabolites in the CSF and plasma samples, which are readily accessible in clinical settings. To test this hypothesis, we first screened FDA-approved dopaminergic drugs and identified the combination of D2 DA receptor antagonist haloperidol and DA transporter blocker methylphenidate to be the best candidate. To test their effect in early PD model, we used 20-week-old MitoPark mice, which had 30% loss of DA neurons at this age. We found that at the resting state, there was no detectable difference in the CSF DOPAC and HVA levels and the plasma HVA level between littermate control and MitoPark mice. After the drug challenge however, the CSF DOPAC and HVA levels increased by more than 100% in control mice, but showed little increase in MitoPark mice. Thus, we have developed a novel lab test that can potentially be used for screening for early PD in high risk population.

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Phenotypic Screening and Functional Genetics in FTD/ALS and Parkinsonâ€™s Disease
Parkinsonâ€™s disease (PD) and Frontotemporal Dementia/Amyotrophic Lateral Sclerosis (FTD/ALS) are progressive neurodegenerative diseases that are neuropathologically defined by the presence of protein aggregates and the loss of specific subsets of neurons. Both diseases are associated with variants in genes that are implicated in lysosome function and proteostasis. Therefore, it is likely that converging disease pathways exist across these subsets of neurodegeneration, and the interrogation of these pathways can be used both to enhance our understanding of these disorders and lead to innovative therapeutic interventions. We are currently utilizing our modified i3Neuron platform to facilitate systematic single and pairwise knockdown (KD) of large panels of PD and FTD/ALS-associated genes through CRISPR interference (CRISPRi) to interrogate gene-gene interactions. Our efforts focus on generating the first transcriptomic and phenotypic genetic interaction maps between the major genetic causes and risk factors of PD and FTD/ALS in iPSC-derived neurons. In our preliminary screen of single-

knockdown targets, weâ€™ve identified that depletion of the vATPase A1 subunit, ATP6V0A1, diminishes lysosomal acidification. Interestingly, this gene lies within a PD GWAS locus, further emphasizing the genetic link between lysosomal pH and PD. Our recent data implicates lysosomal dysfunction as a central pathophysiological consequence of mutations in the FTD/ALS gene GRN due to defects in endolysosomal acidification and/or maturation. Additionally, single or pairwise knockdown of TMEM106B, a known interactor of GRN, has revealed a clear role as a lysosomal modulator, as our preliminary data show a significant shift in lysosomal morphology when TMEM106B is not present in our iPSC-neurons. These promising initial results demonstrate that identifying common pathogenic changes elicited by disease genes can help us understand the underlying converging mechanisms in PD and FTD/ALS. Our continued approach will include screening across several key cellular processes: endolysosomal acidification and maturation, lysosomal ion homeostasis, selective autophagy, organelle quality control, and neuronal survival. Through these efforts, we hope to lay the groundwork for improved molecular understanding and therapeutic discovery.

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Arf1-Ablation-Induced Neuronal Damage Promotes Neurodegeneration Through A NLRP3 Inflammasome-Meningeal Î³Î´ T cell-INFÎ³-Reactive Astrocyte Pathway
Neurodegenerative diseases (NDs) are often initiated from neuronal injury or disease and propagated through neuroinflammation and immune response. In last few years, scientists started to realize the importance of immune cells in NDs. However, most researches have focused on the nonspecific, innate immune cells, such as microglia. Accumulated evidences suggest that the neurodegenerative process involves communications and interactions among neuron, microglia, astrocyte and immune cells. However, the mechanisms by which injured neurons induce neuroinflammation and immune response that feedback to damage neurons are largely unknown. Here, we found when ablated Arf1 in the adult mice (Tamoxifen induce UBC-CreER/Arf1f/f) will cause neurodegeneration phenotype, using the neuronal (Nes-Cre, Thy1-CreER), oligodendrocytes (PLP1-CreER, Pdgfra-CreER, Sox10-CreER), microglia (Cx3CR1-CreER, TMEM119-CreER), astrocytes (GFAP-CreER) and monocytes (LysM-CreER) inducible Cre mice line to cross with Arf1 loxP mice, and ablation of Arf1 in the different brain cells, We found the neuronal specific knockout Arf1 can mimic the whole body Arf1 knockout neurodegenerative

phenotype. We demonstrated that Arf1 ablation in adult mouse neurons resulted in activation of a reactive microglia-A1 astrocyte-C3 pathway in hindbrain and midbrain but not in forebrain, which further caused demyelination, axon degeneration, synapse loss and neurodegeneration. We further found that the Arf1-ablated neurons released peroxided lipids and ATP that activated a NLRP3 inflammasome in microglia to release IL-1Î², which together with elevated chemokines recruited and activated Î³Î´ T cells in meninges. The activated T cells then secreted INFÎ³ that entered parenchyma to activate the microglia-A1 astrocyte-C3 neurotoxic pathway for destroying neurons and oligodendrocytes. We also showed that the neurodegenerative phenotypes of Arf1-ablated mice are strongly ameliorated by deficiency of INFÎ³, Rag1 and NLRP3 but not of TLR4. Finally, we show that the Arf1-reduction-induced neuroinflammation-INFÎ³-gliosis pathway exists in various human neurodegenerative diseases, particularly in amyotrophic lateral sclerosis and multiple sclerosis. This study illustrated perhaps the first complete mechanism of neurodegeneration in a mouse model. Our finding introduced a new paradigm in neurodegenerative research and provided new opportunities to treat neurodegenerative disorders.

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Exosomal MicroRNAs and Proteins are Linked to PTSD and Depression Symptoms in Veterans with mild Traumatic Brain Injury
Sustaining a mild traumatic brain injury (mTBI) can be associated with persistent or worsening neurobehavioral dysfunction years after injury. Finding biomarkers that relate to specific symptoms may provide necessary insights for developing clinical interventions for those most at risk for poor outcomes following a mTBI. Exosomes are nanosized vesicles released by cells and involved in cell signaling through the release of their cargo, which is rich in proteins and microRNAs (miRNAs), and thus, a biomarker source. In this study, we examined relationships between exosomal proteins and miRNAs and persistent post-traumatic stress disorder (PTSD) and depression symptoms in a cohort of Veterans with a remote history of mTBI. We isolated peripherally circulating exosomes from plasma samples and measured cargo levels of 798 miRNAs and nine proteins: Neurofilament light chain (NFL), Tau, phosphorylated Tau (p-tau), Amyloid beta (Abeta) 42, Abeta 40, which are markers linked to neurodegenerative processes; vascular endothelial growth factor (VEGF), an angiogenesis and vascular injury marker; interleukin (IL)-10, IL6, and tumor necrosis factor-alpha, cytokines implicated in inflammatory responses. Proteins were measured using Single Molecule Array technology and miRNA profiles were analyzed using nCounter® miRNA Expression Panels. Subjects (n = 154) were divided into 4 groups: Controls (no TBI history and without symptoms); TBI only (TBI history, but no symptoms); TBI/DEP (TBI history and depression symptoms); TBI/DEP/PTSD (TBI history, depression and PTSD symptoms). For the protein analysis, we observed higher levels of exosomal NFL in the TBI/DEP/PTSD group when compared to control ($p = 0.0286$) and TBI only groups ($p = 0.0018$), and group differences persisted when controlling for number of TBIs and time since last TBI in logistic regression models. NFL also significantly discriminated TBI/DEP/PTSD from TBI only (AUC = 0.88) and control groups (AUC = 0.82). For miRNA analysis, we observed 40 differentially expressed miRNAs when comparing

TBI/DEP/PTSD to TBI/DEP groups. When comparing TBI/DEP/PTSD to TBI only group and controls, we found 28 and 21 differentially expressed miRNAs, respectively. Our findings suggest the potential of NFL and specific miRNAs as prognostic biomarkers in TBI, providing possible mechanisms and signaling pathways underlying the development of persistent neurobehavioral symptoms following head injury.

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Impaired energy metabolism and mitochondrial damage precede rod photoreceptor cell death in retinal neurodegeneration caused by Pde6b (rd1/rd1)
Introduction: Inherited Retinal Degenerative diseases (IRDs) are a major cause of blindness mainly caused by premature death of rod photoreceptor cells in the retina. As a clinically and genetically heterogenous group of pathologies, IRDs can either be congenital or have an early-/mid- onset where progressive degeneration has severe impact in quality of life among patients. Although more than 270 different genes and loci have been implicated in IRDs, their disease mechanisms remain obscure resulting in little hope for therapy. In this study we use a multi-omics approach to elucidate gene network and pathways underlying disease pathogenesis in the rd1 mice model which mimics the human pathology and has the most aggressive form of degeneration. Methods: We designed this unique study to focus on the early stages of the rd1 retina - Postnatal day (P) 2 to P10 - to identify the molecular events in rod photoreceptors before cell death begins at P12. We performed time course RNAseq of purified rods and integrated it with mass-spectrometry based proteomics and metabolomics from rd1 and WT retina. Validations were performed with ultrastructural analysis and functional assays. Results: Transcriptomic analysis revealed co-expression modules of oxidative phosphorylation (OXPHOS) and energy metabolism genes to be significantly associated with rd1. Proteomic comparisons between WT and rd1 retina confirmed differential abundance of core subunits of OXPHOS complexes at P6, with further exacerbation by P10. Electron microscopy analysis of the rd1 retina between P6 and P10 highlighted loss of mitochondrial cristae, corroborating molecular findings of dysregulation of OXPHOS components as cells get close to disease onset. At the same time, metabolomics uncovered reduced flux in central carbon metabolism and additionally showed deregulation of essential biosynthetic pathways such as nucleic and amino acid metabolism. Furthermore, Seahorse assays validated lower mitochondrial reserve capacity and energy metabolism in ex vivo mutant retina. Conclusion: This study for the first time describes molecular changes in rod photoreceptors before they succumb to apoptosis in IRDs. Through an omics driven approach we identify mitochondrial energy metabolism as a molecular converging point, leading us to hypothesize a critical role of metabolism and the mitochondria in IRDs. Identification of these disease related pathways will aid therapy development and disease management.

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Omics - Genomics/Metabolomics/Proteomics
Machine learning integration of quantitative postmortem neuropathology and shotgun proteomics nominates cerebrospinal fluid drivers of neurodegeneration in progressive multiple sclerosis
There is a paucity of in vivo biomarkers for axonal loss in multiple sclerosis (MS) and current surrogate neuroimaging metrics are non-specific and do not fully capture the extent of neurodegeneration observed post-mortem, highlighting an unmet need for disability-relevant biomarkers in MS. Using quantitative neuropathology, our group has shown an association between meningeal inflammation and axonal loss, suggesting that a neurotoxic cerebrospinal fluid (CSF) milieu plays a critical role in disability-relevant pathology. Therefore, we profiled the CSF proteome of our unique human post-mortem cohort of well characterized progressive MS cases. By evaluating the influence of proteomic variation of tissues readily accessible during life (CSF) on pathologic changes (axonal loss) only detectable, at present, after death, we have a unique translational platform to identify key biomarker candidates that associate with axonal loss, and by extension clinical outcome that can subsequently be validated. Using orthogonal partial least squares discriminant analysis (OPLS-DA), we identified a protein signature in the CSF of MS cases that segregates with axonal loss in the post-mortem spinal cord. The dysregulation of leukocyte elastase inhibitor, increased with high levels of axonal loss, was deemed highly significant. Importantly, neurofilament light chain levels, which are often expressed at lower levels in end-stage disease, did not correlate with axonal loss, suggesting this surrogate disability biomarker is not sufficiently sensitive to capture disability progression across the lifespan. To validate our post-mortem results, we performed an aptamer-based proteomic analysis on 246 CSF samples from individuals with non-demyelinating pathology and MS. We were able to confirm that our candidate biomarkers, including leukocyte elastase, were similarly dysregulated in the CSF of MS patients collected in the clinic relative to controls, lending further support to our translational approach. We now aim to identify the distribution and extent of our candidate biomarkers within post-mortem tissue and determine whether the levels of our candidate proteins in the CSF of living MS patients correlate with rate of clinically measured progression. Overall, we leverage a unique platform to identify biomarkers that can be validated in vivo and ultimately provide diagnostic and prognostic value while also highlighting pathogenic mechanisms of neurodegeneration in progressive MS.

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Omics - Genomics/Metabolomics/Proteomics
Regulation of B cell receptor-dependent NF-kB signaling by the novel tumor suppressor KLHL14 in diffuse large B cell lymphoma
Diffuse large B cell lymphoma (DLBCL) is the most common and aggressive form of non-Hodgkin lymphoma. Although more than half of DLBCL is curable with standard immunochemotherapy, activated B cell-like (ABC) DLBCL subtype has a poor prognosis. Thus, identification of subtype specific vulnerabilities based on genomic and molecular features is essential to develop targeted and personalized therapies. From genomic analysis of 574 DLBCL biopsy samples, we identified KLHL14 mutations were highly enriched (29.6%) in the recently defined MCD (MYD88L265P/CD79B mutation)

genetic subtype of DLBCL, the subset of ABC DLBCLs that rely on B cell receptor (BCR) signaling for survival. KLHL14 belongs to the Kelch-like family of proteins that can function as subunits of Cullin-RING ubiquitin ligase complex. Deficiency of Klhl14 in mice results in embryonic lethality while Klhl14 heterozygous mice are viable and show reduction of B-1a B cells, suggesting a role of KLHL14 in B cell differentiation. However, the impact of cancer mutations and the molecular function of KLHL14 in DLBCLs are unknown. To investigate the functional consequences of KLHL14 mutations, we expressed lymphoma-derived KLHL14 mutant isoforms and wild-type (WT) in ABC cells and monitored cell growth. Whereas KLHL14 (WT) was toxic, KLHL14 mutants had little if any effect, suggesting they are loss-of-function variants. Next, we conducted quantitative proteomics and ubiquitinomics to explore the global ubiquitin signaling networks and proteomic dynamics regulated by KLHL14. The BCR subunits were ubiquitylated and those of protein abundance were concomitantly decreased in KLHL14-expressing cells. Mechanistically, KLHL14 promoted the turnover of immature glycoforms of BCR subunits in the endoplasmic reticulum. Conversely, deletion of KLHL14 by an inducible CRISPR/Cas9 system confered relative resistance to the Bruton tyrosine kinase (BTK) inhibitor ibrutinib, suggesting loss of KLHL14 promotes BCR-dependent NF-kB activation and survival. Consistently, our functional genomic studies by CRISPR screens revealed that the viability of KLHL14-inactivated cells relied to a greater extent than KLHL14 (WT) cells on mediators of NF-kB pathway under the pressure of ibrutinib. In summary, we have unconvered a tumor suppressive function of KLHL14. These findings suggest the genetic status of KLHL14 should be considered in precision medicine trials testing inhibitors of BTK and BCR signaling regulators in DLBCL.

