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A BID-MTCH2 love story: Energizing mitochondria until apoptosis do them part?

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Mitochondrial carrier homolog 2 (MTCH2) is currently one of the most enigmatic proteins in mitochondria biology. MTCH2's ligand is the pro-apoptotic BH3-only BID protein, and the love story between these two proteins involves the regulation of diverse cellular processes including apoptosis, cellular metabolism, mitochondrial dynamics, and protein insertion into the outer mitochondrial membrane. Most recently, we found that MTCH2 is involved in: 1) ER-mitochondria phospholipid-transfer, and 2) energizing mitochondria. I will present a model of how these new data offer important clues to solving the BID-MTCH2 enigma.

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A high-throughput screening platform to probe protein/metabolite interactions enables rapid discovery of functional allosteric binding sites for drug discovery

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Metabolites are not passive victims of anabolic and catabolic reactions, but serve to allosterically regulate a myriad of cellular proteins. We know only a small fraction of these binding interactions and their functional implications. As such, proteins are under

the direct control of metabolites and investigation of novel metabolite-protein interactions offers new avenues for drug discovery.

Atavistik Bio aims to evaluate metabolite-protein interactions by screening proteins with their optimized Atavistik Metabolite Protein Screening (AMPS) platform. The AMPS platform is based on the MIDAS platform developed at the University of Utah and employs a proprietary metabolite library composed of ~700 mass spectrometry-validated metabolites observed in humans. The metabolite library is organized into multiple unique screening pools to provide optimal signal, and definitive identification. The high-throughput screening method employs microdialysis to separate purified target proteins from the metabolite screening pools by a semipermeable dialysis membrane, which allows metabolites but not proteins to freely diffuse. If one or more metabolites interact with the target protein, the concentrations of those metabolites increase in the protein-containing chamber relative to the metabolite-containing chamber. Relative metabolite abundance is measured using an established high-throughput, LC-MS technology.

The AMPS platform data enables Atavistik Bio to build an extensive protein-metabolite database map to reveal unique insights into the crosstalk between metabolite-protein pathways. The platform utilizes advanced informatics tools, deep expertise in chemistry and computationally rich structure-based drug design. Atavistik Bio has identified and understood the role of metabolite-protein interactions across biological and disease-relevant pathways driving the discovery of novel therapeutics.

To validate the platform, several proteins were screened through AMPS, and hits for a subset of proteins were further characterized. Functional and structural assessment of metabolite hits revealed orthosteric and allosteric modulators of branched-chain amino acid catabolism, urea cycle cycle enzymes, and fatty-acid oxidation enzymes. This data set validates the utility of the AMPS platform to identify functional binding sites on proteins with the potential to advance small molecule drug discovery efforts.

A Hunt for Mitochondrial Restrictors of Toxoplasma Infection

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As immune signaling platforms, mitochondria are critical in the host defense against intracellular pathogens. Consequently, mitochondria are a target of microbial effectors, several of which alter mitochondrial morphology and function independently of their role in innate immune signaling. This raises the question of whether mitochondria mediate noncanonical cell autonomous defenses during infection. In previous work, we found that host mitochondria compete with the human parasite *Toxoplasma gondii* for fatty acids, thereby restricting parasite growth. Here, we set out to more broadly define how a host cell weaponizes mitochondrial function. To identify mitochondrial modulators of *T. gondii* growth, we performed a mitochondria-specific loss-of-function CRISPR/Cas9 screen. To define the impact of mitochondrial respiration on cellular defenses, we included in our screen conditions of high or low mitochondrial oxidative function. Our results shed light on how mitochondria regulate microbial growth, and conversely, how *Toxoplasma* exploits mitochondrial function to its benefit.

A mitochondrial iron-responsive pathway regulated by DELE1 import

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Accumulating evidence indicates that mitochondrial import of certain proteins is precisely regulated in response to environmental changes and/or various

external/internal insults. Importantly, in many of these cases, the regulation of mitochondrial import is utilized as a communication tool between mitochondria and other cellular compartments to evoke appropriate responses. A well characterized example is the Parkinson's disease-linked mitochondrial kinase PINK1, whose import arrest and stabilization on the outer mitochondrial membrane upon mitochondrial membrane potential loss triggers Parkin-mediated mitophagy. Here, we identified another example of mitochondrial import-coupled stress sensing mechanism. Heme-regulated inhibitor kinase (HRI) is a cytosolic heme-responsive kinase that is activated by heme deficiency. We found iron deficiency is also a strong activator of HRI, and this iron deficiency-induced HRI activation requires the mitochondrial protein DAP3 binding cell death enhancer 1 (DELE1), which was recently identified as an HRI activator upon mitochondrial stress. Notably, the mitochondrial import of DELE1 and its subsequent protein stability is regulated by intracellular iron availability. When iron is available, DELE1 is degraded by the mitochondria matrix-resident protease LONP1 soon after import. In contrast, iron deficiency induces mitochondrial import arrest of DELE1, thereby enabling it escape from the degradation. Stabilized DELE1 on mitochondrial surface interacts with and activates HRI. The lack of this pathway sensitized erythroid cells against iron deficiency, suggesting a novel role of mitochondria as a signaling platform to activate cell survival signal upon iron deficiency in the iron-demanding cell lineage. Our findings demonstrate DELE1 as a mitochondria-resident iron-responsive signaling molecule, and strengthen the general importance of mitochondrial import-coupled stress sensing mechanisms (Sekine Y, Houston R et al., *Mol Cell.*, 2023).

A mitoproteomic analysis of flavivirus-infected cells unveils the proviral role of PLA2G15 and the alteration of the mitochondria-lysosome contacts sites

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Dengue virus (DENV) and Zika virus (ZIKV) are two expanding flaviviruses, whose viral infections constitute major public health concerns worldwide, especially considering that no antivirals are available for these viruses. Published and preliminary work from our research group has demonstrated that both DENV and ZIKV alter the morphology of the mitochondria, which is accompanied by major perturbations in their roles in metabolism, apoptosis, and innate immunity. Considering this, we hypothesized that DENV and ZIKV hijack mitochondrial functions by modulating their composition and interaction with other cytoplasmic components. To assess this, we performed a label-free mass spectrometry-based proteomic analysis of mitochondria isolated from control and DENV- or ZIKV-infected cells complemented with a medium-throughput RNAi screening. We identified SNX33, KDM5A, and PLA2G15 as flavivirus regulators whose mitochondrial association was significantly changed upon infection. Notably, the knockdown or the pharmacological inhibition of the lysosomal phospholipase PLA2G15 impaired viral replication. Interestingly, the infection by DENV and ZIKV altered the expression profile of PLA2G15, implying a direct targeting of this host factor by flaviviruses. In addition, live cell imagining of infected cells showed alterations in the mobility and distribution of lysosomes upon flavivirus infection. This correlated with a decrease in PLA2G15/mitochondria colocalization (in accordance with our mitoproteome data) as well as in the global abundance of contact sites between lysosomes and mitochondria as assessed by proximity ligation assays. The contribution of PLA2G15 to this flavivirus-mediated morphological alteration is currently under investigation. PLA2G15 inhibitors will be challenged in *in vivo* infection models to assess their therapeutic potential against DENV and ZIKV infection.

A novel approach for high content screening at ultrastructural resolution propels a holistic understanding of mitochondrial ultrastructure

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Mitochondria form a complex membrane architecture required for executing the multitude of mitochondrial functions. The architecture is also dynamic and capable of adjusting during differentiation to numerous cell types and under varying physiological conditions. While much is known about the basic machinery that shapes the membrane, many membrane shaping proteins remain unidentified and, moreover, the orchestration of the process and how it fails during mitochondrial-related diseases remain mysterious. Uncovering proteins that affect membrane shaping has, until now, been limited mostly to low throughput approaches that enable high resolution imaging, such as electron microscopy (EM), that is lengthy and laborious.

Recently we created a methodology that enables high-throughput EM in yeast - MultiCLEM (Multiplexed Correlative Light and Electron Microscopy) using cellular barcoding. Bringing MultiCLEM to the next level, we have now developed high-throughput ultrastructure screening in human cells – hMultiCLEM. We established a robust barcoding protocol, universal for a broad variety of cells, which allows us to screen tens of conditions in a handful of EM experiments. Using this technology, we are now performing the first-ever high content screen at ultrastructural resolution to uncover modulators of mitochondrial cristae. Building on siRNA mediated silencing of tens of genes residing in mitochondria we are mapping the breadth of membrane shaping proteins and their regulators. Our work is unveiling the protein networks that drive cristae modulation and should help shed light on how this occurs in the specific contexts of development, metabolism, circadian rhythm, aging and disease states. More broadly, our method propels the EM field towards a new era of high-throughput EM for membrane morphology studies in basic research and clinical context.

A novel role of LRRK2 in iron handling

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Mutations in leucine-rich repeat kinase 2 (LRRK2) cause autosomal dominant Parkinson disease (PD), while polymorphic LRRK2 variants are associated with sporadic PD. LRRK2 is a multidomain enzyme, linked to a number of cellular pathways including autophagy, lysosomal processing, inflammation, and vesicular trafficking. Mitochondrial dysfunction is central to Parkinson's disease (PD) pathogenesis, and LRRK2 mutations affect mitochondrial dynamics, trafficking, and mitophagy. Mitochondria are also central hubs for iron metabolism, being responsible of forging free iron into iron sulfur clusters and

heme groups. which are essential for diverse cellular functions, such as mitochondrial respiration. Iron is essential for life but also a very reactive harmful ion, thus its levels inside the cell are tightly regulated. Here, using RAW macrophages we provide novel evidence connecting the role of LRRK2 in modulating cellular iron handling. Loss of LRRK2 or expression of the pathogenic variant LRRK2 G2019S dysregulate iron homeostasis, causing a defect in iron storage, iron uptake and sensitizing cells to death induced by iron overload. Importantly, the absence of LRRK2 or expression of LRRK2 G2019S impacts also iron recycling (ferritinophagy), and its modulation. We are investigating the mechanisms underlying the requirement for LRRK2 in iron handling through potential interactions with the ubiquitin ligase HERC2, which regulates NCOA4 turnover and ferritinophagy. Overall our results provide new insights of the role of LRRK2 in sustaining iron homeostasis and provides further evidence connecting PD pathogenesis with improper iron handling.

AASDHPPT is the mammalian mitochondrial phosphopantetheinyltransferase required for mitochondrial fatty acid synthesis (mtFAS) function

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The mitochondrial fatty acid synthesis (mtFAS) pathway is an emerging central regulator of mitochondrial oxidative metabolism, with important roles in TCA cycle enzyme function and electron transport chain (ETC) assembly, and implications for cell fate and differentiation signaling. Yet, despite its integral role in mitochondrial metabolism, several components of the pathway are still poorly defined in mammalian cells. mtFAS builds fatty acyl chains in the mitochondrial matrix on an acyl carrier protein (mtACP) that requires a 4'-phosphopantetheine (4'-PP) co-factor. 4'-PP is derived from coenzyme A and added to proteins by a phosphopantetheinyltransferase (PPTase) enzyme. Yeast have two PPTases: Ppt1, which localizes to the cytoplasm and modifies the

ACP domain of the cytoplasmic fatty acid synthase, and Ppt2, a mitochondrial PPTase that is absolutely required for mtFAS function, but the mammalian mitochondrial PPTase has not been defined. AASDHPPT, the only annotated PPTase in mammalian genomes, clusters with mtACP in CRISPR screening data from our lab as well as co-essentiality data from the Broad's Depmap, implying that its essential function is in the mtFAS pathway. However, its necessity for mtFAS activity has never been tested and prior studies localized the enzyme to the cytoplasm, leaving many questions as to how mtACP might be modified prior to mitochondrial import, and/or whether there might be another, as yet undiscovered mitochondrial PPTase in mammals. Here we show that AASDHPPT is required for mtACP phosphopantetheinylation, along with protein lipoylation and ETC complex assembly, two downstream outputs of mtFAS pathway activity. Clonal AASDHPPT mutant cell lines display respiratory growth defects, decreased basal and maximal oxygen consumption, and reduced oxidative TCA cycle activity. We found that AASDHPPT localizes to mitochondria, an effect that is at least partly driven by the N-terminus of the protein. We also tested several AASDHPPT point mutations listed as variants of uncertain significance identified in patients with inborn genetic diseases and found evidence of reduced function. Our data define AASDHPPT as the mammalian mitochondrial PPTase, identify potentially pathogenic gene variants, and lay the foundation for future work aimed at understanding the regulation of this central metabolic pathway.

Acute suppression of mitochondrial ATP production prevents apoptosis and provides an essential signal for NLRP3 inflammasome activation

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During intrinsic apoptosis, mitochondria release stores of cytochrome c that bind cytoplasmic APAF-1 to cause apoptosome formation and caspase-9 activation. Mitochondria are also implicated in the activation of the NLRP3 inflammasome and ensuing cell death by pyroptosis, although their precise role here is incompletely understood. Apoptosis is of low immunogenic potential, while pyroptosis is highly inflammatory and especially the NLRP3 inflammasome stands out not only for its sheer range of endogenous and exogenous activators, but also for its role in immunopathology in a wide variety of diseases. How mitochondria reconcile roles in such functionally divergent cell death pathways as apoptosis and pyroptosis remains elusive. Here we show that when cells are challenged simultaneously with activators of apoptosis and of NLRP3, apoptosis is inhibited and inflammasome activation prevails. Apoptosis inhibition by structurally diverse NLRP3 activators, including small molecules such as nigericin and imiquimod but also physiological activators like extracellular ATP and SARS-CoV-2, was not a consequence of inflammasome activation but rather of their effects on mitochondria. Specifically, we discovered that NLRP3 activators commonly inhibit oxidative phosphorylation (OXPHOS) through distinct mechanisms. OXPHOS inhibition by NLRP3 activators as well as conventional electron transport chain inhibitors or mitochondrial uncouplers disrupted mitochondrial cristae architecture, leading to trapping of cytochrome c. Although OXPHOS inhibition and disruption of cristae architecture were alone not sufficient for NLRP3 activation, this activity was nonetheless required for triggering NLRP3. Thus, NLRP3 activators and other OXPHOS inhibitors have common effects on mitochondria that not only provide a signal for NLRP3 activation, but also simultaneously inhibit apoptosis for stringency in cell death decisions. We further propose a model in which NLRP3 activation requires two simultaneous signals: a mitochondrial signal involving OXPHOS suppression, and an additional cellular signal. Finally, our work points towards a host-protective function of NLRP3 as a guard of mitochondrial function and a sensor for pathogens suppressing apoptosis that can help explain the evolutionary conservation of NLRP3 despite its penchant to instigate pathological inflammation.