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Omics - Genomics/Metabolomics/Proteomics
Bladder Cancers Affecting Transplant Recipients Harbor Diverse Viruses That Associate with Overall Survival
BK polyomavirus (BKPyV) is a ubiquitous virus that establishes a lifelong subclinical infection in the urinary tract. In transplant recipients, BKPyV can reactivate and cause kidney and bladder damage. Recent sequencing studies have revealed that BKPyV sequences are present in approximately 25% of bladder tumors affecting organ transplant patients. This is dramatically higher than the <1% rate of BKPyV detection in muscle-invasive bladder cancers affecting the general population. Like cancer-causing human papillomaviruses (HPVs), BKPyV has been shown to upregulate the human cytosine deaminase APOBEC3B, which has emerged as a source of the second most abundant mutation signature in human cancers. To comprehensively assess the role of BKPyV and other viruses in bladder cancer from solid organ transplant recipients, we used a database linking US transplant and cancer registries to identify and collect 44 archived primary bladder tumors, 5 metastases, and 15 adjacent normal tissue specimens. Whole genome and total RNA sequencing was performed on the samples. From these data, we detected BKPyV DNA and RNA in 18% of primary tumors. In five tumors, there is clear evidence of BKPyV integration into the host cell genome. In a separate set of analyses, BKPyV transcription was detected in 3.7% of non-muscle-invasive early-stage bladder cancer cases affecting the general population. Our surveys also detected other viruses, including high and low risk HPVs, JC polyomavirus

(JCPyV), and anelloviruses. Nearly all tumors had dominant APOBEC3 signature mutations and BKPyV-positive tumors expressed significantly more APOBEC3B transcripts compared to virus-negative tumors or normal tissues. Clinical data indicate that BKPyV-positive tumors are predominantly high grade with invasive behavior. Strikingly, survival was significantly shorter for patients whose tumors harbored BKPyV or JCPyV (9.7 and 8.4 months, respectively) compared to patients with tumors harboring HPV or no detectable viruses (65.8 and 50.2 months, respectively)($p = 0.0004$). This study comprehensively characterizes the genomes and transcriptomes of tumors, revealing several distinct tumor etiologies that affect patient outcome.

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Development of a high throughput NanoBRET screen for inhibitors of the Ras-Raf interaction

The Ras/MAPK pathway (consisting of the Ras/Raf/MEK/ERK proteins) regulates key cellular processes that are required for normal growth and development and which contribute to various human disease states. Activating mutations in Ras are found in approximately 30 percent of all human cancers, delivering a persistent signal to critical effectors, such as RAF, that drive tumor development and maintenance. Moreover, activating mutations in RAF also function as cancer drivers, with BRaf-V600 mutations being the most common. Pharmacological targeting of the core kinase components in the Ras/MAPK pathway has led to the development and FDA approval of multiple kinase inhibitors, including the RAF inhibitors vemurafenib and dabrafenib and MEK inhibitors trametinib and cobimetinib. However, inhibitor-induced RAF dimerization and/or loss of downstream feedback inhibition, especially in the context of Ras mutant cancers, can result in innate or acquired drug resistance. Thus, additional therapeutic approaches are needed. Towards this end, we have utilized the proximity based NanoBRET system to monitor the interaction of RAF with a constitutively activated Ras allele in live cells, with the goal of identifying molecules that can modulate this critical interaction. Utilization of an assay that retains the normal spatial environments of the cell required for signal transmission, greatly increases the opportunity of finding effective compounds as both direct and indirect disruptors will be found. Using this strategy, approximately 280,000 pure compounds and natural product extracts from the NCI repository were screened for drugs/extracts that can either disrupt or prevent the Ras/Raf interaction. From the primary screen, 33 pure compounds and 99 natural product extracts were found to reduce the BRET signal by more than 60 percent, indicating a loss in Ras/Raf binding. To aid in the validation of hits, NanoBRET stable cells were created in order to monitor the interaction under near physiological conditions. Using these cells, confirmatory dose-response curves have been conducted, and studies are ongoing to determine the effects of these compounds on Ras-dependent signaling as well as the mechanism by which these compounds inhibit the Ras/Raf interaction. Thus, with use of the live-cell NanoBRET system, we hope to identify novel, clinically relevant compounds that can inhibit the Ras/Raf interaction and in turn suppress aberrant Ras/MAPK signaling.

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Glucocorticoid receptors are functionally involved in escalated sufentanil vapor self-administration in female rats
The increase in opioid overdose deaths over the last several years has led to the declaration of an opioid crisis and public health emergency in the USA. Over 50% of opioid overdose deaths have been attributed to the synthetic opioid fentanyl. Fentanyl and analogues such as sufentanil are common heroin adulterants, and are often consumed by smoking or vaporizing, which causes rapid absorption into circulation through the lungs. Although females are equally or more prone to opioid addiction than males, research in female subjects has been scarce. Opioids are potent analgesic and euphorigenic drugs; however, dysphoric and stress-sensitized emotional states emerge with opioid dependence during withdrawal. We hypothesized that these negative emotional states of opioid dependence involve an overactivation of stress circuitry in the brain, including extrahypothalamic glucocorticoid receptor (GR) signaling. We tested the hypothesis that GR activity contributes to opioid addiction-like behaviors in female rats. For these experiments, we utilized a rodent model of vaporized opioid self-administration, where rats were trained to lever press for sufentanil vapor in 1 h (short access, ShA) or 12 h (long access, LgA) sessions, 3 times a week. The rats in ShA conditions displayed a stable level of self-administration throughout the experiment. In contrast, the rats in LgA conditions significantly escalated their intake over time and exhibited significantly greater somatic (i.e., physical) signs of opioid dependence. Treatment with mifepristone, a mixed glucocorticoid and progesterone receptor antagonist, significantly reduced sufentanil self-administration in the LgA but not ShA group. To examine

the specific role of GR in this effect, we then utilized a GR-selective antagonist, CORT113176, that is inactive at progesterone receptors. Consistent with our hypothesis, GR-specific antagonism with CORT113176 reduced sufentanil self-administration in female rats tested under LgA not ShA conditions. Together, these findings indicate that GR is engaged in opioid dependence and plays a functional role in opioid addiction-like behavior in female rats. Continued research on GR signaling in both male and female subjects will provide a better understanding of the role of stress systems and sex differences in the etiology of opioid use disorder, and may contribute to the development of new strategies to prevent, diagnose and treat opioid use disorder.

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Evaluating the Toxicological Screening Utility of 3D Spheroid Primary Human Hepatocyte Cultures
Humans are regularly exposed to hundreds of chemicals in the environment that have limited hazard data, placing pressure on regulatory agencies to make safety decisions. While animal studies are considered the benchmark for hazard assessment, they are expensive, time consuming, low throughput, and do not sufficiently predict human toxicity. Toxicology screening holds promise to more efficiently test hundreds of chemicals for hazard identification. However, initial screens relied on two-dimensional (2D) cancer cell-lines and were criticized for the lack of physiological relevance, especially xenobiotic metabolic competence. Three-dimensional (3D) cell culture models are transforming in vitro toxicology through their potential for enhanced translational relevance by exhibiting tissue-like functionality. Primary human hepatocytes (PHHs) are considered the gold standard for assessing hepatic responses to xenobiotics in vitro, but rapidly lose their metabolic competence within hours, limiting their utility in toxicological screening. Our goal was to assess the feasibility and human relevance of PHHs cultured as 3D spheroids in modeling hepatotoxicity. Single-donor spheroids from 5 individual human donor livers were generated in 384-well ultra-low attachment plates. Spheroids from all donor preparations were viable through at least 28 days in culture and maintained physiologically-relevant activities of major drug metabolizing liver enzymes, including CYP3A4. 3D PHHs were exposed in concentration response to a set of reference chemicals for 96 hours and assessed for cytotoxicity. Drugs withdrawn from the market due to liver injury (e.g. troglitazone, cerivastatin) more potently caused cell death in 3D PHHs compared to safer drug analogues (e.g. rosiglitazone, atorvastatin). Aflatoxin B1, a known hepatotoxicant attributed to metabolic activation of toxicity, caused concentration-related cell death in 3D PHHs at significantly lower concentrations than 2D PHHs, consistent with their enhanced metabolic competence. Current work is using high-throughput transcriptomics to profile the biological activity of a diverse set of reference chemicals across donor preparations. This work demonstrates the utility of 3D PHH spheroids to predict liver injury potential and sets the stage for modeling genetic diversity in vitro. 3D PHHs could greatly improve the human relevance and predictivity of high-throughput toxicology screening for the safety evaluation of chemical and drugs.

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Physiology
Sex dimorphism in erythropoietin regulation of fat mass
Erythropoietin (EPO), the cytokine required for erythropoiesis, contributes to metabolic regulation of fat mass and glycemic control. In male mice and ovariectomized female mice on high fat diet (HFD), EPO treatment increased hematocrit, improved glucose tolerance and attenuated body weight gain via reduced fat mass. Estradiol supplementation abrogated the EPO effect on body weight in ovariectomized female mice, providing evidence for estrogen related gender specific EPO action in metabolic regulation. To determine estrogen response in metabolic regulation with EPO treatment, we use the estrogen receptor Î± knockout (ERÎ±KO) mice and mice with targeted deletion of estrogen receptor alpha in adipose tissue (ERÎ±adipoKO). Male and female ERÎ±KO mice on HFD exhibit increased fat mass and glucose intolerance. EPO treatment on HFD increased hematocrit in both wild type (WT) and ERÎ±KO mice and reduced gain in fat mass in male mice as well as female ERÎ±KO mice but did not affect fat mass in female WT mice. The improvement in glucose tolerance and insulin tolerance with EPO treatment during HFD was significantly greater in female ERaKO mice compared with female WT. We previously demonstrated that specific deletion of EPO receptor in adipose tissue in mice decreases glucose tolerance and insulin sensitivity and increases susceptibility to diet-induced obesity. On normal chow diet, EPO was not able to reduce fat mass in female WT and ERaKO mice but EPO improved GTT only female ERaKO mice not female WT. Although no decrease in body weight was observed in female WT and ERÎ±adipoKO with EPO treatment on HFD, female ERÎ±adipoKO also showed significantly improved glycemic control with EPO treatment compared with WT control. EPO treatment decreased white fat associated gene expression, Psat1 and Wdnm-1like in subcutaneous fat pads from both female ERÎ±KO mice and ERÎ±adipoKO on HFD but not WT female mice. These results confirm the role of estrogen in the sex-differential EPO effect on fat mass regulation and suggest cross talk between EPO and estrogen signaling in metabolic homeostasis, fat mass regulation and glucose metabolism via estrogen response in white adipose tissue in female mice.

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Physiology

Î²3-Adrenergic Receptors Regulate Human Brown/Beige Adipocyte Lipolysis and Thermogenesis

Recently, adult humans have been shown to have metabolically active brown adipose tissue (BAT), a heterogenous tissue composed of white, brown, and brown-like (â€œbeigeâ€) adipocytes. Activated human BAT increases resting energy expenditure and ameliorates lipid and glucose metabolism. Thus, understanding the mechanisms of human BAT activation is of great interest for developing strategies to treat metabolic disease. Clinical evidence links the administration of the selective human Î²3-adrenergic receptor (Î²3-AR) agonist, mirabegron (Myrbetriq®), to increased BAT metabolic activity, tissue glucose uptake, increased plasma free fatty acids, and improved insulin sensitivity. Therefore, we hypothesized that the Î²3-AR in human brown/beige adipocytes plays a role in thermogenesis and lipid metabolism. To develop an in vitro model to study human BAT, we isolated primary brown/beige adipocytes from supraclavicular fat (16y female, NCI, NIH). Tissue relevance was established by showing that primary human brown/beige adipocytes expressed brown/beige adipocyte lineage markers, thermogenic genes, and Î²3-ARs, closely recapitulating the profile of human BAT. Next, we tested the functional roles of the Î²3-ARs in human brown adipocytes by silencing the receptor in differentiated cells using siRNA. Silencing ADRB3 expression via siRNA-ADRB3 decreased gene expression of tissue-specific thermogenic UCP1; fatty acid oxidation enzymes; and mitochondrial mass, which was supported by immunostaining and genes encoded by mitochondrial DNA. Functionally, silencing the Î²3-AR lowered basal cAMP levels compared to controls, and it blunted agonist-mediated increases in cAMP levels and lipolysis. Consistent with reduced thermogenic gene expression, mitochondrial mass, and lipolytic capacity that is necessary for BAT thermogenesis, silencing ADRB3 lowered cellular respiration, glucose oxidation, and glycolysis compared to the siRNA-control brown/beige adipocytes. Furthermore, mirabegron stimulated BAT lipolysis and thermogenesis in control adipocytes, but both processes were prevented after silencing Î²3-AR expression. In summary, human brown/beige adipocytes express functional Î²3-ARs that play a pivotal role in proper maintenance of downstream lipolytic and thermogenic machinery. Additionally, mirabegron stimulates lipolysis and thermogenesis via the Î²3-AR, which supports the use Î²3-AR agonists to achieve metabolic benefit in humans.