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AMPK arrest mitochondria in response to low ATP

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Neurons are well-known to require large amounts of ATP to power neurotransmission, which makes them particularly sensitive to defects in mitochondrial function. These cells are often large and branched and so must employ mechanisms to position mitochondria where they are most needed. Mitochondria are moved by molecular motors and are coupled to these motors by the adaptor complex proteins Miro1/2 and Trak1/2. This study investigates a novel mechanism coupling cellular energy levels with mitochondrial position. Using low concentrations of the electron transport chain (ETC) inhibitor antimycin A, we find that ETC-inhibited mitochondria are anchored in place by the actin cytoskeleton. This low concentration of antimycin A reduces oxygen consumption and neuronal ATP levels, but it is not sufficient to depolarize mitochondria, grossly affect mitochondrial shape, or activate the mitochondrial-depolarization sensing kinase PINK1. We show that this mild electron transport inhibition activates the energy-sensing kinase AMPK, which phosphorylates Trak1 to trigger the association of mitochondria with the actin cytoskeleton. This anchoring mechanism may serve to arrest energy-producing organelles in places where they are most needed. Further investigation into the effects of AMPK signaling on neuronal mitochondria will deepen our understanding of the importance of energy homeostasis in healthy and diseased states.

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ATF4 activates a mitochondrial metabolic defense during infection

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In an infected cell, mitochondria are perceived as signaling platforms or reservoirs for danger signals. Whether mitochondria play a broader role in cellular defense remains largely unexplored. We found that the integrated stress response (ISR) activates a mitochondrial metabolic defense to protect against the human parasite Toxoplasma gondii. Infection with Toxoplasma gondii drove an increase in mitochondrial DNA (mtDNA) independently of mitochondrial biogenesis. The increase in mtDNA depended on the host transcription factor ATF4, a key effector of the ISR. ATF4 protected one-carbon mitochondrial metabolism and promoted mitochondrial nucleotide sequestration during infection, thereby limiting parasite nucleotide levels. Cells deficient for ATF4 or the mito 1C enzyme MTHFD2 did not increase mtDNA during infection and were more permissive to parasite replication. ATF4 activation also drove mitochondrial nucleotide and mtDNA levels during infection-independent stress. Thus, ATF4 drives mitochondrial one-carbon metabolism and mediates metabolic immunity against a pathogen.

ATP Synthase Functionality and Dynamics in the Senescent Heart

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Heart disease is the leading cause of death in the elderly population and like all muscles, the heart is a highly energy-consuming tissue. The high energy requirement of the heart is reflected in the abundance of mitochondria in cardiomyocytes and the cristae dense architecture of the organelles. Energy conversion takes place during oxidative phosphorylation (OXPHOS) within the inner mitochondrial membrane (IMM), which is folded to cristae in order to increase the surface area for OXPHOS. The ATP Synthase, or Complex V (CV), is well known for its involvement in ATP synthesis, but it also plays an important structural role. In addition, it has recently been shown that the enzyme can act in reverse mode and hydrolyse ATP. This is increased under pathophysiological conditions, but it also takes place in the absence of stress [1]. The versatile functions of CV are reflected in its spatiotemporal organisation, making single molecule localisation

microscopy in living cells a valuable tool to decipher them under different conditions [2, 3].

In the present study, we were interested in the effects of cellular senescence on CV in cardiomyocytes. We used human induced pluripotent stem cell derived cardiomyocytes as a model system and elicited senescence by application of low doses of doxorubicin. We observed dramatical changes in mitochondrial ultrastructure with reduced cristae density and altered morphology [4]. Performing single molecule tracking of CV, we observed overall reduced mobility, while at the same time the fraction of immobile complexes was also decreased, indicating a re-organisation of CV. This was correlated with functional decline of CV, which was determined by measuring ATP production as well as membrane potential. We found decreased ATP levels while the mitochondrial membrane potential was increased, which indicates a decline in ATP synthesis function in our senescent cardiomyocyte model. Our results build a base for further investigations into the role of reduced CV functionality and dynamics in senescence associated heart disease, but also allow general conclusions on the versatility of CV function.

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Autophagy functions in mitochondrial and nuclear genome maintenance

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Autophagy plays a critical role in maintaining homeostasis of mitochondria. Mitochondria contain their own small multicopy genomes (mtDNA), which are organized in many mtDNA-protein structures (nucleoids) and distributed throughout the dynamic tubular network of mitochondria. mtDNA is replicated by the dedicated mitochondrial DNA polymerase gamma (POLG) in a manner that is asynchronous with the nuclear genome throughout the cell cycle. In our previous work, we discovered a novel function of POLG in mtDNA degradation. Specifically, when autophagy is impaired, POLG switches from synthesis to degradation of mtDNA during starvation causing quantitative mtDNA instability and irreversible loss of respiratory function. mtDNA degradation is mediated by the inherent 5′-3′ exonuclease activity of POLG. Our new work builds on the mechanisms of POLG-mediated mtDNA degradation (POMD) and shows how autophagy plays a key role for the maintenance of the mitochondrial and nuclear genome. Specifically, POMD results in the activation of the nuclear DNA damage response in dependence of mechanisms of cytosolic mtDNA release. Cells depend on autophagy in order to resolve DNA damage during starvation. Our work suggests a model in which continuous POMD in the absence of autophagy causes mtDNA fragment accumulation in the nucleus resulting in compromised nuclear DNA damage resolution.

Bidirectional neuron-astrocyte mitochondrial transfer in Alzheimer´s disease

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Neurons have a complex morphology and consequently face a greater challenge in distributing and maintaining mitochondria throughout their arborizations. Recent studies in our lab using models of Alzheimer´s disease (AD), a type of dementia affecting memory, thinking and behavior, revealed early mitochondrial dysfunctions preceding synaptic disorganization, with severe impairments on mitochondrial anterograde transport and accumulation of dysfunctional mitochondria in the pre-synapse before

overall decay of energy metabolism[1]. Further studies *in vitro* revealed that while healthy astrocytes change their mitochondrial dynamics in response to stressed neurons, with increased expression of mitochondria movement and biogenesis genes, AD astrocytes fail to do so. Using different types of neuron-astrocyte coculture systems – with or without physical contact – we observed an exchange of mitochondria between astrocytes and neurons, which is disrupted in the App^{NL-G-F} knock-in AD mice and potentially contribute to the loss of heathy mitochondrial pool in axonal terminals. We observed that neurons release functional mitochondria via specialized extracellular vesicles (EVs), named mitovesicles, which are further captured by astrocytes. While WT mitovesicles mainly integrate in the host astrocytic mitochondrial network, App^{NL-G-F}-derived mitovesicles show reduced engulfment and integration. Further analysis revealed that despite similar release in number of neuron-derived EVs and median size of 150 nm evaluated by nanoparticle tracking analysis, App^{NL-G-F}-derived EVs show decreased complex-I driven mitochondrial respiration. An upregulation of mitophagy events and related gene expression in App^{NL-G-F} astrocytes in coculture with neurons suggests that dysfunctional fragments of neuronal mitochondria are eliminated by transmitophagy in astrocytes. To understand the neuroprotective potential of this transfer, we treated App^{NL-G-F} neurons with EVs released from astrocytes. While WT astrocytes-derived EVs mitigate mitochondrial respiratory deficits and enhance synaptic ATP in App^{NL-G-F} neurons, mitochondria-depleted WT EVs and App^{NL-G-F} EVs show no protective potential. Interestingly, analysis of App^{NL-G-F} astrocytes-derived EVs indicate decreased expression of inner mitochondria membrane proteins (e.g., OxPHOS subunits), that is not related with altered levels of these protein in App^{NL-G-F} astrocytes, which may suggest deficient incorporation of inner membrane into mitovesicles from App^{NL-G-F} astrocytes. Overall, these data reveal that AD mitovesicles have different functional profiles that influence its transfer and fate in the recipient cell, likely affecting its neuroprotective potential.

1. Naia, L. et al. *Mol. Psychiatry* in press (2023).doi:10.1038/s41380-023-02289-4

Cdk8 regulates mitochondrial dynamics and suppresses a Drosophila model of Parkinsonism

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Drosophila Cyclin-dependent kinase 8 (Cdk8), the orthologue of vertebrate CDK8 and CDK19, is a threonine/serine kinase that is best known to function with the Mediator Complex to a regulate gene expression. Using flies to investigate Cdk8 family function, we found that depletion of *cdk8* severely reduces lifespan and causes bang sensitivity and impaired mobility, as detected in climbing assays. We found that Cdk8 can modulate mitochondrial morphology under physiological conditions in numerous tissues. Depletion of *cdk8* by RNAi leads to an elongated mitochondrial phenotypes and mitochondrial dysfunction characterized by elevated ROS and decreased ATP production. In contrast, elevated expression of Cdk8 leads to increased fission and a fragmented phenotype, like what is seen with modulation of Drp1, a protein required for mitochondrial fission. Defects due to *cdk8* depletion could be rescued by expression of wildtype human CDK19, one of the two Cdk8 orthologs. Of note, cytoplasmically-targeted human CDK19 could similarly rescue depletion of *cdk8*, implicating a non-nuclear function of CDK19/Cdk8. We found that Cdk8 promotes and is required for phosphorylation of Drp1 to allow proper fission of mitochondria. To test whether mitochondrial dysfunction underlies the cellular phenotypes, we raised *cdk8*-depleted flies on a diet containing the clinically approved antioxidant NACA and observed rescue of climbing defects and mitochondrial fusion phenotypes, establishing elevated ROS as one of the key hallmarks of Cdk8 dysfunction. We found a compelling further link between Cdk8 and familial Parkinsonism, which is due to defective mitochondrial quality control. Elevated Cdk8 could rescue mitochondrial and climbing defects in a fly model of Parkinsonism caused by a mutation in the *pink1* gene. Rescued animals displayed restored Drp1 phosphorylation and functional mitochondrial dynamics. These findings point to a potential therapeutic direction for Parkinsonism by promoting Cdk8 activity.

Cell-free hemoglobin induces vascular and mitochondrial dysfunction in the pulmonary endothelium in an oxidation-state dependent manner

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Background: Sepsis results in significant organ damage due in part to a breakdown in endothelial vascular function. Elevated levels of cell-free hemoglobin (CFH) drive endothelial dysfunction in the lungs. CFH generates reactive oxygen species (ROS) via the iron present in the heme moiety, which can be oxidized from ferrous to ferric iron. Recent studies suggest mitochondrial dysfunction may be a key pathway leading to microvascular dysfunction. We hypothesize that CFH induces mitochondrial dysfunction and microvascular endothelial barrier disruption in the lung in an oxidative-state dependent manner. Methods: CFH was converted from ferrous (CFH2+) to ferric (CFH3+) by 8 cycles of 21 min of UV irradiation and validated by spectrophotometry. Primary human lung microvascular endothelial cells (HLMVECs, both male and female donors) were treated with 1.0 mg/mL CFH, or vehicle control, in all experiments unless stated. Barrier dysfunction was measured via electric cell-substrate impedance sensing (ECIS) and express permeability test (XPerT, Dubrovskyi 2013) at 24 h. We quantified mitochondrial superoxide using MitoSOX Red (5 uM) by flow cytometry at 1, 6, and 24 h. In addition, we measured total cellular ROS production by fluorescence microscopy using CellROX Deep Red (5 uM) at 6 and 24 h. Mitochondrial morphology was assessed by transmission electron micrographs taken of HLMVECs treated with CFH at 6 h, and morphology (electron density, roundness, area, circularity, and aspect ratio) was quantified by a blinded reviewer using ImageJ. Activation of the mitochondrial permeability transition pore (mPTP) was assessed by flow cytometry using calcein-AM with cobalt chloride to. Results: Treatment of HLMVECs with CFH generated excess ROS, measured by CellROX and MitoSOX, increased at 6 h compared to vehicle (522 vs 265 MFI, p = 0.006). Morphology analysis demonstrated significant changes to mitochondrial network dynamics including: increased electron density and aspect ratio and decreased circularity and roundness. Activation of the mPTP by CFH resulted in a significant drop in MFI (1295 vs 2197 MFI, p = 0.0069) indicating an increase in mPTP activity compared to vehicle and CFH2+. We did not find any differences by sex. Barrier dysfunction caused by CFH3+ measured by ECIS peaked at 6 h (-1270 vs 262 max TER drop, p<0.0001), while XPerT confirmed dysfunction (9.41e+06 vs 6.53e+05 total fluorescent area, p =

0.0067). Conclusions: The results demonstrate significant perturbations to mitochondrial function and network dynamics caused by oxidized CFH (3+). These findings point towards the ferric (3+) form of hemoglobin as the primary driver of endothelial dysfunction, and the underlying mechanism is directly related to disruption of the mitochondrial network. The association of mitochondrial and vascular biomarkers demonstrates an urgent need to better understand how mitochondrial dysfunction can be prevented to limit vascular damage.

Changes in Glutamine Metabolism and Cellular Stress Response Across Mitochondrial Disorders

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Background: Primary mitochondrial diseases (MDs) comprise a heterogenous group of hereditary and acquired disorders characterized by mitochondrial dysfunction, leading to changes in metabolism and cellular responses. Previously, we identified increased glutamine metabolism and an activation of the integrated stress response (ISR) in dermal fibroblasts from patients with the dilated cardiomyopathy with ataxia syndrome (DCMA). Evidence from *in vitro* models of Complex I deficiency (C1D), Barth Syndrome (BTHS) and Kearns-Sayre Syndrome (KSS) suggests that these alterations may not be unique to a single mitochondrial disorder (DCMA).

Aims & Hypothesis: This study aimed to compare glutamine metabolism and the integrated stress response activation in dermal fibroblasts from healthy individuals and patients with C1D, BTHS, and KSS. We hypothesize significant differences in glutamine metabolism and ISR activation between DCMA, C1D, BTHS, KSS fibroblasts and healthy fibroblasts.

Methods: For metabolism studies, healthy (N=5) and diseased fibroblasts (N=3) were each seeded in 3 wells of a 24-well plate at a density of 2.0 x 10^4 cells/well. After a 24-hour incubation (T=0), the growth media was replaced with DMEM media containing 2mM isotopically labeled glutamine [5-^{13}C 2-^{15}N], 5.5mM glucose, and 10% FBS, followed by a 48-hour incubation. Extracellular media were extracted in a 50% methanol and analyzed using liquid chromatography-mass spectrometry. Metabolite identification and data analysis were conducted using EL-Maven Software and GraphPad Prism. For gene expression, healthy (N=3) and diseased (N=3) fibroblasts are each seeded in three T75 tissue culture flasks. At 90% confluency, cells are detached and pelleted for RNA extraction using Qiagen's RNeasy mini kit. One microgram (µg) of Complementary DNA (cDNA) was synthesized using Promega's GoScript reverse transcriptase kit then diluted 10X with ultrapure water. Diluted cDNA and master mix were loaded into each well of a 384-well plate. Amplification was done using QuantStudio 5 Real Time PCR system and gene expression was computed relative to a housekeeping gene (GAPDH) using the 2-ΔΔCT method.