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Physiology

MicroRNA Regulation of RGS4 in an Asthmatic Mouse Model of Allergic Inflammation

RATIONALE: Allergic asthma is the most common type of asthma. It is triggered by inhaled allergens and affects an approximate of 30 million people worldwide. Many host-derived inflammatory mediators present in asthmatic airways induce bronchospasm by acting on G protein-coupled receptors (GPCRs). GPCRs are negatively modulated by a large group of intracellular proteins called regulators of G protein signaling (RGS) proteins. RGS4, specifically, is expressed in respiratory epithelium and airway smooth muscle, with expression that increases in proportion to clinical severity. Rgs4-/- mice have reduced susceptibility to develop airway hyperresponsiveness compared to wild type (WT) mice. The role of post-transcriptional regulators, such as microRNAs (miRNAs), in RGS4 expression remain unknown. Here, we hypothesized that miRNAs are critical regulators of RGS4 expression and may thereby affect

the phenotype of allergen-challenged mice. METHODS: We challenged WT and Rgs4-/- mice intranasally with PBS or secreted filtrates of Aspergillus fumigatus (Af), a fungus that triggers type 2 immunity and is closely associated with severe asthma. Animals were treated three times per week over a two-week period. Total RNA was extracted from the lungs using the miRNeasy mini kit followed by miRNA sequencing analysis and validation studies. Data analysis was performed using the R software and Ingenuity Pathway Analysis. RESULTS: We identified five differentially expressed and statistically significant miRNAs (miR-3535, miR-677-5p, miR-874-3p, miR-150-3p, miR-574-5p) in the lungs of WT mice compared to Rgs4-/- mice at homeostasis. We also found differentially expressed miRNA signatures in WT and Rgs4-/- mice challenged with Af. In silico pathway analyses identified biological networks (e.g. immune cell trafficking, inflammatory disease and response) affected by RGS4 and exposure to Af that ranged from direct predicted gene targeting to complex interactions with multiple intermediates. Interestingly, there were sex differences in miRNA expression and predicted regulatory networks in PBS-treated Rgs4-/- mice when compared to Af-treated Rgs4-/- mice. CONCLUSIONS: Our results indicate that RGS4 can influence lung miRNA expression in response to Af exposure, indicating that RGS4-dependent miRNA regulation of inflammatory gene expression could affect the predisposition to develop allergic airway inflammation.

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Physiology
E-selectin-dependent neutrophil recruitment in adipose tissue exacerbates steatosis-to-NASH progression
Background Non-alcoholic steatohepatitis (NASH) is a highly prevalent chronic liver disease characterized by lipid accumulation, inflammation and fibrosis. The mechanisms involved in the transition of benign steatosis to NASH have not been fully elucidated. Inflammation in the liver and adipose tissue (AT) is dependent on leukocyte-endothelial cell interactions. However, the interplay between neutrophil recruitment in AT and the progression of NASH is poorly understood. Objective The aim of this study is to investigate E-selectin-mediated neutrophil infiltration in the liver and AT and investigate how its inhibition influences steatosis-to-NASH progression. Methods Wild type (WT) and E-selectin knockout mice (Sele KO) were fed a high-fat diet(HFD) followed by intravenous injection of adenovirus overexpressing Cxcl1(AdCxcl1), to mimic NASH. HFD-fed mice were used as steatosis controls. Human data was obtained from transcriptomics datasets of liver and AT samples from patients suffering from NASH and steatosis. Results E-selectin (SELE) was the highest upregulated adhesion molecule in both liver and AT of NASH patients versus steatosis patients. SELE expression in both AT and liver correlated with the expression of IL1B and CCL2, suggesting that these inflammatory mediators likely contribute to SELE upregulation in NASH. HFD+AdCxcl1 animals also showed increased Sele, Il1b and Ccl2 expression in comparison to control animals. When compared to WT, Sele KO mice showed a significant decrease in ALT serum levels and a lower number of liver MPO and F4/80 positive cells, representing neutrophils and macrophages, respectively. In these animals a significant reduction fibrosis was observed by downregulation of profibrotic markers, such as Tgfb and Acta2 and decreased Sirius Red staining. In AT samples, Sele KO also resulted in a downregulation of pro-inflammatory cytokines (Il1b, Ccl2, Tnfa), which correlated with a decrease in apoptosis. A downregulation of lipolysis markers

and a decrease of serum free fatty acids (FFA) indicated a reduction in lipolysis. This drop in circulating FFA, which aggravate NASH, suggests that E-selectin inhibition in both the liver and AT reinforce a reduction of NASH. Conclusion Overall, our findings suggest that E-selectin plays a dual role in the induction of inflammation in both liver and AT, thereby promoting NASH progression. This molecule might, therefore, represent an effective therapeutic target against this disease.

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The mosquito protein AZ1 has both cytolytic and antiviral properties
Mosquito-borne flaviviruses such as yellow fever, zika, and dengue represent a major burden on human health, with dengue alone being responsible for nearly 50 million infections annually. While the anthropic effects of these pathogens have been well documented, we identified an anti-viral protein from the arthropod host that has received much less attention, potentially passing up avenues for the development of novel therapeutics. The mosquito protein Aedes Zika-related 1 (AZ1) is upregulated in response to both blood meals and infection by flaviviruses such as Zika, suggesting a role in both digestion and viral defense. However, its exact function or mechanism of action for either role is unknown. Solving the structure of AZ1 revealed a striking similarity to the cockroach allergen Bla g 1, which binds a wide range of lipids within a large central cavity. Molecular modeling, circular dichroism, and solution NMR reveal that AZ1 binds fatty acids in a similar manner, with the lipid cargoes exerting a significant stabilizing effect suggesting that they are integral to AZ1â€™s biological function. Indeed, AZ1 was able to lyse red blood cells and to a lesser extent other eukaryotic cells, with its cytotoxicity being dependent on its lipid cargo. Inhibiting the cell membrane repair pathway enhanced lethality, while studies using fluorescent fatty-acid analogues coupled with lipophilic probes for membrane fluidity suggest that AZ1 delivers its lipid cargo into biological membranes, destabilizing them in the process, and resulting in cell death. In a biological context, this could facilitate the breakdown of ingested blood and other cells within the mosquito gut, contributing to AZ1â€™s role in digestion. In terms of antiviral properties, micromolar levels of AZ1 prevented viral infection of Zika and Dengue viruses. This effect displayed similar cargo dependence as cytolysis. Weaker antiviral activity was observed against lentivirus, but was not detectable against adeno-associated virus. The latter is unique among the pathogens tested in that it lacks a lipid envelope, suggesting that antiviral activity of AZ1 is also

grounded in its ability to disrupt biological membranes. These results describe a common mechanism for both putative functions of AZ1 and elucidates the purpose of a protein whose entomological role was unknown, providing a potential avenue for the development of therapeutics to ease the burden of mosquito-borne viruses on human health.

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How enzymes add short and long glutamate chains to tubulin tails to functionalize microtubules
Microtubules, a major component of the cytoskeleton are assembled from dimers of ï¿¡Î²-tubulin. Despite being synthesized from the same building block, microtubules give rise to structures with highly varied geometries and dynamics such as the axoneme of a cilium, the mitotic spindle or the axon of a neuron. This functional diversity is facilitated by multiple evolutionarily conserved post-translational modifications on the C-terminal tails of both alpha-and Î²-tubulin. These constitute a â€˜tubulin codeâ€™, that akin to the histone code, is read by essential microtubule effectors to elicit specific physiological responses. Polyglutamylation is the most abundant modification found in the mammalian brain. It is the reversible addition of single or multiple glutamates to the genetically encoded glutamates of the tubulin C-terminal tails and is catalyzed by the tubulin tyrosine ligase like (TTLL) family of enzymes. The length of the glutamate chains regulates the binding and activity of molecular motors and microtubule associated proteins. TTLLs are thought to be specialized for either initiating or elongating the polyglutamate chain. However, biochemical and mechanistic basis for such segregated activity is lacking. To address this issue, we performed biochemical characterization of TTLL4, and TTLL6 and found that the former is specialized for branch initiation while the latter specifically elongates from these branch points. To further understand the structural basis for this regioselectivity, we solved crystal structures of TTLL6 in complex with initiation and elongation intermediate analogs and identified key active site residues that make TTLL6 a chain elongating enzyme. Through phylogenetic analysis, we engineered mutations in the active site of TTLL6 and converted it into a branch initiating enzyme. We then solved the crystal structure of the mutant in complex with the analogs and found that the engineered mutations provide necessary hydrogen bonds to position the acceptor glutamate in such a way that it favors branch initiation while the corresponding wildtype residues help position the acceptor glutamate favorable for branch elongation. Phylogenetic analyses also revealed that the same active site residues discriminate between initiation and elongation among all TTLLs. In conclusion, our data sheds light on how TTLLs regulate the position and length of the polyglutamate chains on tubulin, thus giving rise to complex microtubule patterns observed in cells.

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Protein Structure/Structural Biology

Structural insights into inhibition of innate immune effector PKR by viral non-coding RNAs

Double-stranded ribonucleic acid (dsRNA) is frequently a hallmark of viral infection. In vertebrates, dsRNA-activated protein kinase R (PKR) is triggered by viral dsRNAs to induce an innate immune response. Activated PKR phosphorylates the alpha subunit of the eukaryotic translation initiation factor 2 (eIF2) thereby blocking translation initiation to curb infection. In the constant arms race between the host and viruses, the latter have evolved versatile RNAs that inhibit PKR to escape the host immune response. Despite the key roles of PKR in innate immunity and in inflammation, it remains poorly understood how different RNAs, through diverse secondary and tertiary structures, can exert opposite regulatory effects on PKR. To understand how RNA inihibits PKR, we have biochemically and structurally charaterized the adenovirus VAI RNA; the first discovered viral non-coding RNA and a potent inhibitor of PKR. We report a 2.7 Å crystal structure of VAI RNA, which shows how a 23 base-pair (bp) apical stem (AS) is extended by a strictly conserved tetrastem to form a coaxially stacked duplex region of 27 bp. A 3-bp pseudoknot at the center of the RNA acts as a topological device to bring the tetrastem spatially close to the AS. Importantly, mismatches in the tetrastem abolished VAI RNAâ€™s ability to inhibit PKR. While the AS alone does not inhibit PKR, fusing the AS with the tetrastem through a direct covalent linkage generates a 27-bp RNA able to block PKR function as efficiently as the entire VAI RNA. Hence we define this region as the minimal core effector for PKR inhibition. This work shows how the VAI RNA juxtaposes the tetrastem with the AS to form a dsRNA region that can tighlty bind PKR while staying under the 30 bp dsRNA length threshold typically required for PKR activation. We also show that the human HIV-I trans-activating TAR RNA inhibits PKR as efficently as VAI. TAR is predicted to form a 24-bp duplex RNA. Importantly, shortening the lower stem of TAR blocks its capacity to inhibit PKR. Taken together, these results reveal how different viruses converge on a similar strategy to block PKR activation via distinct structural elements. PKR is sequestrated in its inactive state by an imperfect 24-27 bp dsRNA segment. In conclusion, this work provides mechanistic insights into PKR control by RNA, expands the principles of discriminating self vs non-self RNA, and inform the design of functional RNA devices to elicit specific PKR activities.

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Protein Structure/Structural Biology

A structural basis for neutralization of Nipah virus via antibody-mediated targeting of the fusion glycoprotein

Nipah virus is a highly pathogenic paramyxovirus capable of causing severe neurologic and respiratory disease in humans. Bat populations act as the natural reservoir of Nipah virus, from which near annual spillover events into humans result in outbreaks with fatality rates reaching 90%. Currently, there are no licensed vaccines or therapeutics against Nipah virus, which, combined with its severe pathology, has led the WHO to classify Nipah as a priority pathogen, underscoring the urgent need for research and development of countermeasures. The Nipah virion surface is decorated with two glycoproteins, termed NiV-G and NiV-F, which allow the virus to attach to and enter host cells, respectively. As the sole proteinaceous antigens on the virus surface, NiV-G and NiV-F are exposed and accessible for host

immune recognition and thus make attractive vaccine targets. Indeed, previous studies have shown that generation of neutralizing antibodies against these glycoproteins is critical to control Nipah virus disease in animal models. In order to identify immunologically vulnerable target sites on the virus surface and to elucidate the molecular basis for antibody-mediated neutralization of Nipah virus, we sought to define the epitopes of monoclonal antibodies against NiV-F using X-ray crystallography and cryo-electron microscopy. We determined the structures of NiV-F in complex with the Fab fragments of two neutralizing antibodies, mAb66 and mAb92. The crystal structure reveals that Fab66 binds an epitope at the most membrane distal apex region of NiV-F. The interaction is light chain dominated and insertion of the CDR3 loop into a shallow depression on the NiV-F surface allows several key tyrosine residues on Fab66 to form stabilizing contacts with NiV-F. Characterization of mAb92 using cryo-electron microscopy reveals an overlapping but distinct epitope also located at the membrane distal region of NiV-F, supporting the hypothesis that this apical region is accessible for immune targeting. Importantly, these membrane distal epitopes are highly conserved among Nipah virus isolates. Experiments to determine whether prophylactic treatment with mAb92 offers protection from a lethal Nipah challenge in hamster models are ongoing to evaluate the in vivo relevance of these epitopes. Combined, this work reveals the membrane distal region of NiV-F as a site of vulnerability on the Nipah virus surface and may inform the design of improved targeted vaccines in the future.