Results: We found that in C1D and KSS patient cells, labelled glutamine (5-^{13}C 2-^{15}N) uptake was not significantly affected (*P*=0.755) compared to healthy control cells. In contrast, BTHS patient cells labelled glutamine uptake is significantly increased (*P*=0.015). The secretion of [5-^{13}C 2-^{15}N] glutamine-derived metabolites was significantly different in C1D, BTHS and KSS compared to healthy controls (*P*<0.05), with each disorder illustrating a unique phenotype. We also found that the mRNA expression levels of several ISR genes is upregulated in DCMA (*P*<0.05), downregulated in C1D (*P*=0.0005, *P*=0.0033 and *P*=0.0003) and unchanged in BTHS and KSS (*P*>0.05).

Conclusions: Our findings demonstrate that abnormal glutamine metabolism is a common feature in several mitochondrial disorders (DCMA, C1D, BTHS and KSS). Furthermore, our gene expression data suggests that the integrated stress response may not always be activated in mitochondrial disorders.

Changes in Mitochondrial Respiratory Chain Super Complexes Across Functional Mammary Gland Development

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Determining the energetics of normal mammary gland development is an essential step toward understanding breast cancer. The changes that occur to the mammary gland during pregnancy and lactation present a uniquely metabolically dynamic environment as the mitochondria rise to the challenge of increasing energetic demands and structural remodeling. While morphological differences in mitochondria across mammary gland development have been noted and we have previously demonstrated that mitochondrial autophagy, termed mitophagy, is a necessary requirement for the lactogenic differentiation of mammary epithelial cells (MECs), the functional change within the mitochondria themselves has not been elucidated. By studying energetic changes in cancer cells via blue-native polyacrylamide electrophoresis (BNPAGE), we have found that bHLH/PAS family transcription factor Singleminded-2 (Sim2s) plays a role in the stabilization of mitochondrial respiratory chain supercomplexes (MRC SCs). Sim2s also plays an essential role in mitochondrial turnover during mammary gland development, prompting the investigation into a potential link between changes in the MRC and mitophagy in normal mammary gland development. Using HC11 cells, a mouse mammary epithelial cell line that undergoes functional differentiation analogous to the mammary gland in vivo, we show here that the MRC undergoes structural changes during differentiation. BNPAGE followed by western blot visualization shows that SCs are most stabilized at 24 hours differentiation, corresponding to the peak of functional differentiation in vivo. Activity as measured by In-Gel activity assay augments this data, with peak SC activity also occurring at 24 hours. With the reduction of Sim2 via sh knockdown, we see that the differentiation-associated changes in MRC are ablated, having a lower prevalence of both structure and activity, indicating the additional role of Sim2 in regulating not only mitochondrial turnover but also the functional difference of mitochondria caused by the increased mitophagy. To determine changes in mitophagy in vivo, we are using the Mitochondria Quality Control (MitoQC) mouse to fluorescently visualize mitochondrial turnover during the change from pregnancy through lactation to involution. Preliminary images show higher levels of turnover in lactation into involution, corresponding with the peak differentiation time points and their subsequent changes in MRC organization. Taken together, this data shows for the first time that mitophagy is necessary for mammary gland differentiation and is driven by the need for increased MRC super complex stability and activity to meet the energy demands of a developed mammary gland.

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Characterization of a novel SR splicing factor in mitochondrial and peroxisomal stress response

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Dysregulated protein translocation can lead to impaired organelle function and loss of cellular homeostasis. Peroxisomes are essential and yet the least studied subcellular organelles, while disrupted peroxisomal import often leads to severe impairment of lipid metabolism and tissue functions. However, our knowledge of how cells cope with peroxisome dysfunction and impaired peroxisomal import is largely missing. Here, through a genome-wide CRISPR screen, we have unveiled SCAF1 as a novel regulator for cellular fitness and mitochondrial homeostasis upon defective peroxisomal import. SCAF1 is a member of the serine/arginine (SR)-rich splicing factor family and is normally localized to nuclei. Intriguingly, SCAF1 is localized to mitochondria under peroxisomal import stress. Mitochondrial localization of SCAF1 is also observed upon mitochondrial stress (e.g., complex I inhibitor treatment). Through protein truncation analysis, we identify a non-canonical mitochondrial targeting sequence (MTS) at the N-terminus of SCAF1. To further dissect the role of SCAF1 in maintaining mitochondrial homeostasis, we perform a proteomics analysis to characterize SCAF1 interactome and find that SCAF1 interacts with many mitochondrial matrix proteins involved in ribosomal assembly and transcription (e.g., DDX28, DHX30, and LRPPRC), highlighting the critical role of SCAF1 in mitochondrial translation and transcription. Aligned with this idea, we find that the knockdown of SCAF1 led to an increase in the expression of mtDNA-coded proteins. Furthermore, SCAF1 knockdown impairs mitochondrial complex I activity and induces apoptosis upon defective peroxisomal import. Together, our data uncover a novel adaptive mechanism for maintaining mitochondrial homeostasis in response to the loss of proteostasis during peroxisomal and mitochondrial stress.

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CPEB3 Couples Synaptic Activity with Mitochondrial Energy Metabolism

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The mammalian brain has very high metabolic requirements owing primarily to the energy intensive nature of synaptic transmission and plasticity. Most of this energy demand is met by oxidative phosphorylation in neuronal mitochondria residing near synaptic sites. How are these distal synaptic mitochondria structurally and functionally maintained far away from the soma and how is their energy output tuned to the fluctuating energy demands of synapses undergoing plasticity, are central unresolved questions.

Local protein synthesis has emerged as the principle mechanism through which the synaptic proteome is both altered and maintained during plasticity when synapses undergo profound structural and functional changes. Cytoplasmic Polyadenylation Element Binding protein 3 (CPEB3) is an RNA binding protein that controls local synthesis of key plasticity related proteins (PRPs) and thereby mediates long term synaptic plasticity and memory. Here we reveal, that in addition to synaptic proteins, CPEB3 regulates the local synthesis of critical mitochondrial proteins, thereby maintaining distal mitochondrial function and coordinating its output with synaptic activity and energy demand.

We found that CPEB3 is highly localized to mitochondria, specifically in distal neurites, where it correlates with local protein synthesis hotspots. Knockdown of CPEB3 led to dysregulated expression of two key mitochondrial proteins: HSP60 and Pink1 in addition to its well established plasticity related targets GluR2 and PSD95, reduced mitochondrial membrane potential and dramatic loss of neurite branching, which has been shown to be dependent on local mitochondrial energy support. CPEB3 expression in the hippocampus is enriched in neurons with higher level of basal synaptic activity such as CA3 pyramidal neurons, PV interneurons and mossy cells in the dentate gyrus, consistent with their higher energy demands. Both CPEB3 expression and mitochondrial markers expression in the hippocampus were enhanced by contextual fear conditioning. Moreover, we found that CPEB3 expression is controlled by Brain Derived Neurotrophic Factor (BDNF) – a major activity dependent neurotrophin in the brain, providing a

mechanistic link between neuronal activity, local protein synthesis and energy metabolism.

Our findings are consistent with a novel, feed forward model, wherein activity dependent BDNF signaling and downstream CPEB3 mediated local protein synthesis at distal mitochondria enables mitochondrial function, which in turn supply the energy and metabolic support needed to sustain highly energy demanding cellular processes such as protein synthesis, neurite branching, synaptic plasticity and memory maintenance. Moreover, the coordinated local translation of both mitochondrial and plasticity related transcripts by CPEB3, ensures a critically important matching between synaptic function, energy demand and energy production at the synapse.

Cystine/Glutamate Antiporter, xCT, Controls Skeletal Muscle Regeneration and Metabolism

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Cysteine is the rate-limiting metabolite in glutathione (GSH) synthesis and plays a critical role in maintaining cellular redox homeostasis. Cystine, the oxidized form of cystine, is transported into cells by the plasma-membrane cystine/glutamate antiporter, xCT, and is subsequently reduced to cysteine. GSH redox is important for skeletal muscle metabolic homeostasis and cell regeneration, and disordered cysteine metabolism has been associated with multiple metabolic and neurodegenerative diseases. Bioinformatic analyses of publicly available datasets revealed that the expression of both xCT and GSH-related genes are negatively correlated with genes involved in myogenic differentiation, leading to our hypothesis that xCT controls skeletal muscle regeneration and metabolism. Subtle gray mice (xCT-mutant) that harbor a mutation in the Slc7a11 gene encoding for xCT, were used for primary muscle cell culture and in vivo experiments. 2-mercaptoethanol supplementation was crucial for xCT-deficient primary myoblast

proliferation but not differentiation, indicating the importance of xCT in cellular redox during proliferation. Moreover, xCT-mutant myotubes differentiated in both normal glucose (5.5mM) and high glucose (25mM) conditions had higher mitochondrial and glycolytic capacities despite lower levels of GSH. To investigate the role xCT in skeletal muscle regeneration, tibialis anterior (TA) muscles from xCT-mutant and wild-type (WT) mice were subjected to intramuscular cardiotoxin injections. Immunostaining of Ki67, MyoD, and Pax7 revealed that satellite cells in xCT-mutant mice have a greater proliferation and activation during early regeneration, with a greater commitment to differentiation at 7 days post-injury. In agreement, at 21 days post-injury, the cross-sectional area of xCT myofibers was larger than that of WT mice. To examine the metabolic implications of xCT-deficiency, xCT-mutant and WT mice underwent a 5-week progressive aerobic training protocol (treadmill running 1h/day, 5 days/week). Prior to exercise training, 5-week-old xCT-mutant mice were more insulin-sensitive than WT control, an effect that was more pronounced following exercise training. High-resolution respirometry analyses of mitochondrial energetics of TA myofibres showed that only WT mice increased CI OXPHOS, CI+CII OXPHOS, and maximal respiration upon exercise training. CI OXPHOS and CI+CII OXPHOS were lower in exercised xCT-mutant mice compared to exercised WT mice. The absence of improved mitochondrial function was partly attributable to a decrease in mitochondrial content, as skeletal muscle citrate synthase (CS) activity was lower in exercised xCT-mutants compared to non-exercised xCT-mutants. In contrast, lactate dehydrogenase (LDH) activity increased in exercised xCT mice, suggesting that xCT plays a role in skeletal muscle metabolic flexibility. Together, results demonstrate that xCT is crucial for maintaining skeletal muscle health. A greater understanding of involved mechanisms may reveal novel therapeutic approaches for skeletal muscle-related metabolic diseases and dystrophies.

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Cytosolic retention of the mitochondrial protease HtrA2 during mitochondrial protein import stress (MPIS) triggers the DELE1-HRI pathway

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Mitochondrial stress inducers, such as the proton ionophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and the ATPase pump inhibitor oligomycin, trigger the processing of DAP3-binding cell death enhancer 1 (DELE1) and lead to the activation of heme-regulated inhibitor kinase (HRI)-directed integrated stress response (ISR) pathway. Previous studies performed using epitope-tagged forms of DELE1 showed that these stresses induced the accumulation of a cleaved form of DELE1, DELE1-S, which interacts with HRI. Here, we report that mitochondrial protein import stress (MPIS) is an overarching stress that triggers the DELE1-HRI pathway, and that endogenous DELE1 could be cleaved into two forms, DELE1-S and DELE1-VS, the latter accumulating only upon non-depolarizing MPIS. We further showed that DELE1 specifically senses MPIS triggered by the inhibition of the TIM23 complex at the inner mitochondrial membrane (IMM). While MPIS can also cause mitophagy induction through engagement of the NLRX1-RRBP1 pathway, we observed that DELE1-HRI and NLRX1-RRBP1 signaling were engaged independently upon MPIS. Surprisingly, while the mitochondrial protease OMA1 was crucial for DELE1 cleavage in HeLa cells, it was dispensable in HEK293T cells, suggesting that multiple proteases may be involved in DELE1 cleavage upon stress. In support, we identified a key role for another mitochondrial protease, HtrA2, in mediating the cleavage of DELE1 into DELE1-VS, and showed that a Parkinson's disease (PD)-associated HtrA2 mutant displayed reduced DELE1 processing ability, suggesting a novel mechanism linking PD pathogenesis to mitochondrial stress. Our data further suggest that DELE1 is likely cleaved into DELE1-S in the cytosol, while the DELE1-VS form might be generated during halted translocation of the protein into mitochondria. Together, this study identifies MPIS as the overarching stress detected by DELE1 and identifies a novel role for HtrA2 in DELE1 processing.

Diabetic sensory neuropathy and insulin resistance are induced by loss of UCHL1 in Drosophila

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Despite diabetic sensory neuropathy (DSN) being one of the most common complications of type 2 diabetes (T2D), the mechanistic association between T2D and DSN at molecular levels remains elusive. Here we identify ubiquitin C-terminal hydrolase L1 (UCHL1), a deubiquitinase highly expressed in neurons, as a key molecule underlying T2D and DSN. Genetic ablation of UCHL1 leads to neuronal insulin resistance and T2D-related symptoms in Drosophila. Furthermore, loss of UCHL1 induces DSN-like phenotypes, including numbness to external noxious stimuli and axonal degeneration of sensory neurons in fly legs. Conversely, UCHL1 overexpression improves DSN-like defects of T2D model flies. UCHL1 governs insulin signaling by deubiquitinating insulin receptor substrate 1 (IRS1) and antagonizes an E3 ligase of IRS1, Cullin 1 (CUL1). Consistent with these results, genetic and pharmacological suppression of CUL1 activity fully rescues T2D- and DSN-associated phenotypes. Therefore, our findings suggest a complete set of genetic factors explaining T2D and DSN, together with potential remedies for the diseases.

Drp1 Controls Mitochondrial Complex II Assembly and Muscle Metabolism

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The Dynamin-related GTPase, Drp1 (encoded by Dnm1l) plays a central role in mitochondrial fission and is requisite for numerous cellular processes; however, its role in muscle metabolism remains unclear. Herein, we show that among human tissues, skeletal muscle exhibits the strongest correlations with the DNM1L gene. Knockdown of Drp1 (Drp1-KD) promoted mitochondrial hyperfusion in the muscle of male mice. This led to reduced fatty acid oxidation, impaired insulin action, and elevated succinate levels in Drp1-KD muscle. Moreover, muscle Drp1-KD reduced Complex II assembly and activity due to diminished mitochondrial translocation of succinate dehydrogenase assembly factor 2 (Sdhaf2). Restoration of Sdhaf2 normalized Complex II activity, lipid oxidation, and insulin action in Drp1-KD myocytes. These findings emphasize the critical role of Drp1 in maintaining mitochondrial Complex II assembly, lipid oxidation, and glucose homeostasis. Collectively, our studies provide a mechanistic link between mitochondrial morphology and skeletal muscle metabolism, holding clinical significance in combatting metabolic diseases.

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Dual Inhibition of GSK-3 and CDKs by AZD5438 Protects Mitochondria From Toxins Associated With Parkinson's Disease

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Parkinson's disease (PD) is a complex neurodegenerative disease associated with the loss of dopaminergic nigrostriatal nerve cells. Neurons require large amounts of energy and lowered energy production in age and disease is associated with neuronal cell death. Parkin and PINK1 regulate mitochondrial quality control and mutations in these genes are associated with PD. We previously reported that UPS genes and GSK-3 and CDK inhibitors improve mitochondrial health and inhibit PINK1-dependent Parkin recruitment to mitochondria. A comparison of GSK-3 and CDK inhibitors and mitochondrial permeability transition pore inhibitors was carried out and AZD5438 was found the most effective at protecting cells from mitochondrial toxins. AZD5438 prevented the fragmentation of the mitochondrial network and loss in mitochondrial membrane potential associated with CCCP treatment. Furthermore, treatment with AZD5438 alone increased the complexity of the mitochondrial network and prevented the rotenone-induced decrease in PGC-1α and TOM20 levels. Further studies found AZD5438 promoted glycolytic respiration, exerted powerful anti-apoptotic effects, and modulated mitochondrial dynamics. Findings in this study could be investigated further using animal models of PD and dopaminergic neurons from human iPSCs with mutations specific to mitochondria.

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Effects of mtDNA mutations in the adaptive immune system

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Effects of mitochondrial DNA mutations that compromise OXPHOS are increasingly being defined in the innate and adaptive immune systems. For example, the heteroplasmic human MELAS (A3243G) and mouse C5024T mutations in tRNA genes are selected against during the development of immunological memory and are associated with several cellular and systemic immunophenotypes. How pathogenic mtDNA mutations influence immunity, is therefore, an area of great interest. In our recent work, analysis of antigen receptor repertoires has revealed C5024T mice to have increased levels of somatic hypermutation in their IgG (memory) repertoire, compared to wild-type controls, in agreement with an exacerbated activation program, and paving the way for more complex human studies highly relevant to infectious disease susceptibility and vaccination. Alongside this work, we have begun multi-dimensional immunophenotyping and functional studies in PBMCs from a cohort of (n = 40) LHON patients who carry homoplasmic G3460A in *ND1*, also known to reduce cellular aerobic capacity. The latest immunological data arising from both heteroplasmic and homoplasmic mutation studies will be presented.

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Elucidating the Impacts of High Amino Acids on Mitochondrial Form and Function

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High amino acids are implicated in numerous metabolic diseases such as Type 2 Diabetes and Cardiovascular Disease. In patients that have a metabolic disease, the increased presence of branched-chain amino acids is often an indicator of such a

disorder. But it remains unclear how high amino acids perturb cellular homeostasis. Previous work from our lab found that elevated intracellular levels of one amino acid, cysteine, perturb mitochondrial function by interfering with cellular iron availability. We also found that elevated intracellular amino acids induce remodeling of mitochondria through formation of mitochondrial-derived compartments (MDCs), which are multi-lamellar protein-sequestering domains generated from the mitochondrial outer membrane. To further understand the impact of amino acids on mitochondrial function and their role in triggering mitochondrial remodeling via MDCs, we developed a yeast strain that lacks specific feedback control of the yeast plasma membrane localized general amino acid permease, Gap1. Unlike wild-type cells which strictly regulate amino acid uptake, this strain imports any proteogenic amino acid to high levels. Using this system, we are systematically testing the impact of elevated single amino acids on mitochondrial structure and function, and determining which amino acids are capable of inducing MDC biogenesis. Our preliminary findings suggest that multiple amino acids beyond cysteine impact mitochondrial structure, indicating that mitochondria are highly susceptible to functional alterations in the presence of elevated intracellular amino acids.

Elucidating the role of Metaxin proteins in mitochondrial function

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Homozygous null mutations in the Metaxin 2 (MTX2) gene contribute to the pathogenesis of Mandibuloacral dysplasia associated to *MTX2* (MADaM), a rare progeroid-like syndrome that gives rise to alterations in mitochondrial network morphology, reduced sensitivity of patient cells to apoptotic stimuli and defects in nuclear architecture. The mechanisms underlying this disease remain poorly understood, emphasising the need to prioritise investigation into the function of MTX2 and its binding partners, MTX1 and MTX3.

MTX1/2/3 are the accessory components of the mammalian Sorting and Assembly Machinery (SAM) complex, one of the two major protein import complexes of the outer mitochondrial membrane (OMM) whose primary function is to orchestrate the biogenesis and insertion of β-barrel proteins into the OMM. The SAM complex also constitutes the OMM cytosol-facing module of the Mitochondrial Intermembrane Space Bridging (MIB) complex, a ~2,200-2,800kDa complex which structurally links the outer and inner mitochondrial membranes and is integral to the maintenance of mitochondrial ultrastructure. MTX1/3 and MTX2 are homologous to SAM37 and SAM35 in yeast respectively, although the extent to which these proteins are functionally akin to their yeast counterparts concerning their accessory contributions β-barrel biogenesis requires further exploration. We sought to explore the consequences of the loss of MTX1/2/3 using CRISPR/Cas9-mediated genome editing in human-derived cell lines to better understand their contributions to mitochondrial function and to elucidate the mechanisms underlying pathologies associated with the loss of MTX2.

Using unbiased label-free proteomic analysis, we uncovered unique changes to the global cellular proteome resulting from the absence of MTX1/2/3. While no significant changes to the stability of known components of the MIB complex was observed, loss of MTX2 conferred a substantial reduction in both MTX1 and MTX3. Conversely, MTX2 levels remained unchanged upon loss of MTX1 and MTX3, suggesting the existence of a hierarchal order of stability amongst these proteins. Furthermore, the loss of MTX1 resulted in a substantial decrease in the steady-state abundance of OMM β-barrel proteins, including TOM40 and VDAC1. *In vitro* mitochondrial import assays corroborated this finding and revealed that the loss of MTX1, and to a lesser extent MTX2, markedly impairs the temporal import and assembly profile of various β-barrel substrates of the SAM complex. To our surprise, loss of MTX3 was inconsequential to the import and assembly of these substrates, suggesting that a degree of functional divergence may exist between the two mammalian homologs of yeast SAM37. Together, these findings have formed the basis for ongoing investigation into the downstream consequences of impaired β-barrel import and stability on both mitochondrial and cellular health in the absence of MTX1/2. Furthermore, we propose that this phenotype may serve as a mechanistic link to secondary characteristics associated with the loss of MTX2 in the context of progeroid-like pathologies.

Establishment of a new cell model to identify drugs curtailing axonal mitochondria depletion in ADOA retinal ganglion cells

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Autosomal dominant optic atrophy (ADOA) is a hereditary optic neuropathy characterized by the progressive bilateral loss of vision for which no treatment currently exists. Mutations in the nuclear encoded mitochondrial protein Optic Atrophy 1 (Opa1) are associated with ADOA which affect primarily Retinal Ganglion Cells (RGCs). Upon RGC death, the optic nerve composed of RGC axons degenerates resulting in blindness. The Scorrano lab has demonstrated that RGCs carrying mutated Opa1 display excess autophagy, accumulation of autophagosomes in axonal hillocks and mitochondrial depletion along axons, all associated with loss of vision in an ADOA mouse model. Remarkably, genetic inhibition of autophagy restored both axonal mitochondria distribution and vision in ADOA mice. We hence reasoned that pharmacological inhibition of pathways connecting ADOA mitochondria to autophagy hyperactivation could restore axonal mitochondrial distribution in ADOA RGCs, ultimately interrupting the pathogenetic cascade that leads to blindness. To this end, we seek to perform a high content imaging-based drug screening to identify compounds rescuing axonal mitochondrial content in ADOA RGCs. Such large-scale experiment however highlighted the technical bottleneck that is to work with primary mouse RGCs. Indeed, isolating primary RGC from mice cannot provide the sufficient amount of biological material that is necessary. To meet this demand, we have generated the first immortalized RGC line which will also facilitate our *in vitro* studies overall.

Exploiting mitochondrial translation to uncover compartment-specific metabolic vulnerabilities

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One of the key characteristics of eukaryotic cell metabolism is the compartmentalization of amino acid synthesis in different organelles. Although a coordinated metabolic exchange across the mitochondrial and cytosolic compartments is critical for cell physiology and disease, techniques that are able to distinctly measure metabolic limitations in both compartments are lacking. We recently harnessed ribosome profiling for sensing restrictive amino acids in the cytosol, and developed diricore, a procedure for differential ribosome measurements of codon reading. Here, we present mito-diricore, a system to monitor mitochondrial and cytosolic translation which in turn is able to uncover metabolic restrictions in both compartments simultaneously. As a first step, we applied mito-diricore to *in vitro* cultures of breast cancer cells exposed to different metabolic challenges and showed that both compartments exhibit non-overlapping amino acid restrictions. Furthermore, mito-diricore revealed that upon glucose starvation cancer cells develop deficiencies in the amino acid glycine in the cytosolic compartment and restrictions in alanine and serine in the mitochondria.

Cancer metastasis depends on cell survival following loss of extracellular matrix attachment. Loss of attachment causes mitochondrial perturbations and increased ROS production. We applied our system to a model of breast cancer cells growing without attachment and uncovered that cells in suspension experience a mitochondria-specific serine limitation. Serine metabolism in the mitochondria is one of the main sources of NADPH which in turn is used to limit mitochondrial ROS and ferroptosis. Indeed, serine starvation inhibits the capacity of cancer cells to grow without extracellular matrix attachment. A mitochondria-focused CRISPR screen revealed that ablation of TXNRD1 enhances the growth of cancer cells in suspension, rescues the serine limitation, and protects detached cancer cells from ferroptosis. Altogether, we establish a system that exploits mitochondrial translation as a sensor of compartment-specific metabolic restrictions and we characterize a novel pathway that links TXNRD1 to mitochondrial serine metabolism. Our system has the potential to explore compartmentalized metabolic programs under diverse physiological and pathological conditions and identify potential mitochondrial metabolic targets for therapeutic intervention.

Exploring a Singleminded-2s / Sirtuin interaction in the regulation of mitochondrial dynamics in normal mammary development and ER+ breast cancer

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Characterizing the mechanisms underlying the regulation of cellular differentiation and mitochondrial dynamics are critical to understanding normal development and breast cancer in the mammary gland. Our previous studies have highlighted the significance of Singleminded-2s (Sim2s), a member of the bHLH/PAS family, in regulating mitochondrial dynamics during mammary gland development and the progression of estrogen receptor-positive (ER+) breast cancer. Sim2s is temporally regulated, with maximal expression occurring during mid-lactation. Cross-fostered pups nursed by mice over-expressing Sim2s under the mouse mammary tumor virus (MMTV-Sim2s) display significantly higher weights by mid-lactation compared to pups nursed by control dams. Overexpression of Sim2s leads to alterations in mitochondrial morphology and dynamics, characterized by increased OPA1 expression and decreased DRP1 levels, resulting in enhanced mitochondrial fusion and elongation. Furthermore, we have extended our investigations to an ER+ breast cancer cell line (MCF7). We have identified SIM2s as a tumor suppressor expressed in mammary epithelial cells, known to inhibit epithelial-mesenchymal transition (EMT) and metastasis. In our previous studies, we discovered that loss of SIM2s expression in the MCF7 cell line promotes mitochondrial fragmentation, as evidenced by a decrease in OPA1 expression and an increase in DRP1 levels. In conjunction with these findings, literature suggests that sirtuins also play a role in the regulation of mitochondrial dynamics similar to Sim2s. Specifically, there is an upregulation of Sirtuin 1 in cancer when Sim2 is lost, while Sirtuin 3 increases during differentiation and normal development progression. Therefore, our aim is to elucidate the potential interaction between Singleminded-2s and sirtuins, a family of NAD+-dependent deacetylases, in the regulation of mitochondrial dynamics. We hypothesize that Sim2s may modulate mitochondrial function through its association with sirtuins, thereby influencing normal mammary gland development and breast cancer progression.

Extracellular Vesicles Rescue Glomerular Endothelial Mitochondrial Dysfunction in Alport Syndrome

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Background: Chronic kidney disease (CKD) affects more than 10% of the population worldwide, and our understanding of the mechanisms in many forms of CKD are poorly understood. Alport syndrome (AS), is a form of CKD caused by mutations in the collagen IVα3, α4, or α5 gene. Even though injury to glomerular endothelial cells (GEC) in AS is well established, the role of GEC in Alport progression has not been elucidated. Here, we describe the role of mitochondria and lipid metabolism in GEC injury in an animal model of AS, and the potential of using amniotic fluid stem cell (AFSC) derived extracellular vesicles, (EVs) as a rescue strategy to restore glomerular homeostasis.

Methods: The phasor approach to fluorescent lifetime imaging microscopy (FLIM) was applied to evaluate the mitochondria and metabolic changes in GEC of AS vs WT mice. GEC isolated by FACS from Tek-tdT reporter AS and WT mice were compared by bulk RNA-seq, lipidomic and flow cytometric analysis. In vitro, silencing experiments on primary human GEC were performed to study the role of fatty acid synthase (FASN) in mitochondrial dysfunction and GEC damage. FASN-carrying AFSC-EVs and control nanoparticles were applied both in vitro and in vivo to restore lipid homeostasis in GEC.

Results: FLIM studies showed strong correlation between the metabolic state of GEC and the age or severity of disease in AS mice. RNA-seq analysis revealed changes in the pathways associated with lipid metabolism and mitochondria function. Mitochondrial dysfunction was confirmed using flow cytometric analysis of the MitoTracker signal in tdTomato expressing GEC. Lipidome analysis revealed high abundance of triglycerides in AS GEC. We confirmed accumulation of lipid droplets in the glomeruli of AS mice and in FASN KO human primary GEC, in vitro. These results suggest potential mitochondrial dysfunction in GEC. AFSC derived EV treatment restored lipid homeostasis in GEC, both in vitro and in vivo.

Conclusion: We report for the first-time mitochondrial dysfunction in Alport GEC, and the ability of AFSC-derived EVs to rescue this phenotype. Better understanding of the metabolic changes in AS GEC could lead to the development of targeted new therapies for other forms of CKD.