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Psychology and Psychiatry
Translatable THC withdrawal symptoms in male and female mice
Cannabis is the most widely used illicit drug and our societal climate towards legalization makes it critical to understand its neural actions. Delta-9-tetrahydrocannabinol (THC) is the major psychotropic component in cannabis and acts on the brainâ€™s endocannabinoid system to produce its abuse and addiction potential. Cannabis use disorder (CUD) is accompanied by several withdrawal symptoms (WS) including irritability, mood changes, hypophagia, but the most pronounced is sleep disruption in early withdrawal. Sleep disruption and other WS contribute significantly to relapse and are a major barrier in the treatment of CUD. There is little clinical research on this matter and much of it is confounded by pre-existing conditions and a sex-bias towards males, making preclinical research increasingly pertinent. Classically, THC WS are difficult to observe in rodents, and are predominantly elicited via treatment with a cannabinoid receptor-antagonist (precipitated), as opposed to abrupt drug cessation (spontaneous) that more appropriately models human withdrawal. Our laboratory has previously reported a role for endogenous cannabinoid signaling in sleep in mice, and we now aim to determine if spontaneous THC withdrawal-induced sleep disruption and other WS can also be modeled in mice. Using polysomnographic recordings we measured sleep-states before, during, and after chronic injections of THC or vehicle, and found that THC treated mice showed altered sleep architecture similar to that seen in human cannabis users, including: 1) augmented slow-wave (SWS) sleep after the first THC injection which normalizes by the last injection of a THC-treatment known to induce tolerance; and 2) destabilization of SWS and rebound of REM sleep during the first three days of withdrawal that normalizes to control levels after six days of withdrawal. Behaviors associated with motivation (lever

pressing for reward) and anhedonia (sucrose preference test) showed alterations that map to sleep-disruption time points. Additionally, each of these metrics show sex differences and may be explained by withdrawal-timepoint and region-specific alterations in dopamine release (measured via fast-scan cyclic voltammetry, ex-vivo) within the striatum, a brain region at the nexus of both drug addiction, motivation, and sleep. These are the first murine models of directly translatable cannabis WS, and facilitate further preclinical research examining neural mechanisms of CUD and cannabis withdrawal.

Ashley Cornell
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Psychology and Psychiatry
I hope they didnâ€™t see that: Social anxiety severity and the quality of recent peer interactions affect brain response following errors in the presence of peers
Pediatric social anxiety (SA) is a common illness that involves a fear of negative evaluation. SA youth report concerns about making mistakes in the presence of peers, yet the neural correlates underlying this common worry remain unclear. Further, while self-report measures reliably quantify SA symptoms, they fail to account for potentially important person-specific factors, such as the quality of recent peer interactions. Given the salience of such interactions to adolescents, we must account for them to understand how adolescent subjects process a socially stressful experience, such as making an error in the presence of a peer. Using a novel paradigm, we test associations among SA symptoms, the quality of recent peer interactions measured using ecological momentary assessment (EMA), social context (i.e., peer observation), and error-related brain response. Method. In the current analysis, 48 youth (8-18 years old, M=14.06 ± 2.67), 25 of whom met criteria for at least one anxiety disorder, completed a cognitive control task during fMRI. Participants completed half of the task while they believed they were being observed by a same-age, same-sex peer and half of the task while alone. All participants completed 1-week of smartphone-based EMA to assess the quality of recent peer interactions from â€œvery positiveâ€ to â€œvery negative.â€ Analyses used the average quality across the week. The SA subscale score from the SCARED was used as a measure of SA severity. SA severity and quality of recent peer interactions were continuous, between-subject variables in the analysis while social context (peer, alone) and trial type (error, correct) were repeated, within-subject variables. Age was a covariate. Results. Whole brain analyses (p<.005, k>50) revealed a significant SA X interaction quality X social context X trial type interaction in several prefrontal regions (e.g., IFG, dlPFC), cingulate cortex, amygdala, and superior temporal gyrus (STG) - regions implicated in error monitoring (cingulate, IFG), responses to threat (amygdala), and social cognitions (STG), among others. Follow-up tests revealed that youth with higher SA and more negatively rated interactions with peers showed greater activation in these regions when committing an error in the presence of a peer, compared to alone. Conclusions. These findings highlight the importance of framing our understanding of SA-related differences in brain response within the context of person-specific social experiences.

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The human hippocampus plays a time-limited role in retrieving autobiographical memories
The hippocampus is a medial temporal lobe structure that is required for memory encoding, but its role during retrieval is fiercely debated. The standard model of consolidation predicts a time-limited role for the hippocampus when recalling past events, hypothesizing that over time, retrieval becomes â€œconsolidatedâ€ to the neocortex. On the other hand, the competing multiple trace/trace transformation theories posit indefinite involvement of the hippocampus regardless of the age of a memory. Lesion evidence remains inconclusive, and the conclusions one can draw from fMRI have been limited by reliance on covert (silent) recall. This obscures dynamic, moment-to-moment content of retrieval and leaves only an impoverished sense of oneâ€™s internal experience during remembering. Here, we capitalized on advances in fMRI denoising to employ overtly spoken recall, overcoming fMRIâ€™s historical shortcomings and allowing clear access to the content of memories as they are recalled and described. Forty participants retrieved recent and remote memories, describing each for approximately two minutes. The content of each memory was identified following the common Autobiographical Interview procedure, and each recalled detail was modeled using a general linear model approach. Subsequently, BOLD activity associated with the recall of recent and remote events was directly compared. Bilateral posterior hippocampal and parietal regions exhibited temporally-graded activity, with more recent events evoking greater activity than more recent events, consistent with predictions of the standard model of consolidation. Analyses assessing the task-based connectivity between these regions and others associated with mental scene construction exhibited a similar gradient, again supporting the standard modelâ€™s prediction of reduced hippocampal interaction with the neocortex over time. Beyond addressing a longstanding debate in cognitive and systems neuroscience, the current results highlight the potential use of overt speech to more naturalistically probe cognitive processes in fMRI environments.

Anahit Mkrtchian
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Ketamine modulates fronto-striatal circuitry in depressed and healthy individuals
Unlike any existing antidepressant treatments, ketamine has rapid-acting antidepressant properties and particular efficacy in treating motivational dysfunction in depression. However, the precise neural mechanisms underlying ketamineâ€™s beneficial effects remain unknown. In particular, ketamine improves motivation-related symptoms in depression, but can also elicit symptoms of motivational impairment in healthy individuals, suggesting that it might have different effects in health and disease. This study examined whether ketamine affects the brainâ€™s reward system, which is known to drive motivational behavior. Considering that inflammation, as measured by C-reactive protein â€“ has shown to influence neural and behavioral motivational processes, we also examined if inflammation may underlie some of these changes. These questions were assessed in the context of a double-blind,

placebo-controlled, crossover trial of ketamine in 33 individuals with treatment-resistant major depressive (TRD) disorder and 25 healthy volunteers (HVs). Resting-state functional magnetic resonance imaging (rsfMRI) was acquired two days post-ketamine and post-placebo infusions and was used to probe functional connectivity in reward circuits using striatal seeds. Ketamine increased fronto-striatal functional connectivity in TRD participants towards levels observed in HVs, while shifting the connectivity profile in HVs towards a state similar to TRD participants under placebo. These effects were largely observed in the absence of inflammatory (C-reactive protein) changes and were associated with both acute and sustained improvements in symptoms in the TRD group. Ketamine thus normalized fronto-striatal connectivity in TRD participants but disrupted it in HVs independently of inflammatory processes. These findings highlight the importance of the reward circuitry in ketamineâ€™s mechanism of action. Specifically, shifts in fronto-striatal circuitry may represent a neurobiological circuit-level mechanism driving ketamineâ€™s beneficial effects on motivational symptoms, which may contribute towards mechanistically-informed treatments in the future.

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Memorability of Words in Arbitrary Verbal Associations Modulates Memory Retrieval in the Anterior Temporal Lobe

Despite large individual differences in memory performance, human observers consistently remember some stimuli better than others. Failure in recognizing commonly memorable items is indicative of early-onset Alzheimerâ€™s in older adults. It remains unknown, however, why stimuli vary in their memorability and how the human brain utilizes memorable information to facilitate memory processes. The lack of knowledge on these issues has limited further application of stimulus memorability property for early detection of memory-related disorders. To fill this gap, this study combines online cloud-sourced data, laboratory experiments, computational modeling, and direct recordings from the human brain to investigate the neurocognitive mechanism for the memorability of verbal stimuli. First, we tested a commonly used neuropsychology cue-recall verbal memory task on 2,623 online Amazon Mechanical Turk participants, using arbitrarily constructed word pairs from 300 common nouns. We found that certain words were correctly retrieved across individuals irrespective of the arbitrary cues used to initiate memory retrieval. Second, we modeled word memorability based on the intrinsic properties of words. We found that memorable words shared greater semantic similarity with other words on average. These words situated more centrally in the semantic network, such that they could be easily accessed from any arbitrary starting points during memory search. Third, we implemented the same verbal memory task in 30 participants who underwent intracranial electroencephalogram recording for seizure monitoring. Behaviorally, we replicated the memorability property of words from the online study and found that memorable words were retrieved faster. These words came to mind so easily that they led to more intrusion errors when retrieval failed. Neurally, we found that successful retrieval of memorable words resulted in faster reinstatement of neural activity in the anterior temporal lobe (ATL). Collectively, our data provide a novel neurocognitive account for the memorability of associative verbal content. That is, verbal memorability is determined by intrinsic properties of words

from our everyday linguistic experiences, such that the ATL is wired to prioritize memorable verbal content to facilitate memory retrieval. Ultimately, our findings shed light on how memorable failure for memorable verbal content is indicative of functional declines in the ATL in memory-related disorders.

Omar Al-Louzi
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Progressive multifocal leukoencephalopathy lesion and brain parenchymal segmentation from MRI using serial deep convolutional neural networks
Background: Progressive multifocal leukoencephalopathy (PML) is a rare opportunistic brain infection caused by the JC virus associated with significant morbidity and mortality. There are no validated automatic methods for quantification of PML lesion burden or brain volume loss on MRI. We designed an automated PML lesion/brain parenchymal segmentation method using a proposed approach that we have dubbed â€œJCnetâ€. Methods: We retrospectively analyzed PML patients recruited at the NIH Neuroimmunology Clinic. MRI scans were acquired on a Siemens Skyra 3T scanner. Four standard brain MRI sequences were used: 3D fluid-attenuated inversion recovery, 3D T1-weighted, T2-weighted and proton density sequences. We implemented a 3D patch-based method consisting of 2 consecutive fully convolutional neural networks (CNNs), each with a feature pyramid architecture, for PML brain and lesion segmentation. JCnet was compared to validated methods of brain/lesion segmentation, including FAST (FMRIB's Automated Brain Segmentation Tool) and Lesion-TOpology-preserving Anatomical Segmentation (LTOADS). Dice similarity coefficients (DSC) were calculated to measure segmentation accuracy compared to manual labelling. All comparisons were performed using Wilcoxon matched-pairs signed-ranks test. Results: A total of 32 PML patients (mean age 53years, SD 14; 12 female) were analyzed. Mean time between clinical PML onset and MRI acquisition was 0.7 years (range 0.5 monthsâ€“3.7 years). The dataset was split into 20 training and 12 testing cases. On average, a smaller patch sizes achieved a lower classification accuracy compared to a larger size for brain parenchymal (mean DSC 0.986 vs. 0.991, p<0.005) and lesion segmentation tasks (mean DSC 0.801 vs. 0.881, p<0.005). Using JCnet resulted in a 4.5% absolute improvement in PML brain segmentation compared to FAST (mean DSC 0.991 vs 0.945, p<0.005), and 1.6% compared to LTOADS (mean DSC 0.991 vs 0.975, p<0.005). A notable 54% absolute improvement in PML lesion segmentation was obtained by JCnet in comparison to LTOADS (mean DSC 0.881 vs 0.341, p<0.005). Conclusions: We implemented an end-to-end deep learning method for fully automated segmentation of lesion volume and brain parenchymal loss in PML. By tracking quantitative measurements of PML-related brain changes, this approach provides a window for clinicians and scientists to accurately monitor PML in vivo and its response to experimental therapies.