FAM210A is a translational regulator of mitochondrially encoded proteins

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Mitochondria contain their own DNA and their own gene expression machinery. Family With Sequence Similarity 210 Member A (FAM210A) is a protein of 272 amino acids that has been discussed as a potential inner membrane fission factor and has later been suggested to regulate mitochondrial translation. It has also recently been implicated in the regulation of the ratio between the short and long forms of the GTPase OPA1, a key player in mitochondrial inner membrane dynamics.

We further characterized the function of FAM210A and verified its role as a regulator of mitochondrial translation. Ablation of FAM210A reduces the abundance of respiratory chain complexes I-IV, so that both nuclear and mitochondrial encoded proteins are severely depleted. Complex V, however, remains unaffected. Mitochondria from FAM210A knockout cells show highly fragmented networks and aberrant inner membrane architecture. This study establishes a link between mitochondrial translation and the intricate architecture of the mitochondrial inner membrane.

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Familial Parkinson's Disease Linked Mutations of PINK1 Lead to Altered Mitochondrial Processing

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Parkinson's disease (PD) is a devastating neurodegenerative disease characterized by a loss of dopaminergic neurons located in the substantia nigra. Mitochondrial dysregulation has been observed in numerous neurodegenerative diseases, including PD, due to the high energy requirements of neuronal tissues.

Genetic mutations in the gene encoding for PINK1, have been associated with cases of early onset PD. In a neuroprotective role, PINK1 accumulates on he outer membrane od damaged mitochondria, flagging them for destruction, while in healthy cells PINK1 is turned over by a protease in the inner mitochondrial membrane - PARL. PD-associated mutations of PINK1 are found near the PARL cleavage site and are predicted to lead to a loss of PINK1 cleavage.

Although *in vitro* analysis using recombinant protease and substrate has been used in the lab, numerous factors affecting protease activity such as lipid composition and interacting proteins are difficult to account for. Therefore, cellular analysis was conducted to assess cleavage and localization of PINK1 PD variants.

To reveal the etiology of PINK1 mutations, confocal imaging and western blot analysis of various cell lines transfected with PD linked PINK1 variants were performed to examine mitochondrial retention of the protein. The PINK1-R98W PD variant displays high retention of the protein in the mitochondria. However, *in vitro* cleavage analysis shows no cleavage defect for the R98W variant, suggesting this variant has defects in mitochondrial trafficking. Ultimately, elucidating the mechanism of cleavage of PINK1 PD-variants by PARL will provide rational for therapeutic drug design to combat PD.

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Functional gene networks uncover impaired mtRNA catabolism as a novel mechanism of cellular zinc toxicity

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Recently, multiple groups have characterized the ubiquitously expressed SLC30A9 transporter as a mitochondrial zinc exporter essential for the maintenance of mitochondrial function. Loss of SLC30A9 leads to mitochondrial swelling and zinc accumulation, impaired respiration, and decreased abundance of mitochondrially-encoded electron transport chain (ETC) proteins in human cells. Despite having a significant consequence to cellular fitness, the mechanism of zinc-induced mitochondrial dysfunction following loss of SLC30A9 activity remains unresolved. Moreover, elevated

levels of intracellular zinc also lead to swollen mitochondria and impaired respiration, suggesting that this axis may be of potential relevance to general zinc toxicity.

To address this gap, we analyzed multiple functional genomics datasets to identify mitochondrial genes and processes associated with SLC30A9. We discovered a robust association between SLC30A9 and mtRNA catabolism in human cancer cells. Specifically, following cell line covariance normalization of the DepMap dataset, the predominant coessential partner of SLC30A9 is REXO2. REXO2 encodes a mitochondrial DEDD exoribonuclease that catalyzes the final step of mtRNA turnover.
Additionally, SLC30A9 knockdown induces perturbations to mitochondrial transcript abundances in a similar manner to several mtRNA catabolism genes, including REXO2. These transcript profiles are distinct from those of mitochondrial ribosome and complex I subunit knockdowns, both of which have been postulated to be axes of mitochondrial zinc toxicity. The DEDD protein family employs a two-ion mechanism for the hydrolysis of oligonucleotides. Notably, Zn^{2+} is not sufficient for the RNase activity of REXO2 and has been shown to inhibit multiple DEDD enzymes. To determine if zinc alters REXO2 activity, we purified recombinant human REXO2 and measured the ability of the protein to degrade short RNAs in the presence of various divalent cations. Our preliminary data indicate that low levels of Zn^{2+} have a potent inhibitory effect on REXO2 activity in the presence of the catalytic cations Mn^{2+} and Mg^{2+}. Additionally, we found that SLC30A9 and REXO2 knockdown cells exhibit impaired respiration and destabilization of multiple ETC complexes on similar timescales. Based on these results, we propose a novel axis of zinc toxicity in human cancer cells, in which elevated mitochondrial zinc following SLC30A9 depletion inhibits REXO2, disrupting mitochondrial RNA turnover and transcription of ETC complex subunits, ultimately leading to impaired respiration.

GJA1-20k-Mitochondrial Dynamics Regulate Exercise-Induced Cardiac Preconditioning

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Cardiac ischemic preconditioning is a phenomenon in which brief ischemic episodes provide endogenous protection against tissue death in response to myocardial infarction. Exercise induces high metabolic demands and also mediates preconditioning protection, however the mechanism by which this occurs is not well understood. It has been established that GJA1-20k, an internally translated isoform of the gap-junction protein Connexin-43 (Cx43), mediates ischemic preconditioning by inducing protective mitochondrial biogenesis, fission and metabolic quiescence, however the role of GJA1-20k in exercise mediated preconditioning is unknown. The goal of this study is to determine if exercise-induced preconditioning is mediated by GJA1-20k expression and mitochondrial localization.

Wild-type C576/BL mice were subject to 90-minute acute exercise (AE) via swimming, or no exercise training (NE). At 12-14 weeks of age, mice were sacrificed. Whole-heart samples were prepared for western blot analysis using a standard lysis buffer and mitochondrial fractionation was performed. Western blot analysis indicated increased GJA1-20k expression relative to total Cx43 in AE compared to NE littermates (1.0 +/- 0.1 vs 3.0 +/- 0.2, p=0.0079, n=5,5). Western blot analysis of mitochondrial and cytoplasmic fractions of ventricular tissue demonstrated increased GJA1-20k expression in the mitochondria of the AE group relative to mitochondria of the NE. As Cx43 trafficking to the intercalated disc is dependent on GJA1-20k expression, we examined disc-localized Cx43 in AE and NE mice and found that exercise regulated Cx43 disc-localization patterns.

These data indicate that exercise induced increases in GJA1-20k expression, GJA1-20k mitochondrial content, and regulates Cx43 trafficking patterns in mouse cardiomyocytes. Further work will focus on elucidating the mechanism by which exercise regulates GJA1-20k expression and the pathways involved in exercise mediated preconditioning.

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Human CCDC51 and yeast Mdm33 are functionally conserved inner membrane mediators of mitochondrial morphology that demarcate a subset of fission sites

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While mitochondrial fission and fusion dynamics have been extensively studied for over 20 years, it has been a long-standing question of how mitochondrial fission is coordinated across two membrane bilayers. The yeast inner membrane protein Mdm33 is required for normal mitochondrial morphology and has been implicated in mitochondrial fission via its involvement in lipid homeostasis pathways. However, the precise role of Mdm33 remains unclear and it is not thought to be conserved in metazoans. Using a bioinformatic approach, we identified a candidate human mitochondrial protein with similar domain architecture to Mdm33, the inner membrane protein CCDC51. We find that CRISPRi-mediated stable depletion of CCDC51 causes accumulation of aberrant lamellar mitochondria like those found in yeast Δ*mdm33* cells. Transient knockdown of CCDC51 leads additionally to mitochondrial hyperfusion and accumulation of interconnected nets, suggesting altered fission dynamics contribute to the morphology defect of stably-depleted cells. Conversely, and as in the case of Mdm33, overexpression of CCDC51 promotes Drp1-dependent mitochondrial fragmentation. Using live-cell confocal microscopy, we determine that both yeast Mdm33 and human CCDC51 concentrate in discrete foci on the mitochondrial membrane that are spatially and temporally linked to a subset of mitochondrial division events. Finally, we show that exogenous expression of CCDC51 can partially rescue the mitochondrial morphology defect of yeast Δ*mdm33* cells, demonstrating they are true functional analogs. Together, these data indicate that Mdm33 and CCDC51, despite negligible primary sequence homology, work in a similar way to promote normal mitochondrial morphology. Further, these data raise the possibility that CCDC51/Mdm33 play an important auxiliary role on the mitochondrial inner membrane in support of mitochondrial fission dynamics.

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Human mitochondrial protease OMA1 eliminates arrested protein import intermediates upon depolarization of the inner mitochondrial membrane

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Most mitochondrial proteins are synthesized as precursors in the cytosol and require an effective import into the organelle. To pass through translocation channels in mitochondrial membranes, such precursor proteins must be largely unfolded. However, protein misfolding occurs even in physiological conditions in healthy cells. Misfolded proteins can become arrested in translocases, impairing protein import and mitochondrial function. Both cytosolic and mitochondrial quality control mechanisms can act on the precursors stuck in a passage. The toxicity of misfolded proteins and mitochondrial dysfunction are pivotal factors in many pathological conditions constituting hallmarks of aging-related degenerative diseases. Thus understanding of molecular mechanisms that resolve protein import failure is essential for advancing diagnostic and therapeutic approaches. To discover such responses in human cells, we used fusion protein designed to stall at an intermediate step of import. We used a cell line model with an inducible expression of a fusion protein consisting of an inner mitochondrial membrane-directed domain (ATP5MG, complete protein) and a fluorescent tag including a super folder GFP. The stable fold of GFP did not allow completion of the fusion's translocation, while its N-terminal part was built into the inner mitochondrial membrane, resulting in a stable arrest.

We found out that the degradation of the import-blocking model protein depends on the fitness of mitochondria. In particular, our results revealed that depolarisation of the mitochondrial inner membrane activates the proteolytic processing of model protein by mitochondrial proteases. After inhibiting metalloproteases, the degradation of arrested protein significantly decreased. Our experiments provide evidence for the role of inner membrane protease OMA1 in releasing stalled protein from the translocase. We have observed that OMA1 cleaves model protein inside the mitochondria, allowing the retro-translocation of its arrested fragment to the cytosol. Checking the further fate of the released protein, we have confirmed the participation of cytosolic factors VCP and proteasome in its further degradation. The mechanism we found differs significantly from these described in fungi, where ATPase-driven extraction of blocked protein is directly coupled with proteasomal processing.

Upon evaluation of mitochondrial morphology, we observed that arrested protein import intermediates strongly altered cristae organization by tethering outer and inner

mitochondrial membranes. Notably, activating proteolysis by an uncoupler of the membrane potential resulted in significant restoration of the cristae structure. Thus, clogging-induced damage is not permanent and can be effectively counteracted by cellular quality control machinery.

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Hyperinflammatory antibacterial innate immunity and elevated lung immunopathology in a model of PolG-related mitochondrial disease

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Mitochondrial diseases (MtD) are a clinically heterogeneous group of disorders caused by respiratory chain dysfunction and metabolic failure. These diseases often result from pathogenic mutations in genes that function in oxidative phosphorylation or mitochondrial DNA (mtDNA) maintenance, are often debilitating, and have limited treatment options with no cures. Environmental exposures, such as microbial infections, appear to exacerbate the stepwise progression of MtD. MtD patients are also more susceptible to recurrent infections from opportunistic respiratory pathogens including *Pseudomonas aeruginosa* (PA). These infections can quickly spiral out of control, leading to sepsis and unrestrained inflammatory responses. The underlying immune alterations in MtD that impair antibacterial activity and enhance immunopathology are unclear, and thus there is a critical need for mechanistic research to advance immune-focused therapies for MtD. Mutations in the mtDNA polymerase gamma (PolG) represent the most prevalent single-gene cause of MtD. Using a faithful mouse model of PolG-related MtD, we have uncovered novel immune alterations in PolG mutant animals, including elevated IFN-I signaling in myeloid cells that mirrors that seen in clinical samples collected from MtD patients. In addition, we have found that PolG mutant mice exhibit increased myeloid cell infiltration and cytokine production in the lung after instillation of live PA. Consequently, PolG mice more readily succumb to PA infection due to prolonged hyperinflammation in response to PA challenge. Mechanistic studies revealed that elevated IFN-I signaling drives PolG macrophages to undergo increased pyroptotic cell death during PA infection, which is driven by elevated expression and activity of caspase-

11. Finally, we have found that chronic IFN-I signaling in PolG mutant represses activation of the transcription factor nuclear erythroid 2-related factor 2 (Nrf2), which orchestrates both antioxidant and anti-inflammatory responses. Stabilizing Nrf2 with the FDA-approved drug, dimethyl fumarate (DMF), was effective at reducing IFN-I responses and pyroptosis in PolG macrophages exposed to PA. Future work will characterize how reduced Nrf2 activity promotes hyperinflammatory innate immune responses, contributing to acute lung injury that limits control of PA infection in PolG-related MtD. This research may pave the way for the development of innovative Nrf2-based therapies to mitigate infection-related immunopathology in PolG-related MtD or other mitochondrial disorders.

Identification of inflammatory cell death through IL-17 induced Mitochondria-Associated ER Membranes formation in Chondrocytes

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Osteoarthritis (OA) is a major cause of disability in the elderly population and represents a significant public health problem and socioeconomic burden worldwide. This study investigated the therapeutic effect of direct mitochondrial transplantation (MT) on knee osteoarthritis. Interleukin (IL)-17 is an inflammatory cytokine and is known to be a key pathogenic factor in rheumatoid arthritis, but its role in osteoarthritis remains poorly understood. In this study, we investigated the effects of IL-17 on osteoarthritic cartilage and chondrocytes. Increased catabolic factors and decreased anabolic and chondrogenic factors in Il-17-treated osteoarthritis patient-derived chondrocytes. Furthermore, we found that IL-17 synergistically with IL-1beta induced mitochondrial damage in chondrocytes in the osteoarthritic environment and induced Mitochondria-Associated ER Membranes (MAM) formation by adhesion of mitochondria to the ER. The formation of MAMs was found to excessively induce apoptosis by AnnexinV/PI experiments, increasing markers of ferroptosis and necroptosis, and was inhibited by 2-APB, which regulates MAM formation and Ca2+ release from the ER. In MIA induced OA rat model, intra-articular administration of 2-APB or MSC-derived mitochondria resulted in chondroprotective effects, inhibition of bone loss, and reduction of inflammatory cell death markers, as confirmed by IHC experiments. All above results suggest that modulation of IL-17 could attenuate osteoarthritis through inhibition of MAM formation-induced inflammatory cell death.