Erin Beck
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NINDS
Radiology/Imaging/PET and Neuroimaging
A new MRI method for visualizing subpial cortical lesions at 3 tesla
Background: Cortical demyelination is common in multiple sclerosis (MS) and can be extensive. Cortical lesions contribute to disability independently from white matter lesions and may form via a distinct mechanism. However, current magnetic resonance imaging (MRI) methods at 3 tesla (3T) are insensitive to cortical, and especially subpial, lesions. Subpial lesions are well-seen on T2*-weighted imaging at 7 tesla (7T), but T2*-weighted methods on clinically relevant 3T scanners are limited by low lesion-to-cortex contrast and cerebrospinal fluid (CSF)-to-lesion contrast. Objectives: To develop a T2*-weighted sequence with suppressed CSF signal to improve subpial cortical lesion visualization. Methods: We developed a new MRI sequence, Inversion Recovery Susceptibility Weighted Imaging with Enhanced T2-Weighting (IR-SWIET, acquisition time 5min). We compared cortical lesion visualization independently on IR-SWIET (median of 4 acquisitions), Magnetization-Prepared 2 Rapid Acquisition Gradient Echoes (MP2RAGE), Double Inversion Recovery (DIR), Susceptibility Weighted Imaging (SWI), and Phase Sensitive Inversion Recovery (PSIR) images for 10 adults with MS. We also identified cortical lesions with a multimodal reading of IR-SWIET (median of 2 acquisitions), MP2RAGE, and FLAIR images for each case. Lesions identified on 3T images were verified on â€œgold standardâ€ 7T T2* and MP2RAGE images. Results: Cortical, and particularly subpial, lesions appeared much more conspicuous on IR-SWIET compared to other 3T methods. A total of 101 subpial lesions were identified on IR-SWIET (average per-participant sensitivity vs 7T 29%, SEM 8%) vs 36 on MP2RAGE (5 +/- 2%), 17 on FLAIR (2 +/- 1%, p=0.048), 28 on DIR (6 +/- 2%, p=0.03), 42 on SWI (11 +/- 5%, p=0.01), 13 on PSIR (4 +/- 2%, p=0.02), and 380 on 7T. When a combination of IR-SWIET, MP2RAGE, and FLAIR images were used, a total of 147 subpial lesions (30 +/- 5%) were identified, versus 83 (16 +/- 3%, p=0.004) on a combination of DIR, MP2RAGE, and FLAIR. Leukocortical lesions were better visualized on MP2RAGE. Conclusions: Subpial lesions are better visualized on IR-SWIET compared to previously reported 3T methods, while leukocortical lesions are well visualized on MP2RAGE. Thus, the two sequences are synergistic and the use of both improves cortical lesion visualization. IR-SWIET may allow for improved MS diagnostic specificity and a better understanding of the clinical implications of cortical demyelination.

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Radiology/Imaging/PET and Neuroimaging
Characterization of CNS involvement in Ebola-Infected Macaques using Magnetic Resonance Imaging, 18F-FDG PET and Immunohistology
The Ebola (EBOV) virus outbreak in Western Africa resulted in residual neurologic abnormalities in survivors. Many case studies detected EBOV in the CSF, suggesting that the neurologic sequelae in survivors is related to viral presence. In the periphery, EBOV infects endothelial cells and triggers a â€œcytokine stormâ€. However, it is unclear whether a similar process occurs in the brain, with secondary neuroinflammation, neuronal loss and blood-brain barrier (BBB) compromise, eventually leading to lasting neurological damage. We have used in vivo imaging and post-necropsy immunostaining to elucidate the CNS pathophysiology in Rhesus macaques infected with EBOV

(Makona). Whole brain MRI with T1 relaxometry (pre- and post-contrast) and FDG-PET were performed to monitor the progression of disease in two cohorts of EBOV infected macaques from baseline to terminal endpoint (day 5-6). Post-necropsy, multiplex fluorescence immunohistochemical (MF-IHC) staining for various cellular markers in the thalamus and brainstem was performed. Serial blood and CSF samples were collected to assess disease progression. The linear mixed effect model was used for statistical analysis. Post-infection, we first detected EBOV in the serum (day 3) and CSF (day 4) with dramatic increases until euthanasia. The standard uptake values of FDG-PET relative to whole brain uptake (SUVr) in the midbrain, pons, and thalamus increased significantly over time (p<0.01) and positively correlated with blood viremia (pâ‰¤0.01). On MF-IHC, EBOV antigen (VP40) co-localized with endothelial cells as expected, but also with neurons (NeuN), which is consistent with direct neuronal infection and has not been shown before. Many of the infected neurons were apoptotic (cleaved caspase-3/PARP1 staining co-localizing with NeuN and VP40) and showed increased expression of glucose transporters (mainly GLUT-3). Elevated GLUT-3 levels could explain the increased FDG uptake in these regions. On MRI, significant increases in pre-contrast T1-relaxation times with decreased post contrast T1 values suggested BBB disruption and leakage. This was confirmed by MF-IHC showing extravascular albumin staining. Using imaging and histopathology, we showed that acute EBOV infection in macaques leads to BBB disruption, neuronal infection, apoptosis and increased GLUT3 expression. Our findings shed light on the pathophysiology of CNS involvement in EBOV that could explain residual neurologic manifestations in survivors.

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Subgroups of cigarette smokers demonstrate the hidden cost of sustained attention in nicotine abstinence
Nicotine withdrawal syndrome (NWS) manifests as negative reinforcing affective and clinical disruptions leading to unsuccessful smoking cessation attempts and relapse. Identifying subgroups of smokers may better our understanding of potential treatment targets, tailored treatments and improved outcomes. To date, affective and subjective measures of NWS have been used to subgroup smokers. In this study, we have implemented a novel subgrouping based on cognitive performance on the parametric flanker task (PFT). N=49 smokers underwent functional magnetic resonance imaging (fMRI) twice â€” during baseline nicotine satiety and again after about 48hrs of biologically verified nicotine abstinence. The cognitive performance on the PFT was used to categorize the smokers into good (above 70% accuracy) and poor (below 70%) subgroups. While the good performers were better on the task, they showed increased errors of omission (EOM, indicative of failures in sustained attention) during nicotine abstinence as compared to the poor performers. fMRI analyses revealed neurobiological differences between the subgroups. The good performers showed increased activation in brain regions of the salience (detecting and filtering salient stimuli) and the frontoparietal (facilitating flexible interaction between control regions) networks compared to poor performers during both nicotine satiety and abstinence. A functional connectivity analysis was performed using these regions to identify potential circuits in the brain underlying the cognitive disruptions of NWS. Higher functional connectivity was

observed between regions responsible for attentional control and the aggregation of sensorimotor information in the good performers. To summarize our findings: 1) when performing the task, the good performers show higher accuracy than the poor performers during satiety and abstinence 2) paradoxically, the good performers appear to be susceptible to attentional lapses during abstinence (indicated by the increased EOM) while the poor performers seem to be protected from these disruptions (no change in EOM) 3) the good performers display greater recruitment of salience and attentional brain regions but the failure to sustain this recruitment during nicotine abstinence appears to cause lapses in their non-selective attention. These results highlight a new way to subgroup smokers to study and mitigate the cognitive disruptions of the NWS.

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RNA Biology

Developmentally regulated microRNA decay of the mir-35 family is seed sequence dependent

The mir-35 family of microRNAs (miRNA) in Caenorhabditis elegans are maternally contributed as well as zygotically expressed in early embryos. Expression of the mir-35 family, which consists of 8 miRNAs, mir-35-42, is essential for viability; complete loss of the mir-35 family results in embryonic lethality. mir-35 family abundance is developmentally regulated and is sharply decayed at the end of embryogenesis. While there is much known about the biogenesis and functions of miRNAs, very little is known about the decay mechanisms of miRNAs. Because of this tight regulation of the mir-35 family during development, this family of miRNAs is an interesting candidate for studying the mechanisms of miRNA decay. The mir-35 family has two defining characteristics: the first is a shared, family-specific seed sequence (nucleotides 2-8 at the 5â€™ of the miRNA), and second, mir-35-42 are preferentially loaded into the Argonaute protein ALG-2, rather than the more studied ALG-1. We are interested in ascertaining whether either of these characteristics play a role in the regulated decay of the mir-35 family. To examine if the mir-35 family turnover is seed sequence-specific, we mutated the seed sequence of the mir-35 miRNA via CRISPR and monitored levels of the mutant mir-35 as it compares to wild type mir-35. We detected a perdurance of mutant mir-35 past embryogenesis demonstrating the necessity of the seed sequence in regulating mir-35 family decay. We found that the mir-35 seed mutants were loaded into ALG-2 similarly to wild type mir-35, indicating that altered Argonaute loading is not the cause of the altered turnover of the mir-35 seed mutants. Therefore, ALG-2 loading is not sufficient for regulated turnover since mutant mir-35 is misregulated despite its normal loading into ALG-2. Having shown that the seed sequence is necessary for mir-35 decay, we next interrogated whether the seed is sufficient for the regulated decay of the mir-35 family. We mutated all residues of mir-35 outside of the seed region and observed that these miRNAs are decayed fairly similarly to wild type mir-35. Thus, the seed is largely sufficient to elicit developmentally timed decay. Overall, our findings contribute to a better understanding of the regulation of mir-35 family abundance in early development. By elucidating the mechanisms of mir-35 family decay, this research will offer insight into the broader mechanisms that couple miRNA decay to developmental progression.

Shuaikun Su
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RNA Biology
A dual-activity topoisomerase complex interacts with PRRC2A to regulate stress granules dynamics and neural development
DNA topoisomerases are vital enzymes that solve DNA topological problems during DNA metabolism. These enzymes catalyze DNA strand passage reactions to relax supercoils resulted from unwinding of duplex DNA by replication, transcription and other machinery. Unlike the well-characterized DNA topoisomerases, the roles of topoisomerases in RNA metabolism remain largely unknown. Our group recently discovered that topoisomerase 3B (TOP3B) is the only topoisomerase in animals that possesses dual activity for both DNA and RNA. Increasing evidence shows that TOP3B forms a complex with TDRD3 and FMRP, which associates with polyribosomes and RNA stress granules (SGs) to promote synapse formation. Importantly, TOP3B mutations in human have been linked to mental dysfunction, such as schizophrenia and autism. However, the mechanism remains unclear. Here we present evidence that TOP3B-TDRD3 interacts with a SGs component, PRRC2A, to regulate mRNA levels and neural development. First, our IP-MS analysis identified PRRC2A (Proline rich coiled-coil 2â€‰A) and its fly ortholog nocte as a top hit in proteins that coimmunoprecipitate with TOP3B-TDRD3. PRRC2A has been reported as a m6A reader that regulates mRNA stability and neural development. Interestingly, our genetic studies in fly show that Tdrd3 knockout enhanced the neurological defects in the eyes of nocte RNAi mutants, while Top3b knockout suppresses the defects. Moreover, while nocte RNAi mutant flies exhibit significantly reduced mRNA and protein levels of a synaptic gene, chp, in neurons, the Tdrd3-nocte double RNAi mutant display further reduction. Second, we studied whether TOP3B-TDRD3 is important for SGs assembly, as is PRRC2C (a homolog of PRRC2A). We found that SGs in human HeLa cells inactivated of either TOP3B or TDRD3 have normal kinetics of formation, but faster disappearance, indicating that TOP3B-TDRD3 is needed for SGs disassembly. Finally, we found that a de novo single nucleotide variant (SNV) of TOP3B, C666R, which was identified in an autism patient and had reduced RNA binding and topoisomerase activity, significantly reduces localization of TOP3B to SGs. A similar finding was also observed for a TOP3B mutant lacking its RNA binding domain (RGG-box) and activity. Together, these data suggest one mechanism by which TOP3B-TDRD3 complex regulates neural development and prevent mental dysfunction could be to work with PRRC2A/nocte to regulate RNA SGs dynamics and neural mRNA levels.

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RNA Biology
Glucose-induced Spot 42 small regulatory RNA encodes a 15-amino acid protein SpfP that blocks Crp-dependent activation
The 109-nucleotide Spot 42 RNA, whose expression is induced by glucose upon cAMP receptor protein

(CRP) de-repression, is one of the best characterized base pairing small RNAs (sRNAs) in Escherichia coli. During growth on glucose, Spot 42 represses the synthesis of transporters and enzymes involved in the utilization of non-preferred carbon sources by base pairing with corresponding mRNAs. Thus, under glucose limiting conditions, many genes repressed by Spot 42 are transcriptionally activated by CRP, while the gene encoding Spot 42 is repressed. This feedforward loop promotes gene repression when the preferred carbon source is available and delays gene activation when the preferred carbon source disappears. We have recently shown that Spot 42 also encodes a 15-amino acid protein SpfP, adding another level of complexity to the regulatory network. We created a plasmid to express a codon scrambled SpfP construct (SpfP-scram) to examine the function of the small protein in the absence of base pairing. Consistent with the elimination of base pairing activity, translational fusions to Spot 42 base pairing targets showed regulation by full length Spot 42 but not SpfP-scram. Interestingly, overexpression of SpfP-scram also led to a defect for growth on galactose similar to Spot 42, suggesting that the sRNA and protein impact the same pathway. To identify protein partners for SpfP, we wanted to generate a tagged derivative of 15-amino acid long SpfP, a challenge when most epitope tags are larger than the small protein. To address this challenge, we expressed SpfP in a strain carrying a plasmid encoding an orthogonal tRNA synthetase capable of loading a suppressor tRNA with the non-native amino acid, p-azido-phenylalanine, which can be biotinylated. We found that SpfP biotinylated in this way co-purifies with the CRP transcription factor. In line with this observation, SpfP overexpression blocks CRP dependent activation of the galactose operon. Together, these data show that Spot 42 is a dual-function RNA where both the sRNA and small protein promote preferential utilization of glucose by downregulating genes of secondary carbon source metabolism. This work also provides the first example of a small protein that modulates the activity of a transcription factor.