Identification of novel mitophagy regulators in a high-throughput imaging siRNA screen

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Mitochondria can be targeted for lysosomal degradation via the autophagy pathway in a process denoted as mitophagy. This occurs in all cells under basal conditions and may also be upregulated during development and in response to stressors such as mitochondrial membrane depolarisation and hypoxia. The mechanisms underlying mitophagy are still poorly characterized, but previous work have implicated the existence of specific mitochondrial determinants that are recognized by the autophagy machinery to promote selective degradation of mitochondria.

To unravel mitochondrial determinants important for hypoxia-induced mitophagy, we have carried out an siRNA-based imaging screen targeting mitochondrial and mitochondria-associated genes in U2OS cells expressing a matrix-located mitophagy reporter (NIPSNAP$^{1-53}$-EGFP-mCherry, referred to as iMLS) under hypoxia-mimicking (deferiprone-induced) conditions.

As expected, the screen demonstrated that the depletion of many mitochondrial proteins resulted in increased mitophagy, likely due to perturbed mitochondrial homeostasis. Most interestingly, 39 mitochondrial proteins were required for hypoxia-induced mitophagy, including components of the import machinery, replication complex, iron-sulphur cluster biosynthesis and the respiratory chain. Data from our current characterisation of some of these candidates and their mitophagy-regulating mechanisms will be presented. Our study has identified potential regulators of mitophagy, offering promising therapeutic targets for diseases linked to mitochondria dysfunction.

Identification of The Yeast Mitochondrial Zinc Importer

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Zinc is the second most abundant transition metal in the cell after iron. Moreover, about 10% of all proteins bind zinc, including the famous zinc finger motif, prevalent in many transcription factors. For mitochondrial function, zinc is essential due to its central role in the superoxide dismutase, mitochondrial transcription factors, ribosomes and components of the electron transport chain. Surprisingly, although zinc importers have been identified in most organelles, how zinc is imported into mitochondria remains a mystery in all organisms. While the mammalian mitochondrial calcium uniporter (MCU), has been implicated in zinc import under pathological conditions, no zinc-dedicated importer has been found in mitochondria so far. Thus, we used *Saccharomyces cerevisiae* (hereafter referred to as yeast), which lacks the MCU, as a model organism to study zinc import into mitochondria. A multiple sequence alignment of all yeast mitochondrial membrane proteins of unknown function, revealed a set of candidate proteins which are related to known metal transporters. By measuring the levels of different metals in cells overexpressing these candidates, we identified a novel mitochondrial zinc importer which retains its zinc transport capacity also when expressed exogenously in bacteria. Moreover, we show that the zinc transport function is retained through evolution in the human homolog. Hence, our work uncovers the first mitochondrial zinc specific importer, opening up new opportunities in understanding and manipulating mitochondrial functions.

Identifying Novel Mechanisms of Quality Control in Autophagy-deficient Cancer Cells

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Autophagy is a cellular recycling process that regulates organelle quality control. A specific form of autophagy that maintains mitochondrial health is called mitophagy. Previous work from our lab shows that autophagy-dependent cancer cells can acquire resistance to autophagy inhibition and exhibit normal mitochondrial function. This indicates that cells can survive without autophagy and mitochondrial quality control. Previously, our lab showed that autophagy-deficient cells upregulate an autophagy-independent form of mitochondrial degradation known as Mitochondrial-Derived Vesicles (MDVs). These tiny vesicles bud off the mitochondria and directly traffic to the lysosome, bypassing autophagosomes. However, upstream regulatos of MDVs remain largely unknown. To understand how autophagy-deficient cells maintain their mitochondrial health without autophagosomes, we performed a genome-wide CRISPR screen using mitochondrial-localized pH-sensitive probes to identify novel regulators of mitochondrial delivery to the lysosome. This screen uses mCherry-GFP-Fis1 which localizes to the outer membrane of mitochondria and we have shown that ratiometric flow cytometry can quantify the amount of mitochondria that are in an acidic environment like a lysosome. The top hit from this screen was MEMO1, which is a known regulator of microtubule activity and could be important for trafficking of Mitochondrial-Derived Vesicles. An individual guide RNA targeting MEMO1 confirmed that MEMO1 knockdown decreased the percentage of mitochondria that traffic to lysosomes in cells lacking the core autophagy gene, ATG7. We are currently elucidating the mechanisms by which MEMO1 regulates mitochondrial quality control and testing if MEMO1 directly regulates MDVs. These mechanisms could be important in cancer, aging, and neurodegenerative disease which have dysregulated mitochondrial homeostasis pathways.

In vitro and in-cell investigations into metabolic control of mitochondrial gene expression

Reardon, Sean

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The human mitochondrial DNA (mtDNA) is a ~16 kb circular DNA encoding 13 essential OXPHOS proteins, 22 mitochondrial tRNAs, and 2 mitochondrial ribosomal RNAs. mtDNA is compacted by a dual transcription factor/compaction protein, mitochondrial transcription factor A (TFAM), whose binding and compaction is proposed to regulate processes involving mtDNA like transcription and replication. Possible modes for regulating TFAM include changing its expression, degrading it, or adding post-translational modifications (PTMs) such as phosphorylation by protein kinase A (PKA) and nonenzymatic acetylation by acetyl-CoA that may tune TFAM affinity for DNA. PTMs on TFAM would represent an efficient and cost-effective way to modulate mtDNA compaction and gene expression in response to the changing cellular energy needs and metabolic stress conditions. While these modifications have been detected in cells, there is yet to be a targeted quantification of TFAM PTMs specifically. We investigated these modifications to TFAM *in vitro* to test the impact of PTMs on TFAM's compaction and transcription initiation functions. We demonstrated that DNA-bound TFAM is less susceptible to both enzymatic phosphorylation and non-enzymatic acetylation. Surprisingly, we showed using electrophoretic mobility shift assays that pre-phosphorylated or pre-acetylated TFAM compacted circular double-stranded DNA comparably to unmodified TFAM. Additionally, we provided an in-depth analysis of TFAM's lysine reactivity towards acetyl-CoA using isotopic labelling and liquid chromatography-tandem mass spectrometry. Finally, we showed that phosphorylation and acetylation of TFAM increased the processivity of transcription through TFAM-imposed barriers on DNA, but that TFAM bearing either modification maintained full activity in transcription initiation. We conclude that phosphorylation by PKA and nonenzymatic acetylation by acetyl-CoA are unlikely to occur on TFAM that are bound to the mtDNA (Reardon and Mishanina, *J Biol Chem* 2022). Driven by the *in vitro* discoveries, we are currently investigating the possibility of PTM regulation of TFAM in cells, in particular how changes in cellular metabolism impact mitochondrial transcription. We

are employing super-resolution microscopy to visualize and quantify mitochondrial mtDNA and RNA under various metabolic conditions, as well as qPCR to assess mitochondrial transcription output.

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Infection-induced peripheral mitochondria fission drives ER encapsulations and inter-mitochondria contacts that rescue bioenergetics

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The dynamic regulation of mitochondrial shape at the interface between fission and fusion is critical for cellular responses to internal and external stimuli. In homeostatic cells, two modes of mitochondrial fission, midzone and peripheral, were shown to provide a decision fork between either mitochondrial proliferation or clearance of damaged mitochondria, respectively. However, the relationship between specific mitochondria shapes and functions remains unclear in many biological contexts. While commonly associated with decreased bioenergetics, fragmented mitochondria were paradoxically found to exhibit elevated respiration in several disease states, including infection with the prevalent pathogen human cytomegalovirus (HCMV) and metastatic melanoma. Here, we aim to elucidate how HCMV infection remodels membrane contact sites to promote mitochondria fragmentation concurrent with increased respiration. We integrated live, super-resolution microscopy, cryotomography, and molecular assays to show how HCMV infection selectively fragments mitochondria and circumvents host autophagy to promote increased mitochondrial bioenergetics. First, we find that the HCMV protein pUL37x1 specifically promotes fission at the mitochondrial periphery, while concurrently suppressing mitochondria fusion. While peripheral fission was previously shown to decrease bioenergetics and promote mitophagy in uninfected cells, we show that during HCMV infection peripheral progeny maintain bioenergetic activity and evade degradation. We further find that peripheral progeny preferentially form mitochondria-ER encapsulations (MENCs). MENCs are stable and asymmetric ER-mitochondria associations, enriched with the membrane contact site protein PTPIP51, that we recently found to form during HCMV infection. Using IP-MS and live-cell metabolic sensors, we show that PTPIP51 suppresses mitophagy while elevating

mitochondrial respiration and calcium intake. We further establish that MENCs facilitate stabilization of pro-viral inter-mitochondria contact sites, which accumulate as infection progresses and allow the exchange of bioenergetic potential between mitochondria. We place these findings in a broader context, using our developed targeted mass spectrometry assay to globally profile alterations in membrane contact site proteins upon infection with different HCMV strains. We find that both laboratory-adapted and low-passage, clinically relevant HCMV strains induce similar MENC formation in either fibroblast or epithelial cell types. Expanding the scope of our study, we assessed MENC frequency and function in metastatic melanoma cells, which, similar to HCMV, exhibit mitochondria fragmentation concomitant with increased respiration. By comparing multiple melanoma cell lines with different levels of metastatic potential, we find that highly metastatic cells form more MENCs and that the mitochondria within these MENCs display elevated bioenergetics. In summary, here we address the fundamental question of how, in certain biological contexts, mitochondrial fragmentation leads to increased bioenergetics. This question is relevant for several critical energy-demanding pathologies, including viral infection and cancer. We report the discovery of a mechanism where a viral infection promotes peripheral mitochondrial fragmentation and then leverages ER-mitochondria and inter-mitochondria membrane contacts to stabilize and elevate bioenergetic output. We demonstrate the relevance of this mechanism in HCMV infection and broaden the scope of our findings by showing the formation of MENCs in metastatic melanoma cells. Hence, our study adds another example of the importance of studying a viral infection for uncovering basic biology mechanisms that are relevant in different contexts.

Interleukin 21 Promotes Intestinal Inflammation by Disrupting Mitochondria-ER Crosstalk in Regulatory T Cells

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Background: Regulatory T cells (Tregs) are a specialized subset of immune cells required for maintaining self-tolerance and suppressing immune-mediated diseases such as inflammatory bowel disease (IBD: Crohn's disease and ulcerative colitis). IBD is a debilitating, relapsing, and incurable inflammatory condition that damages the gastrointestinal tract. Notably, Tregs acquire an inflammatory-like phenotype in a subset of refractory IBD patients; however, mechanisms that regulate the derivation of inflammatory-like Tregs and their contribution to disease pathogenesis have remained elusive. We hypothesize that microenvironmental factors can alter cellular metabolism, resulting in Treg defect and refractory IBD. **Methods:** Human Tregs derived from healthy donors were characterized via microarray, immunoblotting, fluorescent-activated cell sorting, mass cytometry by time of flight, metabolomics, electron microscopy, Seahorse Flux analysis, and confocal microscopy in response to IL-21 stimulation. IL-21-induced transcriptional changes in Tregs *in vitro* were validated in a publically available single-cell RNA sequencing (scRNA-seq) dataset from inflamed ileal Crohn's disease tissue of anti-tumor necrosis factor (TNF) responders and non-responders. The *in vivo* functionality of wildtype (WT) and *Il21* receptor-deficient (*Il21r*-/-) Tregs were examined in animal models of colitis induced by the adoptive transfer of CD4+CD45RBhigh (pathogenic) T cells into *Rag1*-/- (T- and B cell-deficient) mice. **Results:** Cell imaging analyses revealed a unique enrichment of mitochondria-ER appositions in Tregs, and its disruption led to a glycolytic switch from oxidative phosphorylation (OXPHOS) and an inflammatory response evidenced by the expression of a plethora of inflammatory cytokines. Through an *in vitro* challenge of human Tregs, we identified IL-21 as the unique signal capable of inducing an inflammatory response. Moreover, the integration of targeted transcriptomic and metabolomic data revealed the activation of many metabolic processes, including glycolysis, in response to IL-21. Notably, IL-21-induced metabolic transcriptional signatures were more enriched in Tregs from ileal Crohn's disease lesions of anti-TNF non-responders than that of the responders, implying a potential role for IL-21-induced metabolism in driving Treg defect and therapy resistance. Mechanistically, cell-imaging and bioenergetic analyses showed disruption of mitochondria-ER interaction and function, as evidenced by glycogen synthase kinase 3β (GSK3β) activation and glycolytic switch from pyruvate-mediated OXPHOS metabolism. Linking impaired mitochondrial pyruvate metabolism to inflammatory cytokine expression, inhibition of glycolysis, GSK3β, or supplementation with membrane-permeable methyl pyruvate abrogated IL-21-induced metabolism and the consequent inflammatory response. Finally, in contrast to WT Tregs, *Il21r*-/- Tregs efficiently treated murine colitis, as evidenced by the reduction

in clinical signs (disease activity index), weight loss, intestinal tissue inflammation and damage (mouse colon histology index), and serum inflammatory cytokines.

Conclusion: IL-21 can incapacitate Treg metabolic state and physiology by inducing mitochondria-ER dysfunction.

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Intracellular Bacterial Replication during UTI alters Mitochondrial Metabolism

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Urinary tract infection (UTI) is among the most prevalent urologic diseases, caused primarily by uropathogenic Escherichia coli (UPEC). Infection by UPEC and other prevalent uropathogens, like Klebsiella pneumoniae and Pseudomonas aeruginosa, is characterized by a transient intracellular stage during which bacteria invade superficial epithelial (facet) cells and divide within the cytosol to form multicellular communities called biofilms. After replicating in the cytosol, bacteria exit the intracellular biofilm – killing the bladder epithelial cell in the process – and disseminate to naïve urothelial cells or to the upper urinary tract. While in the intracellular biofilm state, bacteria evade innate immune responses and the effects of antibiotics. Although the steps of the intracellular cycle have been well-characterized, there has been a paucity of knowledge regarding how uropathogens alter host cell metabolism as they expand inside the bladder cell environment. This lack of knowledge limits our ability to prevent or effectively treat UTI. In this work we present data demonstrating that both UPEC and Pseudomonas use specific quinol oxidases and cytochromes to respire intractellularly. For UPEC, we show that intracellular oxygen scavenging by the bacteria alters mitochondrial physiology by reducing the efficiency of mitochondrial respiration, stabilizing the hypoxia inducible transcription factor HIF-1, and promoting a shift toward aerobic glycolysis. Analysis of mitochondrial electron transport chain efficiency in intracellularly infected urothelial cells revealed an increase in proton leak and decreased coupling efficiency relative to mock infected cells, indicating intracellular infection impairs mitochondrial respiration efficiency. To further characterize the influence of intracellular infection on mitochondrial physiology, we performed structured illumination microscopy to analyze mitochondrial network morphology. In contrast to the punctate mitochondria observed in mock

infected cells, we observe tubular mitochondrial networks in intracellularly infected cells. Mitochondrial size and major axis length are increased in intracellularly infected cells relative to mock infected cells, indicating intracellular infection leads to an enhancement in mitochondrial fusion. Invasion of several bacterial pathogens into human cells has been shown to promote mitochondrial fusion, and this fusion both enhances mitochondrial respiratory efficiency and supports innate immune defense. We then performed Nanostring profiling interrogating the transcriptional consequences of bacterial replication on host metabolism. We demonstrate a stark shift from mitochondrial energy metabolism to glycolys, with specific increases in host hypoxia and glycolysis genes and a corresponding decrease in the abundance of transcript encoding the mitochondrial pyruvate importers MPC1 and MPC2. Moreover, we observed increased abundance of transcript encoding PDK1 – a kinase that inactivates pyruvate dehydrogenase and antagonizes oxidative phosphorylation. Finally, we observe suppression of apoptosis in UPEC-infected cells, a consequence due to the activity of HIF-1, that we propose benefits the bacteria, as it extends the life of the host cell in which they replicate. In sum, our results reveal the metabolic basis for intracellular bacterial pathogenesis during urinary tract infection and identify subversion of mitochondrial metabolism as a bacterial strategy to facilitate persistence within the urinary tract.

Investigating the role of mitochondrial respiratory chain supercomplex formation in the mitochondrial dependency of triple negative breast cancer

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The mitochondrial respiratory chain (MRC) serves many key functions including, maintaining energy balance, recycling redox cofactors, superoxide and reactive oxygen species (ROS) production, and regulation of cell survival. These functions are each associated with hallmarks of cancer and thus, mitochondrial biology is altered in many tumor contexts and has been shown to be a therapeutic vulnerability in pre-clinical cancer models. Our group has previously demonstrated that increased mitochondrial respiration promotes chemoresistance in the highly aggressive triple negative breast cancer (TNBC) subtype, and that inhibition of MRC complex I or mitochondrial fusion in TNBC cells surviving chemotherapy delayed tumor regrowth. Neoadjuvant chemotherapy (NACT) is a mainstay treatment for TNBC due to the lack of targeted therapy options available currently. However, in 45% of patients residual tumor remains after NACT leading to a substantially poorer prognosis after surgery. Therefore, there is a critical need to deeply understand the mitochondrial mechanisms driving residual tumor cell survival to inform future targeted therapeutic development strategies. Examination of OXPHOS in cultured human TNBC cell lines revealed cells surviving DNA-damaging chemotherapies had significantly increased oxygen consumption rates (OCR) while cells surviving microtubule-stabilizing (taxane) chemotherapies had decreased OCR. We found that 'upstream' of respiration, both glucose flux into the TCA cycle as well as NAD/NADH were similar across all treatments. However, 'downstream' products of respiration, including ATP production and signaling pathways involving NAD^+ and reactive oxygen species (ROS) were significantly different between the therapy types. Interestingly, superoxide dismutase (SOD2) protein expression was elevated after both DNA-damaging agent and taxane treatment, yet ROS levels were only significantly elevated after treatment with taxanes. Given the differences in MRC output (OXPHOS, NAD^+ signaling, and ROS production) with similar MRC input (glycolysis and TCA cycle activity and NAD^+/NADH) we sought to explore the differences in the formation and function of MRC complexes after chemotherapy treatment. The MRC consists of four complexes that exist both individually and in aggregates known as supercomplexes (SCs). Examination of MRC formation revealed cells surviving DNA-damaging agent treatment had slight increases in high-order complex I (CI)-containing SCs, while cells surviving taxanes did not. Taken together, these findings suggest taxane treatment leads to increased superoxide production associated with reduced CI-containing SCs while DNA-damaging agent treatment mitigates ROS by promoting CI-containing SC formation. Further investigation of these findings is paramount to understanding the differential mechanisms of chemotherapy resistance between DNA-damaging agent and taxane treated TNBCs.

LETMD1 is a novel regulator of mitochondrial protein import in brown adipocytes

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Brown adipose tissue (BAT) can dissipate energy as heat through the process of non-shivering thermogenesis, thereby presenting a powerful agent against the growing global obesity epidemic. We and others have recently shown that LETM1-domain containing 1 (LETMD1) is a cold-induced mitochondrial protein essential for mitochondrial cristae structure and thermogenic function[1-5]. However, the detailed mechanism underlying the biological and physiological functions of LETMD1 in BAT remains unclear. We have now generated a UCP1driven brown adipocyte-specific (Letmd1UKO) mouse model to confirm a cell autonomous function of LETMD1 in maintaining the structure and function of brown adipocytes. Notably, we observed abnormal mitochondrial protein aggregation and an increased insoluble fraction of mitochondrial inner membrane and matrix proteins in the Letmd1UKO brown adipocytes, leading to elevated mitophagy. Using TurboID proximity labeling, we identified several proteins in the translocase of the inner membrane (TIM) complex as potential interacting partners of LETMD1. Ongoing efforts are elucidating how the interaction between LETMD1 and candidate proteins regulates protein import and assembly. These results provide insights into how LETMD1 mediates BAT thermogenesis and mitochondria homeostasis. The information may also lead to the identification of new therapeutic targets for combatting obesity and metabolic disorders.

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Loss of Mitochondrial Integrity Drives a Type 1 Interferon and Senescence Associated Secretory Phenotype Gene Signature Associated with Aggressive Phenotypes in Pancreatic Ductal Adenocarcinoma

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Altered metabolism and tumor promoting inflammation are main hallmarks of cancer. What lies at the intersection of these hallmarks is the multifunctional organelle, the mitochondria. The unique structure and function of the mitochondria allows them to be main gatekeepers of both intrinsic and extrinsic metabolic and immune signaling pathways. As such, they play a critical role in adapting to oncogenic stress and promoting cell survival pathways. Our lab has recently identified a cohort of human tumors with downregulated expression of Mitofilin (MIC60), a structural protein in the organizational scaffold of the mitochondrial membrane (MICOS). Downregulation of MIC60 is associated with severe mitochondrial dysfunction and increased expression of an 11-gene type 1 interferon and senescence associated secretory phenotype (IFN/SASP) gene signature.

Clinically, expression of this gene signature across multiple tumor types correlated with poor patient outcome. Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignancy with a 5-year survival rate of ~12%, necessitating novel diagnostic and therapeutic approaches. Our data shows that loss of MIC60 in two human PDAC cell lines promoted cell invasion and motility *in vitro*. Additionally, the loss of MIC60 expression in murine cancer cell lines is associated with accelerated tumor growth in an orthotopic mouse model. Given that expression of this gene signature correlates to poor outcomes in PDAC patients, we decided to investigate in these models if the loss of MIC60 correlates with increased expression of the IFN/SASP gene signature. Our data demonstrate that in multiple murine and human PDAC models, loss of MIC60 is correlated with increased IFN/SASP gene expression and more aggressive phenotypes overall. Interestingly, this is associated with a significant increase in p50/p65 nuclear localization and increased expression of NF-kB target genes. Further mechanistic experiments are needed, but our data suggests that the loss of MIC60 in PDAC could lead to the activation of survival pathways that ultimately lead to more aggressive phenotypes. Clinically, these 'MIC60 low' tumors are associated with more aggressive disease variants, local inflammation, and treatment refractory disease (FOLFIRINOX). Therefore, understanding the link between MIC60 loss, IFN/SASP gene expression, and NF-kB activation is a crucial next step in discovering novel therapeutic strategies for aggressive variants of PDAC.

Machine learning methods uncover potential roles of the mitochondrial carrier family in cancer glutathione metabolism

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Background: Alterations of metabolism are a major hallmark of cancers, and mitochondria are crucial in these alterations. Transport of metabolites across the inner mitochondrial membrane (IMM) is of key importance and primarily facilitated by solute carrier family 25 (SLC25) proteins. The functions of approximately 30% of the SLC25 transporters are currently unknown.

Our **goal** was to develop a machine learning (ML) approach to annotate functions for the unknown mitochondrial carriers. For this task, we created a framework to leverage multi-omics data and biological knowledge for annotation of functions. Using a cancer cell line multi-omics dataset, we apply this approach to glutathione (GSH) metabolism in cancer which is essential for redox homeostasis, drug-resistance, and proliferation of tumor cells.

Results: We find that this approach accurately annotates known proteins involved in GSH metabolism, and outperforms a comparable state-of-the-art deep learning model, DeepGOPlus, in annotating this function. Amongst the proteins most likely related to GSH metabolism are the known mitochondrial GSH transporter, SLC5A39, the Mg-ATP transporter, SLC25A24, and the orphan transporter, SLC25A43. The proteins were further investigated using *in silico* structural analysis which reveals similarities in substrate binding residues between SLC25A39, SLC25A24, and SLC25A43, implying similarities in substrate transport.

Additionally, this method predicts other proteins known to be involved in redox metabolism but have yet to be associated with GSH metabolism. The most probable proteins include the mitochondrial calcium uniporter (MCU), ATP-binding cassette family B6 (ABCB6), and Pyruvate Carboxylase (PC). We also show that this framework can be applied robustly with accurate annotation of characteristics beyond GSH metabolism, such as mitochondrial localization, transporter activity, and metabolism of other substances.

Implications: This work provides a framework for uncovering potential functions of proteins from existing biological knowledge and multi-omics data which we apply to a cancer cell line dataset for uncovering potential roles of proteins in cancer GSH metabolism. We expect that the predicted functions from this model, as well as the application of this framework to other functions beyond those considered here, will

provide a useful tool for hypothesis generation of novel protein functions in different biological conditions.

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Mcl1 plays a functional role in mitochondrial iron uptake by stabilizing Mitoferrin-2

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Mitochondria-derived Iron-Sulfur (Fe-S) clusters are one of the ancient co-factors driving essential biochemical reactions including electron transport and enzymatic catalysis. In non-erythroid cells, iron transport to the mitochondria through SLC25A28 (Mitoferrin 2; Mfrn2) is the first crucial step for Fe-S cluster biosynthesis, and yet how it is regulated is not clear. By doing an unbiased genome-wide siRNA screen to assay for Fe-S harboring aconitase enzymatic activity, as a proxy for measuring mitochondrial iron levels, we have identified Mcl1, a BCL2-family protein with essential roles in cancer malignancy and mitochondrial dynamics to play a functional role in mitochondrial iron uptake. Our findings show that by manipulating endogenous Mcl1 levels by siRNA or Mcl1-specific inhibitors at steady-state, there is loss of aconitase activity, mitochondrial iron deficiency, loss of Mfrn2 protein levels and alterations in the cytosolic iron-signaling response. We validated these findings in multiple cellular model systems including primary mouse hepatocytes derived from WT mice. Furthermore, by ectopically expressing fluorescently tagged form of Mfrn2, we have found that Mcl1 inhibition results in lysosomal targeting of mitochondria derived vesicles containing Mfrn2-GFP cargo for degradation. Altogether, we have discovered a novel functional role of Mcl1 in stabilizing Mfrn2 and regulate iron transport to the mitochondria during steady-state conditions.

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Mcrip2 regulates the expression of mitochondrial pathways at the post-transcriptional level and is required for brown adipose tissue function

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In response to changing energy demands, mitochondrial networks undergo dynamic remodeling, (e.g., biogenesis, mitophagy, fusion or fission, and regulated expression of proteins), to help cells and tissues adapt. Many of these adaptive processes are regulated by the Peroxisome proliferator-activated receptor Gamma Coactivator-1 co-regulators (PGC-1α/β), which work with Estrogen Related Receptors (ERRα/β/γ) to activate the transcription of genes important for mitochondrial biogenesis, oxidative metabolism, and other pathways. In brown adipose tissue (BAT), β-adrenergically-activated PGC-1α and ERRs enable the transient induction of transcriptional programs in response to cold. How such transcriptional increases are consolidated into long-term elevations in genes encoding mitochondrial proteins, and specifically in proteins required for thermogenesis in BAT, is unclear. We show here that MAPK Regulated Corepressor Interacting Protein 2 (Mcrip2) is a PGC-1/ERR-regulated, cold-inducible gene that enhances BAT oxidative metabolism gene expression at the post-transcriptional level. Transcriptomic and proteomic studies of BAT collected from WT and Mcrip2 BAT-specific KO mice ($Mcrip2^{BAT-KO}$) indicate that Mcrip2 is required selectively for the expression of genes related to mitochondrial function, particularly genes involved in oxidative metabolism and mitochondrial translation pathways. Accordingly, $Mcrip2^{BAT-KO}$ mice have lipid-laden BAT, decreased oxidative phosphorylation complex I & II activity, increased sensitivity to cold, and gene-specific defects in cold-induced gene expression compared to WT littermates. Diminished mitochondrial function in $Mcrip2^{BAT-KO}$ mice is accompanied by elevated expression of mitochondrial stress response markers. Loss-of-function approaches in brown adipocyte cultures show that Mcrip2 promotes the expression of branched chain amino acid and fatty acid oxidation genes, as well as of uncoupling protein 1 (Ucp1), and that it does so at a post-transcriptional level. Altogether, our findings highlight a critical role for Mcrip2 in building the mitochondrial oxidative capacity necessary for BAT thermogenesis, and suggest a pathway by which mitochondrial adaptation to cold exposure, via PGC-1α/β and ERRs, can be regulated at a post-transcriptional level.

MCT1 facilitates direct mitochondrial oxidation of lactate in the heart

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The heart ranks among the highest lactate-consuming organs in mammals. However, the intricacies of cardiac lactate metabolism remain poorly understood. Here, we unveil the novel role of MCT1 in facilitating direct mitochondrial lactate oxidation in cardiac tissue. Employing 13C-tracers in mice, primary cultured cardiomyocytes, and isolated mitochondria, revealed that lactate enters the mitochondria and contributes to the

tricarboxylic acid (TCA) cycle independently of the mitochondrial pyruvate carrier (MPC). We established that MCT1, a cell membrane lactate transporter, can localize within the inner mitochondrial membrane, facilitating this direct import of lactate into the mitochondria. Furthermore, we determined that this process significantly contributes to the mitochondrial NADH pool in a TCA cycle-independent manner. To gain further insight into the *in vivo* significance of these metabolic findings, we subjected MCT1 cardiac-specific knockout mice to acute and chronic cardiac injuries, which resulted in a profound loss of normal cardiac function. This underscores the critical role of MCT1 and lactate metabolism in maintaining cardiac performance and adaptability under both physiological and pathological conditions. Collectively, these insights carry substantial implications for comprehending cardiac metabolism, laying the groundwork for the development of innovative therapeutic strategies in the context of cardiac hypertrophy and related cardiovascular pathologies.