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RNA Biology
Translation of the 3â€™ untranslated regions of messenger RNAs during the RNase L mediated antiviral response
Viral infections often trigger the RNase L pathway, which plays an important role in the innate immune response. During viral infection, oligo-adenylate synthase (OAS) recognizes double stranded RNA (dsRNA) and then produces a small molecule, 2â€™-5â€™-oligoadenylate (2-5A). 2-5A subsequently

activates and dimerizes the latent endoribonuclease, RNase L. This activated form of RNase L then cleaves single stranded regions of viral and host RNAs and this activity is thought to promote clearance of the virus or apoptosis. In addition, RNase L was reported to interact with proteins involved in translation termination and ribosome recycling (removal) at stop codons, such as ATP-binding cassette E1 (ABCE1) and eukaryotic release factor 3 (eRF3). Therefore, RNase L could also be involved in regulating translation termination and/or recycling, in addition to its canonical RNase role. In cells where termination or recycling are deficient, ribosomes accumulate at stop codons and then translate 3â€™ untranslated regions (UTRs) of messenger RNAs (mRNAs). To assess whether RNase L dimerization influences ribosome termination and/or recycling, and potentially triggers translation of 3â€™UTRs, we assayed the distribution of ribosome footprints at the global and local (individual gene) level by performing ribosome profiling on human cells that had been treated with 2-5A or poly I:C (dsRNA mimic). We found that RNase L activation leads to substantial accumulation of ribosomes in 3â€™ UTRs, but not in RNase L KO cells, suggesting specificity for this pathway. Data from in vitro dual luciferase assays combined with evidence of 3-nt periodicity of the ribosome footprints found in the 3â€™UTR indicate that these ribosomes are engaged in active translation. We therefore favor a model where activation of RNase L inhibits recycling of the small 40S subunit and allows it to reinitiate translation downstream in the 3â€™UTR. While the function of the synthesized 3â€™UTR peptides is unknown, we propose that they could be presented by MHC I molecules on the cell surface. These MHC I-3â€™UTR peptide complexes are potentially recognized by the immune system as â€œnon-selfâ€ and could therefore enhance viral clearance.

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Stem Cells - General and Cancer
CRISPR/Cas9 Targeted MAGT1 Gene Addition into XMEN Patient Hematopoietic Stem Cells and Peripheral Blood T Cells
Mutations in MAGT1 gene encoding for the magnesium transporter 1 cause a â€˜X-linked immunodeficiency with magnesium defect, Epstein-Barr virus infection, and neoplasiaâ€™ (XMEN). The resulting glycosylation defect impairs the expression of key immune regulatory proteins like CD28, CD70, and NKG2D, receptor critical for the cytotoxic function of natural killer (NK) and CD8+ T cells. Impaired antiviral and anti-tumor cytotoxicity leads to chronic EBV infections and development of lymphoproliferative disorders. There is currently no specific therapy available and potentially curative hematopoietic stem cell (HSC) transplant is associated with a high risk of fatal hemorrhagic complications. We assessed the feasibility of using CRISPR/Cas9 technology and rAAV6 delivery of the MAGT1 cDNA to correct XMEN patient CD34+ HSCs and T cells. Mobilized peripheral blood CD34+ HSCs from XMEN patient (IRB-approved protocol) were gene-edited (GE) with Cas9 mRNA/sgRNA and AAV donor. Using a cocktail of agents to enhance targeted integration (TI) and to maintain HSC â€˜fitnessâ€™, we achieved highly efficient gene correction (50-60% TI) and excellent cell viability (> 95%) post-electroporation (EP). GE XMEN CD34+ HSCs showed correction of NKG2D expression and NK cytotoxic activity of in vitro-differentiated NK cells (20-40%) compared to naïve XMEN cells (5-10%) which was comparable to healthy donor (HD) controls (30-40%). In vitro-differentiated T cells from GE

XMEN CD34+ cells using the Artificial Thymic Organoid system had similar level of NKG2D expression as normal T cells. Next, we performed transplantation studies (IACUC approved protocol) and observed robust engraftment (mean 10%, up to 40%) at 16 weeks after transplantation of XMEN (GE, naïve) and HD CD34+ HSCs into newborn immunodeficient mice. Transplanted mice showed highly corrected NKG2D-expressing CD8+ T (~75%) and NK (~35%) cells in peripheral blood, spleen and thymus. Molecular analysis confirmed high TI (50-60%) in mice bone marrow human CD45+ cells indicating persistence of GE HSCs following transplant. In parallel, we applied the same approach to XMEN T cells. Interestingly, we observed increasing percentages of GE cells following in vitro culture, highlighting an important survival advantage of corrected cells. In summary, we demonstrate a robust gene correction approach with efficient TI of MAGT1 cDNA in XMEN patient T cells and CD34+ HSCs, sustained engraftment and multi-lineage differentiation capabilities.

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Stem Cells - General and Cancer
Outer retina reconstruction using a tissue engineered transplant
Degeneration of outer retina (photoreceptors/PRs and retinal pigment epithelium/RPE cells) is the most common cause of vision loss and blindness in diseases of the retina. Similar PR/RPE damage is seen in eye injuries (e.g. laser-induced RPE/PR damage in the civilian population or laser and blast injuries in a battlefield). Currently, there is no treatment for such degenerative eye conditions. Here, we have developed an induced pluripotent stem cell (iPSC) derived outer retina cell replacement therapy to directly replace degenerated PRs and RPE. This works leverages our recently approved Phase I trial to test the safety of iPSC-derived RPE-only patch in macular degeneration patients. The iPSC-RPE-patch is developed by growing a confluent monolayer of polarized RPE cells on a biodegradable poly (lactic-co-glycolic acid) (PLGA) scaffold. iPSC-derived PRs are grown on top of the RPE-patch to form a 3D outer retina tissue. This close proximity of RPE and PRs allows correct polarization and co-maturation of both cell types. To test in vivo functionality of this tissue, we developed a micro pulse laser induced RPE/PR damage pig model. A 532nm laser is specifically absorbed by RPE pigment, causing RPE and adjacent PRs to die of the laser heat. 48 hours post the laser injury, we transplanted a 4x2 mm PLGA-RPE-PR patch under the laser-damaged retina. Animals (N=4 pigs) were evaluated by live imaging of the eye including Optical Coherence Tomography (OCT) that allows us to monitor retina structure and Adaptive Optics (AO) that allows us to track individual PRs. Our results show that the PLGA/RPE/PRP patch was able to protect overlying lasered retina from dying. OCT shows improved retinal thickness and preserved structure over the lasered retinal area with the transplant as compared to lasered only area. AO analyses of the transplanted areas shows an increase of PR density compared to untreated areas. Lastly, histology confirmed the integration of human RPE and PR in the back of pig retina. RPE cells continue to express RPE-lineage markers like Microphthalmia-associated transcription factor (MITF), and photoreceptors express ARRESTIN (ARR3) and other PR markers. This work provides the first ever dual-RPE/PR transplants to treat degenerative eye diseases and civilian or battlefield eye injuries.

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Stem Cells - General and Cancer
NAD+ intervention preserves hematopoietic stem cell function in telomerase-deficient bone marrow.
Telomeres cap the ends of chromosomes, and prevent chromosome ends from eliciting a DNA damage response. Telomere attrition is a major feature underlying hematopoietic stem cell (HSC) dysfunction in aging and bone marrow failure syndromes, including Dyskeratosis congenita (DC). Patients with DC often experience early mortality as result of bone marrow failure, which occurs by the age of 30 in 80-90% of patients. NAD+ is a critical metabolic cofactor that is depleted with aging. Our lab recently demonstrated NAD+ depletion in primary fibroblasts from patients with DC and in tissues from a telomere deficient mouse model that lacks the gene encoding telomerase reverse transcriptase (TERT). We hypothesize that the NAD+ precursor, NR, could benefit patients with DC and promote healthy aging by maintaining telomere integrity and preventing concomitant HSC dysfunction. To test our hypothesis, we utilized late generation TERT KO mice, which exhibit phenotypes resembling those observed in DC patients, including critically short telomeres, elevated levels of telomere dysfunction induced foci (TIFs), and HSC impairment, including early HSC exhaustion and B-lymphopenia. To determine the effects of NR on HSC function in TERT KO mice, we treated late generation TERT KO mice with vehicle or NR (12mM in drinking water) for 10 months. Subsequently, we tested NAD+ levels in the bone marrow using an NAD cycling enzyme-based assay. Significantly higher NAD+ levels were observed in bone marrow from NR-treated compared to vehicle-treated mice, supporting the potential of NR to induce cell-autonomous changes that would impact HSC function in TERT KO mice. To determine the cell autonomous effects of NR, we transplanted bone marrow from vehicle- or NR-treated late generation TERT KO into lethally irradiated (myelodepleted) congenic recipients. Our results demonstrate significantly increased numbers of HSCs in the bone marrow and B-lymphocytes in the blood of recipients of NR-treated compared to vehicle-treated TERT KO donors. Furthermore, we demonstrated decreased TIFs in bone marrow derived from NR-treated compared to vehicle-treated mice. These findings indicate that NR supplementation induces cell-autonomous changes that preserve telomere integrity and benefit HSC function in the setting of telomere impairment. Our results support our hypothesis that NR may be beneficial for HSC function in patients with bone marrow failure syndromes associated with telomere impairment.

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Stem Cells - General and Cancer
A New Polypharmacology Strategy to Increase Reproducibility and Maturation of Functional Cerebral Organoids Derived from Human Pluripotent Stem Cells
Self-organizing cerebral organoids from human pluripotent stem cells (hPSC) recapitulate brain development in a dish and hold great promise for disease modeling, drug screening, and tissue engineering. However, current protocols are hampered by uncontrolled cell death upon organoid formation, organoid-to-organoid heterogeneity, and lack of standardization. In this study, we demonstrated that a combination of four small molecule compounds, termed the â€œCEPTâ€ cocktail, dramatically improves cell survival and provides cytoprotection by minimizing cellular stress during organoid formation. Specifically, measuring ATP production and percentage of the live/dead cells demonstrated that the ROCK inhibitor Y-27632, currently the most widely used reagent to improve cell survival upon organoid formation, is deficient in preventing cellular stress and apoptosis, leading to insufficient organoid development. Western blot analysis revealed that Y-27632-generated organoids indeed showed significant DNA damage and endoplasmic reticulum (ER) stress. In contrast, CEPT-treated organoids were devoid of DNA damage and ER stress with higher levels of structural proteins. Improved morphology of CEPT-generated organoids, as compared to Y-27632, was confirmed by using H&E staining and immunohistochemistry. Furthermore, single-cell transcriptomic data showed that improved cell survival at early stages of organoid formation has long-lasting consequences affecting not only total cell numbers and organoid size, but also enhanced neuronal differentiation along with molecular signatures representing neuronal subtypes of different cortical layers. Compared to a large publicly available human transcriptomic data set (n = 84,863), CEPT-generated organoids indicated a significantly higher correlation to the datasets from the developing human brain. Moreover, bulk RNA sequencing analysis across individual organoids from 3 different hPSC lines revealed that CEPT treatment resulted in higher experimental reproducibility compared to Y-27632. Based on calcium imaging, CEPT-generated cerebral organoids displayed spontaneous functional activity, which was further enhanced upon the addition of GABAA receptor antagonist Bicuculline. Overall, our study identified uncontrolled cell death at the onset of organoid formation as a critical quality control determinant and demonstrated that the CEPT cocktail improved morphogenesis, neuronal differentiation, and overall reproducibility of cerebral organoids.

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Patient mutation in CHCHD10 causes cardiomyopathy and OMA1-dependent mitochondrial fragmentation
Mutations in the paralogous mitochondrial proteins CHCHD2 (C2) and CHCHD10 (C10) have been recently identified as autosomal dominant causes Parkinsonâ€™s disease, ALS/FTD, and myopathy. The function of these proteins, as well as the mechanism by which their mutations lead to disease, however,

remain unknown. We describe a family with myopathy and dilated cardiomyopathy due to a dominant C10 G58R mutation, extending the clinical spectrum of C2/C10 related disorders. To model the mutation, we generated a C10 G58R knock-in (KI) mouse. We show that the heterozygous C10 G58R mouse recapitulates the myopathy and cardiomyopathy phenotype seen in the family. Muscle wasting was evident from weaning, with persistent decreased body weight. Behaviorally, the C10 G58R mouse displayed significant motor and balance impairment when assessed for grip strength, rotarod balance and time until fatigue on a treadmill. Functional cardiac analysis demonstrated decreased ejection fraction and AV conduction block. Ultrastructural examination of the heart demonstrated fragmentated mitochondria with cristae abnormalities. We identified cleavage (and inactivation) of the mitochondrial-shaping protein OPA1 by the peptidase OMA1 as the cause of the cristae abnormalities in C10 G58R KI mice. OMA1 has been very recently shown to be the peptidase which initiates the pathway that relays mitochondrial stress to the cytosol, leading to the activation of the integrated stress response. Consistently, we see activation of the mitochondrial integrated stress response (mt-ISR) concurrent with OMA1 activation in affected tissues of the C10 G58R mouse. Similar activation of OMA1 and the mt-ISR was observed in cardiac and muscle tissue from the proband. MtDNA deletions were also detected in the C10 G58R mouse, replicating findings in affected members of the family. In summary, we extend the clinical spectrum of CHCHD2/CHCHD10 related disorders to include cardiomyopathy and generate a faithful mouse model to study mechanisms underlying the pathogenesis of dominant CHCHD2/CHCHD10 mutations. We identify OPA1 processing by activated OMA1 as a potentially important step in disease pathogenesis and a target for therapy.

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Epigenetic aging in hematopoietic stem cell transplantation is associated with poor outcomes in patients with severe aplastic anemia: results from DNA-methylation GrimAge

Cellular aging in hematopoietic stem cell transplantation (HCT) is important in the context of immune reconstitution and age-related complications. Recently, several DNA-methylation (DNAm) based biomarkers of aging known as â€œepigenetic clocksâ€ have been introduced as novel tools to predict cellular age. Here, we used the recently published lifespan-associated epigenetic clock known as â€œGrimAgeâ€ to assess the associations of donor pre-HCT DNAm age, and its post-HCT changes, with outcomes among patients with severe aplastic anemia (SAA). The study included 732 SAA patients who underwent unrelated donor HCT and for whom a donor pre-HCT DNA sample was available; 41 also had a post-HCT sample collected at day 100. We used Illumina Infinium whole-genome MethylationEPIC array data to calculate GrimAge and GrimAge acceleration (a measure of the deviation from expected cellular age for chronological age). For statistical analyses, we used Cox proportional hazards regression models. Donor pre-HCT GrimAge was highly correlated with chronological age (r=0.85, <0.001), and showed minimal GrimAge acceleration (median= -0.5 years, range= -10 to 16). In multivariable analyses adjusted for recipient age and race, transplant year, conditioning regimen intensity, Karnofsky performance score, human leukocyte antigen (HLA) match, and disease subtype (acquired vs. inherited), we found similar effects for donor chronological age and pre-HCT GrimAge on post-HCT survival (hazard

ratio [HR]=1.01, 95% confidence interval [CI]=1.00-1.03 for both). For donors with extreme GrimAge acceleration (i.e., DNAm age exceeded chronological age by 10+ years), elevated risks of chronic graft vs. host disease (GvHD; HR=2.4, 95% CI=1.21-4.65) and possibly post-HCT mortality (HR=1.79, 95% CI=0.96-3.33) were observed. In the subset with post-HCT samples, we observed a significant increase in GrimAge in the first 100 days post-HCT (median change=12.5 years, range=1.4-26.4). Increased GrimAge post-HCT was associated with inferior survival (HR=1.11, 95% CI=1.02-1.21). Exploratory analyses suggested that increased post-HCT aging was independently associated with <8/8 HLA donor-recipient match and acute GvHD. Cellular aging may be an important biomarker for predicting post-HCT outcomes in SAA patients, though validation is needed. If validated, studies identifying factors associated with cellular aging in HCT may guide treatment decisions and patient follow-up strategies.

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Tumor Biology and Metastasis
Combination of hetIL-15 with chemotherapy in triple negative breast and pancreatic cancer mouse models increases tumor necrosis and alleviates metastatic disease
Introduction: We evaluated the potential therapeutic benefit of conventional chemotherapy combined with hetIL-15 immunotherapy in two mouse models of pancreatic and breast cancer. IL-15 is an important cytokine that stimulates the proliferation and cytotoxic functions of CD8+ T cells and NK cells. We have produced the native heterodimeric form of IL-15 (hetIL-15), which has advanced in clinical trials due to its anticancer activities. Study design and methods: We assessed the efficacy of hetIL-15 immunotherapy in combination with the chemotherapeutic agents gemcitabine or doxorubicin on the growth of primary pancreatic and breast tumors, and on the metastatic disease. We used the transgenic KPC model of pancreatic cancer (LSL-KrasG12D/+;LSL-Trp53R172H/+;Pdx-1-Cre) and the orthotopic 4T1 model of triple negative breast cancer (TNBC) model. We analyzed the tumor infiltrating lymphocytes (TILs) by flow cytometry and immunohistochemistry (IHC) and evaluated the extent of necrosis on the primary tumors by H&E and cytokeratin staining. We also evaluated the lung metastatic burden by histology. Results: The combination of hetIL-15 and doxorubicin significantly delayed 4T1 tumor growth. In addition, histological analysis showed an increase in the percentage of the necrotic areas of the primary tumors in the combination group. hetIL-15 treated groups also showed increased necrotic areas of the primary pancreatic tumors, although tumor growth showed no significant change compared to the control group. Flow analysis of TILs showed increased CD8+/CD4+ T cell ratios in tumors of hetIL-15 treated groups in both cancer models, which was confirmed by IHC. Importantly, the evaluation of the metastatic disease showed that hetIL-15 treated animals in both cancer models had reduced metastatic foci and in some cases were completely disease free. Chemotherapy did not significantly reduce the numbers of the total metastatic lesions but reduced their size. Conclusions: hetIL-15 immunotherapy combined with conventional chemotherapy shows additive effects in both breast and pancreatic mouse

models, in terms of the primary tumor necrosis and the alleviation of the metastatic disease. This study may facilitate rapid clinical translation using combinations of drug candidates already advanced to clinical trials.

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Tumor Biology and Metastasis
TNIK, a novel activator of FAK and YAP signaling, is a therapeutic target in Lung Squamous Cell Carcinoma.
Distal chromosome 3q amplification (3q26-29, also known as the 3q amplicon) is the most frequent genomic alteration in lung squamous cell carcinoma (LSCC). Analysis of LSCC samples from the TCGA reveals that nearly 50% of LSCC patients harbor the 3q amplicon that includes the resident protein kinase gene TNIK. Recent studies have pinpointed TNIK as a potential oncogenic driver in cancer cells with distal 3q amplification; however, the therapeutic potential of TNIK remains unexplored. We found that TNIK was highly expressed in LSCC cells with the 3q amplicon, while its expression was modest in cells that lacked the 3q amplicon, consistent with data from the TCGA. To evaluate TNIK as a target in LSCC, we generated stable, doxycycline-inducible cells expressing shRNA to deplete TNIK from LSCC cells and conducted functional assays to measure cell proliferation and survival. TNIK knockdown or inhibition of its kinase activity with a small molecule inhibitor significantly diminished the viability of LSCC cells with 3q amplification in vitro and in cell line-derived xenograft mouse models. We also observed that TNIK inhibitors significantly abrogated the growth of LSCC patient-derived xenografts and showed that TNIK inhibition induced apoptotic cell death in LSCC cells that harbor the 3q amplicon. Importantly, TNIK depletion or catalytic inhibition in LSCC cells that lack the 3q amplicon had no significant effect on cell survival. Finally, we used a combination of bioinformatics and proteomic analysis (RPPA, peptide mapping, and mass spectrometry) to define the underlying mechanisms driving TNIK-mediated cancer cell survival. We identified the tumor suppressor MERLIN as a novel TNIK substrate and determined that TNIK phosphorylates MERLIN at serine 13 and 315. We also show that TNIK is required to maintain FAK activation and stabilize the YAP transcription factor, two oncogenic pathways inhibited by MERLIN. In conclusion, our results demonstrate that TNIK maintains survival of LSCC cells through modulation of a novel TNIK-MERLIN-YAP/FAK signaling pathway and validate TNIK inhibitors in pre-clinical models of LSCC, including patient-derived xenografts. Our studies highlight the protein kinase TNIK as a promising therapeutic target for the treatment of LSCC patients with distal chromosome 3q amplification.

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Tumor Biology and Metastasis

Can Cycles of Low Caloric Intake Alter the Metastatic Processes Regardless of a Reduction of the Primary Tumor Growth?

Aging is the major risk factor for most chronic diseases, including cancer. Dietary interventions have long been utilized to slow the aging process and to protect against cancer, one of the major causes of mortality in the US. Chronic caloric restriction (CR) improves survival and cancer protection, but poor adherence to CR has led to the development of less stringent dietary strategies that offer the same benefits. The fasting mimicking diet (FMD) is a plant-based diet aimed at providing adequate micronutrients (such as vitamins and minerals) while minimizing the need for water only fasting. The diet is administered over periodic cycles (4 days twice a month), with a profound reduction in calories of one day of 50%, followed by 3 days of 70% reduction, returning to unlimited food access (also known as ad libitum (AL) feeding). The FMD diet promotes healthspan in mice and humans and protects against primary tumor growth, with or without chemotherapeutics. Due to the FMD diet being protective against primary tumor growth, we hypothesized this dietary regime would be protective against metastases and show similar primary tumor growth rate as CR. To address this question, female mice were injected with a highly aggressive triple-negative mouse breast cancer line. Mice were divided into 3 dietary groups: an AL control group (mice had continual access to food), chronic CR (continuous consumption of 20% fewer calories vs. AL), or animals given 2 cycles of the FMD regime. Tumor growth rate was fastest in the AL control group, followed by the FMD group, with the slowest growth rate in the chronic CR group. Metastatic tumor growth in the lungs was used to assess the effectiveness of these dietary interventions. The stage of the metastases was more advanced (based on size) in AL and FMD groups compared to the chronic CR group. This finding is important because it highlights the need for further investigation into the cancer-specific benefits of CR alternatives, such as FMD.

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Vascular Disease and Biology

New model cell lines provide insight into the molecular mechanisms underlying ectopic calcification

Calcification is the process by which a tissue forms calcium phosphate deposition. Physiological biomineralization occurs in skeletal and dental tissues, but can also develop pathologically in soft tissue, even in the absence of systemic mineral imbalance. Ectopic vascular calcification (VC) promotes aortic stiffening, occlusive lesions (e.g., atherosclerotic plaques), and aortic valve stenosis, resulting in an increased risk for cardiovascular morbidity and mortality; >65% of the over-70s manifest some degree of VC. However, the lack of standardized, and genetically-tractable cultured cell models is impeding research into both the underlying molecular basis of this disease as well as the design and testing of therapeutic strategies. Here, we describe our establishing this much-needed model. Using a bespoke immortalized mouse coronary endothelial cell (MEC) line, we recapitulated ascorbic acid-stimulated VC (recorded by Alizarin Red staining). We used CRISPR to create an MEC subline in which we eliminated the Pi-transporter, XPR1 (Xenotropic and Polytropic Retrovirus Receptor 1). We found this novel XPR1-KO line exhibited perturbed cellular inorganic phosphate (Pi) homeostasis: cellular Pi efflux is inhibited, total Pi is elevated, and importantly, the degree of ascorbic-acid stimulated calcification is increased. Furthermore, XPR1 is known to have an SPX domain which has an absolute functional requirement to

bind the inositol phosphate signal, â€œIP8â€ (bis-diphosphoinositol tetrakisphosphate). Thus, we created another MEC line by CRISPR-based KO of PPIP5K (the inositol phosphate kinase activity that synthesizes IP8). These PPIP5K-KO cells precisely phenocopied the disturbance to Pi homeostasis and increased calcification by the XPR1-KO cells. Such data indicate that compromising missense mutations in PPIP5Ks might expand the repertoire of genetic factors that underlie the pathology of ectopic mineralization. Moreover, by using quantitative nanoparticle tracking analyses, we attributed hyper-calcification to 5-fold higher secretion of calcifying matrix vesicles from both the XPR1-KO and PPIP5K-KO lines. Thus, we have created informative and tractable cell lines for the VC research community which: (i) recapitulate ectopic calcification; (ii) show a novel mode of regulation by IP8; (iii) can facilitate design of potential therapeutic interventions.

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3-D bioprinted RPE/Choroid tissue to discover the role of choroidal macrophages in tissue growth and degeneration

Purpose: Choroidal vasculature is one of the densest capillary networks in the body and has high rates of blood circulation. Nanoscale fenestrations in chorio-capillary endothelial cells allow passage of macromolecules to retinal pigment epithelium (RPE) cells that form the outer blood retina barrier of the eye. Macrophages secrete pro and anti-angiogenic cytokines that fine-tune vasculature during development. Here we developed a properly oriented human induced pluripotent stem cell (iPSC) derived 3D RPE/choroid model that behaves similar to the native tissue. This model allows us to study the role of macrophages in choroidal development, which cannot currently be studied in humans. Methods: Endothelial cells, choroidal fibroblasts, and ocular pericytes were encapsulated in a collagen-derived gel and bioprinted on a degradable PLGA scaffold with added hydrogels to facilitate microvascular network formation. IPSC-RPEs were seeded on the apical side of the scaffold 7 days after choroid bioprinting. Primary pro-angiogenic (M2), anti-angiogenic (M1), and mixed macrophages were added to the choroid on the day of printing, 7 days post printing, or 28 days post printing. Confocal microscopy, quantitative cytokine analysis, trans-epithelial resistance measurements, and flow cytometry were used to analyze vascular and RPE components. Results: Pro-angiogenic M2 macrophages increased and stabilized vascular growth. In contrast, the anti-angiogenic M1 macrophages increased vascular growth only when added on day 0 but decreased vascular growth and led to RPE and vasculature degeneration when added at day 7 post-RPE seeding. When added at 28 days, the macrophages induced cytokine expression consistent with Wet Form-AMD, most notably high VEGF, PDGF, and CCL22 expression from the apical RPE tissue. They also increased TNF-a associated signaling (in M1 treated tissues) or abolished it completely (M2 treated tissues), which is consistent with their reported behavior. Conclusions: Macrophages in this model system influence the growth/survival of choroidal vasculature and RPE cells in a M1-M2 polarization-dependent manner and show the ability to induce pathological conditions as well. This 3D in-vitro model enables investigations into the role of immune mechanisms influencing choroidal development and ocular pathologies such as age-related

macular degeneration and choroideremia that affect the choroid but are difficult to examine with existing animal models

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Vascular Disease and Biology
Expression and sub-cellular localization of alpha and beta globin in the endothelium of human resistance arteries
Recent studies in mice revealed alpha globin (HBA1 and HBA2), but not beta globin (HBB), to be expressed in resistance arteries where it has been proposed to regulate vascular reactivity by associating with endothelial nitric oxide synthase (eNOS, NOS3) and restricting nitric oxide signaling across the myoendothelial junction (MEJ) in small arteries. The aim of our study was to determine to what extent this model for endothelial alpha globin holds true in human resistance arteries. We obtained human resistance arteries from omentum tissue collected from ten patients during clinically indicated surgery at the NIH Clinical Center (protocol 13-C-0176). Arteries 80-200 um in diameter were dissected, cannulated, and perfused with cold PBS to remove blood. We measured globin and NOS3 gene expression (gene transcripts per 1ng mRNA ± SEM) using reverse transcriptase-droplet digital PCR (RT-ddPCR): HBA1 (2658 ± 1190), HBA2 (3319 ± 1667), HBB (1831 ± 725), and NOS3 (408 ± 49). In contrast, transcripts from an erythroblast specific gene SLC4A1 were low in vascular tissue (4.2 ± 2.3) but abundant in whole blood (4960 ± 1948). Western blot detected alpha globin and beta globin protein present in a 1:1 ratio. Multiphoton microscopy of omentum arteries showed a broadly autofluorescent complex that localized to the MEJ. Fluorescence lifetime imaging microscopy (FLIM) of these complexes revealed distinct peak lifetimes for autofluorescence and the fluorophore-labeled antibodies against alpha globin, beta globin, and eNOS. We have identified endogenous expression of both alpha and beta globin in human resistance arteries as measured by RT-ddPCR, Western blot, and FLIM. Alpha and beta globin appear to co-localize with each other and form a complex with eNOS at the myoendothelial junction. This sharply contrasts with previous findings in mouse resistance arteries where alpha globin alone was expressed. We therefore propose a new model for the human vasculature in which alpha globin and beta globin form endothelial hemoglobin that interacts with eNOS. The addition of beta globin could stabilize alpha globin-eNOS interactions and provide additional heme iron groups catalyzing the dioxygenation of nitric oxide into nitrate, thereby increasing the ability of this globin complex to restrict the diffusion of nitric oxide between endothelium and vascular smooth muscle.

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Vascular Disease and Biology
Temporally Distinct Myeloid Cell Reponses Mediate Damage and Repair After Cerebrovascular Injury

Despite effective methods to restore blood flow, ischemic and hemorrhagic strokes can have devastating outcomes in humans. These injuries are often associated with cerebral edema and a potent inflammatory response that influence injury progression and resolution. Little is known about innate immune dynamics and the precise neuroanatomical contribution of resident versus peripherally derived myeloid cells during different stages of cerebrovascular injury from inception to resolution. To gain insights into this process, we developed a novel model of isolated cerebrovascular injury using transcranial ultrasound that enabled real-time evaluation of how myeloid cells respond anatomically and temporally to vascular damage. We studied the dynamics of the acute injury response using two-photon laser scanning microscopy and observed that microglia rapidly extended processes and reconstituted the blood brain barrier immediately after injury. This response depended on ATP-induced ATP release via connexin hemichannels and subsequent purinergic receptor signaling. The second phase of the immune response to cerebrovascular injury was characterized by a massive invasion of peripheral myelomonocytic cells that invaded the brain within one day, promoting edema and fatal cerebral herniation. Blocking entry of these cells with anti-adhesion molecule (LFA-1/VLA-4) therapy prevented edema and promoted survival but also impeded brain repair if continued for more than a day. Despite promoting early edema, we found that infiltrating myeloid cells were required for proper brain remodeling and vascular repair at later time points post-injury. In fact, we routinely observed complete revascularization of damaged brain tissue within ten days of injury. This reparative process was mediated by infiltrating CCR2+ classical monocytes and a newly identified population of VEGF-expressing microglia. Inhibition of either population prevented cerebral revascularization and led to long term neurological dysfunction. Collectively, our findings demonstrate that cerebrovascular injury induces a divergent myeloid cell response that can contribute to both damage and repair depending on time, location, and cellular origin. The temporal progression and anatomy of these myeloid cell responses must be considered when developing therapies to reduce acute damage and promote repair following cerebrovascular injury.

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Virology - DNA, RNA, and Retroviruses
The early bacteriophage T4 protein MotB, an unsual compactor of DNA, disrupts host DNA structure and gene expression and stimulates packaging of phage DNA
The occurrence of antibiotic-resistant bacteria has accelerated an interest in investigating phageâ€“host interactions. Understanding the mechanisms by which bacteriophages have evolved to take over their host and the hosts have responded with them would be beneficial to develop new antibacterial strategies. The lytic bacteriophage T4 employs multiple early proteins to take over the Escherichia coli host, but the functions of many of these proteins are still unknown. Previously, we have characterized one of the T4 early genes, motB. MotB is toxic when expressed in E. coli, and the presence of MotB increases T4 phage fitness. DNase I footprinting analyses have shown that MotB binds to both unmodified host and T4 modified [(glucosylated, hydroxymethylated-5 cytosine, (GHme-C)] DNA in a sequence-nonspecific manner. In addition, binding to DNA is improved when both MotB and H-NS, an E. coli histone-like protein, are present. Here we demonstrate how MotB interacts with DNA. Atomic Force

Microscopy revealed that MotB compacts the DNA, forming a â€˜spaghetti monsterâ€™-like structure. Live cell imaging confirmed that expression of MotB causes DNA compaction with H-NS and significant cell elongation. Furthermore, flow cytometry data indicate cell lengthening and a decrease in DNA content, suggesting that MotB may inhibit DNA replication. RNA-seq analyses indicate that MotB does not affect T4 gene expression, but its presence significantly affects host gene expression, including derepressing many H-NS-repressed host genes. Finally, we find that MotB significantly stimulates DNA packaging into T4 phage heads in vitro. We speculate that MotB improves phage fitness through its compaction of host DNA and/or by improving the packaging of phage DNA.

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Virology - DNA, RNA, and Retroviruses
The regulatory role of the virome in intestinal immunity
The microbiome, made up of fungi, bacteria and viruses, regulates immunity. While considerable work elucidated the role of the bacterial microbiome in shaping the immune response to pathogens, the role of the virome in immunity is largely unexplored. Over 90% of the viruses in the virome are bacteriophages (phages) that infect the bacterial microbiome; these phage populations correlate with mammalian disease states, without changing the bacterial microbiome. For example, the gut virome of patients with inflammatory bowel disease had an expansion in phage diversity relative to healthy controls. However, these correlative studies could not elucidate whether expanded phage diversity caused the dysregulated immune response. Thus, we sought to understand how phages modulate mammalian immunity. Identifying a mechanism that phages control intestinal immunity is challenging as natural bacterial communities contain numerous phages; therefore, specific immune phenotypes cannot be attributed to phages. To circumvent this issue, I developed a highly controlled experimental approach allowing me to formally link defined phages to specific immunological outcomes. I first identified bacteria lacking integrated phages and developed an 11-member bacterial community that represents the diversity of the bacterial microbiome. We then colonized germ free mice with this defined bacteria community, and the pups from these mice were then colonized with or without two specific phages (Qb and crAss001). At homeostasis, phage colonized mice had a reduction in Type 1 immunity, specifically fewer IFNg producing CD4+ and CD8+ T cells in the large intestinal lamina propria. Interestingly, this reduction in Type 1 immunity occurred in parallel to an increase in expression of genes associated with Type 2 immunity such as mucins in the intestinal epithelium and GATA3 in CD4+ T cells and regulatory T cells. This reduction in IFNg was maintained during a model of acute viral gastroenteritis, murine norovirus (MNV), as CD4+ and CD8+ T cells from both the small and large intestinal lamina propria as well as the intraepithelial lymphocytes from the small and large intestine produced lower levels of Type 1 cytokines in phage colonized mice. Additionally, phage colonized mice had higher burdens of MNV in the intestine compared to mice without phages. Together, these results support the idea that unexpectedly, phages may play an important role in the regulation of mucosal immune responses.

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Virology - DNA, RNA, and Retroviruses
Identification of a Novel Dengue Virus Receptor in Aedes Aegypti Mosquitoes
Dengue virus (DENV), an arthropod-borne virus (arbovirus), is primarily transmitted by Aedes aegypti (Ae. aegypti) mosquitoes and causes an estimated 100 million cases of dengue fever and 22,000 deaths annually according to the CDC. There are four serotypes of DENV in circulation and although infection with one strain will lead to long-term protection against the infecting serotype, a secondary infection with a different strain promotes greater risk for dengue hemorrhagic fever (DHF) which can be fatal. There is currently no vaccine approved for naïve individuals, as the only approved vaccine is known to cause an increased risk of developing DHF. Therefore, limiting vector infection is crucial to restrict the transmission cycle of DENV. The surface DENV E glycoprotein present on mature virus particles is responsible for receptor interactions. Studies in vertebrate cells have identified multiple receptors on a variety of cell types, but a definitive receptor in mosquitoes has yet to be identified. The mosquito midgut epithelium is the first tissue that DENV encounters during infection through receptor-mediated endocytosis. We isolated membrane proteins from serum fed mosquito midguts and utilized 2D-far western analysis and mass spectrometry to identify multiple putative receptors for DENV in Ae. aegypti. Previous studies have shown that DENV proteolytic processing mediated by midgut trypsins during blood digestion influences the rate of DENV infection in Ae. aegypti. With this knowledge we treated DENV with trypsin before probing during far western assays and observed a change in which DENV only interacted with one potential receptor: pDENVRec. This protein is highly conserved and has orthologs in both culicine and anopheline mosquitoes. We were able to express and purify recombinant pDENVRec for use in BIAcore assays. The results showed that recombinant pDENVRec interacts with DENV and binds with high affinity. To further characterize this receptor, we generated an antibody targeting pDENVRec and validated its specificity for use in IFAs to localize pDENVRec expression in the midgut. We are currently generating a KO mosquito line to determine the effect of eliminating pDENVRec on DENV infection. These results suggest that pDENVRec is an important protein in the mechanism of DENV infection and can potentially be a target for future strategies to block not only DENV infection, but potentially additional flaviviruses like Zika virus and yellow fever virus.

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Virology - DNA, RNA, and Retroviruses
A genome-wide CRISPRa screen reveals helicase with zinc finger 2 as a potent cellular inhibitor of Ebola virus
Ebola virus (EBOV) causes outbreaks of viral hemorrhagic fever in humans with a case fatality rate of 60%. EBOV is a major public health concern because of its extreme virulence, its capacity for human-to-human transmission, and the unpredictability of EBOV emergence. The host type I interferon (IFN) response is critical in controlling EBOV replication at the cellular level, but the specific host proteins that have direct antiviral activity against EBOV, termed restriction factors, are unknown. To identify new host

restriction factors for EBOV, we employed a genome-wide CRISPR transcriptional activation (CRISPRa) screen in human liver cells. In this CRISPRa screen, endogenous gene expression is induced by a nuclease-dead Cas9, recruited transcriptional activators, and guide RNAs that target promoter regions (10 guides/gene, ~210,000 guides in total). To identify putative viral inhibitors, CRISPRa cells were infected with EBOV whose replication is cytopathic, and surviving cells were subjected to RNAseq. After selection with EBOV, approximately 300 genes were significantly enriched in surviving cells. The top antiviral gene candidate was helicase with zinc finger 2 (Helz2), which is a known IFN-stimulated gene. Helz2 proved to be a remarkably potent restriction factor for EBOV by blocking release of infectious virus by more than 1000-fold compared to the control. To identify the mechanism of Helz2 inhibition, viral RNA was measured over a single round of infection during Helz2 overexpression. Helz2 interfered with EBOV RNA replication, while virus binding and initial entry into cells were not affected. Immunofluorescence of Helz2 CRISPRa cells during infection showed that viral inclusion bodies, defined as the sites of viral RNA replication, were smaller and less developed in the presence of Helz2. Furthermore, Helz2 was shown to bind to the EBOV VP35 protein, a cofactor for the virus RNA polymerase and the major IFN antagonist in infected cells. Thus, Helz2 disrupts early viral RNA replication and may also function to increase the ability of virus-infected cells to sense infection and produce IFN. This work identifies a specific point of vulnerability of EBOV during RNA replication and thereby reveals a strategy to design antivirals that mimic Helz2 function. Furthermore, validation of additional â€˜hitsâ€™ from this screen will expand our understanding of the antiviral gene repertoire of the cell against EBOV.

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Virology - DNA, RNA, and Retroviruses
A conserved motif functionally links the antiviral activity of IFITM3 with its oligomeric state in membranes
The interferon-inducible transmembrane (IFITM) proteins inhibit infection by a broad range of enveloped viruses, including Influenza A virus (IAV), Zika virus, and SARS coronavirus. IFITM3 restricts virus entry by inhibiting fusion pore formation between cellular and viral membranes. It has been proposed that virus entry arrest by IFITM3 requires its oligomerization, yet functional proof was lacking. Here, we identified a previously uncharacterized 91GxxxG95 motif in the intracellular loop of the CD225 domain of IFITM3. The GxxxG motif is known to mediate the oligomerization of many cellular proteins. We found that a single point mutation (i.e. G95L) prevented IFITM3 from restricting fusion driven by IAV and VSV-G without affecting IFITM3 protein stability. To determine whether G95L impacts IFITM3 oligomerization, we generated pairs of fluorescently-tagged IFITM3 to measure FRET with fluorescence lifetime imaging (FLIM) in living cells. The expression of wildtype IFITM3-YFP and wildtype IFITM3-mCherry in cells resulted in potent decreases in donor (YFP) lifetimes, indicative of FRET via IFITM3 oligomerization. However, G95L IFITM3-YFP and G95L IFITM3-mCherry expression did not result in FRET, signifying a failure to oligomerize. Our results were confirmed by co-immunoprecipitation experiments using FLAG- and myc-tagged IFITM3. These results reveal that G95 in the intracellular loop of IFITM3 is critical for IFITM3 oligomerization and that oligomerization is associated with antiviral function. While

the precise mechanism by which IFITM3 inhibits virus fusion remains controversial, multiple reports showed that IFITM3 enhances lipid packing (order), thereby making membranes more rigid. Therefore, we utilized a fluorescent membrane order probe (FlipTR) in living cells expressing antiviral IFITM3 (wildtype) or the non-antiviral variant of IFITM3 (G95L). We found that wildtype IFITM3 enhances membrane rigidity in living cells while G95L IFITM3 does not. Lastly, amphotericin B, an antifungal heptaene previously found to counteract the antiviral function of IFITM3, reduced membrane rigidity induced by IFITM3. Overall, our findings demonstrate that the antiviral function of IFITM3 is functionally linked to its capacity to oligomerize and to increase membrane rigidity. We are now performing experiments to test whether enforced oligomerization of G95L IFITM3 rescues its antiviral function and the results of these experiments will be presented.