Metabolic Remodeling Through Mitochondrial Transfer: A Key to Enhanced Immune Responses

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Horizontal transfer of mitochondria is a recent discovery with physiological and pathological implications. Our previous research unveiled a novel mechanism of mitochondrial exchange involving the removal of mitochondria from cardiomyocytes and their uptake by cardiac tissue-associated macrophages. Furthermore, recent publications have demonstrated that the transfer of mitochondria and their associated molecules serves as a signaling mechanism, regulating Treg differentiation and promoting communication between T cells and dendritic cells. However, several questions remain: i) Can terminally differentiated lymphoid cells uptake exogenous mitochondria? ii) What are the underlying mechanisms of horizontal mitochondrial transfer? iii) Can we manipulate aberrant immune responses through mitochondrial exchange?

Our project aims to investigate the mechanisms that underpin mitochondrial transfer, metabolic reprogramming of the cells and how this might regulate T cell differentiation. We have evaluated the role of mitochondrial exchange in intercellular communication in the adaptive immune system by studying the impact of the uptake of exogenous mitochondria on T cell biology. Firstly, we have used animal models with different mitochondrial genetics to investigate how mitochondrial variability controls T cell reprogramming. Mitochondrial heterogeneity in CD8 T cells promotes enhanced mitochondrial respiration and exacerbates adaptive immune responses. Here, we describe for the first time the transfer of mitochondria between CD8 T cells as well as mitochondrial features as their performance, repopulation, and viability in cells. To that end, we used single-cell analyses and a combination of protein isoforms, along with mitochondrial membrane and matrix protein labeling, alongside genetic tools, to demonstrate the active uptake of exogenous mitochondria by T cells. These acquired mitochondria not only maintain their functionality within the recipient cell but also undergo replication, playing a pivotal role in driving metabolic remodeling processes.

The results we obtained through adoptive cell transfer and immunization experiments in mouse models demonstrate that the uptake of functional exogenous mitochondria by lymphocytes improves their metabolic performance and boosts adaptive immune responses. These findings not only shed light on the interplay between mitochondrial variability and intercellular mitochondrial exchange but also open new avenues for innovative T cell immunotherapy strategies.

Microproteins mediate Localized Mitochondrial Translation to drive OXPHOS

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Mitochondrial **s**mall-open reading frame **e**ncoded micro**p**roteins (SEPs) are key regulators and components of the electron transport chain (ETC). Assembly of ETC complexes, in particular Complex I assembly, is a costly bioprocess that is tightly coupled to nutrient availability and redox status. Serine is a major source of 1-carbon units for anabolic growth and redox buffering, and its availability determines the levels of Complex I biogenesis. How mitochondria actively co-ordinate 1-carbon availability to Complex I levels is unknown. Using a genome-wide CRISPR screen against a comprehensive collection of SEPs, we find that a lnc-RNA encoded microprotein CRISTA is required for growth under oxidative conditions when the 1-carbon folate cycle is inhibited. CRISTA potentiates mitochondrial serine import via direct interactions with the serine transporter SFXN1 in the inner mitochondrial membrane. Its C-terminus furthermore interacts with the mitochondrial ribosome to form a SFXN1-CRISTA-mitoribosome triad that is enriched for *mt-ND5* mRNA encoding a core enzymatic and structural subunit of Complex I. Deletion of CRISTA reduces mitochondrial serine uptake, leading to reduced levels of folate intermediates. This in turn impairs 5-taurino-methyl(thio)uridinylation of mitochondrial tRNAs (tm^5U and tm^5s^2U), leading to a specific loss of ND5 translation and a stall in Complex I biogenesis. Conversely, inhibition of the folate cycle destabilizes CRISTA, leading to an attenuation of Complex I assembly in response to reduced folate cycle metabolites. In mice, loss of CRISTA causes post-implantation lethality. Our work uncovers a novel and essential mechanism mediated by a microprotein that bridges 1-carbon flux to Complex I biogenesis, and establishes a prototypical example of localized mitochondrial translation controlled by metabolites through the physical apposition of mitoribosomes with metabolite transporters.

MICU1 loss interferes with mitochondrial fusion; role of peri-mitochondrial actin formation

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Introduction: Mitochondria are multifaceted organelles that provide energy for the main cellular processes. Mitochondrial Ca^{2+} (Ca^{2+}_m) signaling and mitochondrial fusion are central to organelle bioenergetics and quality control. Ca^{2+}_m uptake is mediated by the mitochondrial calcium uniporter complex (mtCU), consisting of a pore, MCU+EMRE, and gatekeepers, MICU1 and MICU2. Ca^{2+}_m signaling has been linked to mitochondrial fusion dynamics but the underlying molecular mechanisms remain elusive. We hypothesized that MICU1 and MICU2 are relevant in mitochondrial fusion through their role in Ca^{2+}_m homeostasis. **Material and Methods:** We used skin fibroblasts derived from patients null for MICU1 and MICU2 and to separate short-and long-term consequences, we have created acute and chronic MICU1 or MICU2 KO mouse embryonic fibroblasts (MEFs) using a CRE-Lox recombination system. *MICU1*$^{fl/fl}$ or *MICU2*$^{fl/fl}$ MEF cells were infected with a CRE or Null adenovirus for 72 hr to induce the recombination. To follow mitochondrial fusion dynamics, infected *MICU1*$^{fl/fl}$ or *MICU2*$^{fl/fl}$ MEF cells were transfected with a mitochondrial-targeted photoswitchable proteins, mtPAGFP /mtDsRed or mtDendra2. For Ca^{2+} measurements, cells were transfected with a mitochondrial matrix-Ca^{2+} sensor (mtRCaMP). Rhodamine Phalloidine was used to stain the Actin filaments. **Results**: MICU1 loss caused mitochondrial fusion inhibition, while MICU2 loss stimulated mitochondrial fusion events in both patient and MEF cells. Rescue of MICU1 and MICU2 restored the mitochondrial fusion activity. Loss of MICU1 but not MICU2 led to elevated resting Ca^{2+}_m and to a peri-mitochondrial actin polymerization in MEF cells. Destabilization of the actin cytoskeleton by inhibiting the activity of the protein ARP2 using the drug CK666 or by siRNA showed restoration of mitochondrial fusion activity during acute or chronic loss of MICU1. CK666 also affected the fusion activity in control cells by decreasing the kiss-and-run and stabilizing the complete fusion events.

Discussion: Our results show that MICU1 loss together with Ca^{2+}_m dysregulation causes mitochondrial fusion impairment, and peri-mitochondrial actin polymerization. Peri-mitochondrial actin polymerization might be a factor in fusion inhibition. MICU2 loss fails to affect Ca^{2+}_m and increases mitochondrial fusion activity, indicating that only MICU1 has pro-fusion activity and MICU2 might limit MICU1's availability. The specific involvement of Ca^{2+}_m in the MICU1-linked fusion and peri-mitochondrial actin is investigated in MCU-deficient cells.

Mief1-dependent mitochondrial fission coordinates the transcriptional and metabolic responses to extracellular forces

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Cells in tissues are constantly exposed to multiple forces including including extracellular matrix elasticity, compression and shear stresses. Tissue-scale mechanical properties can instruct cell behavior by regulating proliferation, metabolism, and cell fate in physiological and diseased conditions. Our knowledge of the underlying mechanisms remains fragmentary, with specific pathways mediating specific responses to specific mechanical cues. We recently discovered that mitochondrial dynamics is broadly regulated by forces and spatial confinements, in vitro and in vivo. High levels of forces maintain high actomyosin tension in cells, which in turn promotes the phosphorylation of the MIEF1 fission factor. Phosphorylation of MIEF1 limits DRP1 recruitment to mitochondria and promotes a filamentous mitochondrial morphology. Strikingly, mitochondrial fission is not only a general response but also a general mechanotransduction mechanism by which cells "read" extracellular forces. We found that DRP1 and MIEF1 regulate both mitochondrial functions as well as a multi-tiered retrograde response involving the YAP-TAZ, SREBP1-2, and NRF2 transcription factors to coordinate proliferation, cell fate choices, lipogenesis, and antioxidant metabolism in response to mechanical cues. We thus propose that mitochondria fulfill a unifying signaling function by coordinating complementary responses to the mechanical tissue microenvironment.

MIMAS, a mitochondrial multifunctional mega-assembly integrating metabolic and respiratory biogenesis factors of mitochondria

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The inner mitochondrial membrane is extraordinarily protein-rich and harbours a multitude of protein complexes, including the oxidative phosphorylation machinery, protein translocases and complexes that maintain the membrane architecture. We have discovered a novel mega-assembly, MIMAS, that has escaped scientific scrutiny so far, likely due to its large size as well as its multifunctional nature. MIMAS comprises a specific set of proteins with diverse functions that were not previously known to assemble into large complexes. Uniquely, the integrity of this complex depends on the non-bilayer lipid phosphatidylethanolamine that appears to be synthesized directly at MIMAS. Our results suggest that MIMAS compartmentalizes the inner membrane to integrate specific steps in respiratory chain biogenesis and metabolic processes in mitochondria.

Mitigating Cytokine-Induced Beta Cell Dysfunction: Investigating the Effect of Butyrate on Mitochondrial Function

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Background: Type 2 diabetes (T2D) is caused by insufficient insulin secretion from dysfunctional beta cells due to metabolic and inflammatory stress. Mitochondria play an essential role in glucose-induced insulin secretion as ATP is the main mediator of insulin exocytosis. In T2D, proinflammatory cytokines such as IL-1β induce beta cell dysfunction by reducing glucose-stimulated insulin secretion and inducing mitochondrial defects. Changes in the gut microbiota have been associated with T2D and insulin secretion. Importantly, gut microbial metabolites such as short-chain fatty acids (SCFAs) improve metabolic health and beta cell function, however the molecular mechanisms and potential role of mitochondria are poorly understood.

Objectives: In this project, we investigated the role of mitochondria in IL-1β inhibition of glucose-stimulated insulin secretion and how the SCFA, butyrate, prevents IL-1β-induced dysfunction of mitochondria and beta cells.

Methods: Pancreatic islets were isolated from C57BL/6 mice and exposed to IL-1β in the absence or presence of butyrate for 10 days to mimic low-grade inflammation in T2D. Beta cell function was determined by measuring glucose-stimulated insulin secretion. Gene expression and oxygen consumption rate were evaluated by RNA-sequencing and the Seahorse XF Analyzer, respectively. Mitochondrial membrane potential and mitochondrial mass were measured by flow cytometry using TMRM dye and MitoTracker Green, respectively.

Results: Butyrate prevented IL-1β-induced impairment of glucose-stimulated insulin secretion and normalized insulin content reduced IL-1β. IL-1β regulated several genes related to mitochondrial function, including fusion, fission, mitophagy, oxidative phosphorylation and generation of reactive oxygen and nitrogen species. Many of the IL-1β-induced changes in gene expression were counteracted by butyrate. For example, butyrate downregulated the antioxidant enzyme glutathione peroxidase 2 (Gpx2) by 8-fold, suggesting that butyrate prevents oxidative stress. Moreover, IL-1β was found to reduce mitochondrial oxygen consumption in response to glucose and increased proton leak and nonmitochondrial respiration. Butyrate prevented these effects of IL-1β. Butyrate also normalized the mitochondrial membrane potential decreased by IL-1β.

Conclusion: Together, our results suggest that butyrate may prevent IL-1β-induced beta cell dysfunction by improving mitochondrial function and reducing the production of reactive oxygen species.

Mito Hi-Ness: A novel fluorescent genetic probe for live-cell imaging of mitochondrial DNA

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Mitochondria contain their own unique genome composed of circular, double stranded DNA. Mitochondrial DNA (mtDNA) is present in hundreds of copies within an individual cell and interacts with proteins to form nucleoid complexes. Nucleoids are compact roughly spherical structures (about 100 nm in diameter) that are localized in the mitochondrial matrix. Direct visualization of nucleoid localization, organization, and dynamics within living cells is critical for revealing new insights about their fundamental role. Yet optical imaging of nucleoids within living cells is challenging. While some DNA dyes (e.g., PicoGreen^{TM} and SYBR^{TM} Gold) can be used to visualize mtDNA for short-term live cell imaging, they do not always label every mtDNA nucleoid. Further, as these probes intercalate DNA, they can impair mtDNA function during live cell imaging. When considering fusion proteins, over-expressing mtDNA nucleoid proteins can affect mtDNA regulation, also limiting the usefulness of this approach. To address the need for improved probes, we build on a novel genetic reporter for nuclear DNA (HI-NESS) that is based on the DNA-binding domain of H-NS, a bacterial nucleoid-associated protein (1). We adapted the approach by designing a reporter that contains a mitochondrial targeting sequence and demonstrate specific labeling of nucleoids within the mitochondrial network in cell lines including U-2 OS. We compare the localization and dynamics relative to SYBR Gold and, of note, we find that the specificity of the probe is also affected by the choice of the fluorescent protein. We also show that Mito HI-NESS has minimal effect on total mitochondrial copy number and composition as assayed by western blots and qt-PCR. Overall, the Mito HI-NESS probes represent a promising new tool for investigating the fundamental biology of nucleoids within the living cell.

(1) Rashid FM, Mahlandt E, van der Vaart M, et al. Nucleic Acids Res. 2022;50(2):e10. doi:10.1093/nar/gkab993

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Mitochondria- and ER-associated actin are required for mitochondrial fusion

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Mitochondria play a crucial role in the regulation of cellular metabolism and signalling. Mitochondrial fission and fusion control these processes by balancing respiratory and metabolic functions, transferring material between mitochondria, and removing defective mitochondria. Mitochondrial fission occurs at sites of contact between mitochondria and the endoplasmic reticulum (ER). These sites serve as a platform to recruit the fission machinery, including the fission GTPase DRP1 responsible for the scission of the mitochondrial tubule. Importantly, when fission is experimentally stimulated, DRP1 recruitment and activation at these sites require the formation of actin filaments in a manner that depends on the ER-localised formin INF2. Similarly, actin was suggested to be present at sites of mitochondrial fusion, but its role in this context remains entirely unexplored.

Here, using our recently characterized actin chromobody probes, we show that mitochondria-associated actin is recruited to both fission and fusion sites under unstimulated conditions, and that this precedes the recruitment of the ER and ER-associated actin. Importantly, selectively preventing the formation of actin filaments on either mitochondria or the ER with organelle-targeted deAct probes disrupts both mitochondrial fission and fusion. We also demonstrate that fusion but not fission is dependent on Arp2/3-dependent actin polymerization, whereas both fission and fusion are dependent on INF2-dependent actin polymerization. Finally, our work identifies two types of fusion events, with actin selectively required for tip-to-side fusion but not tip-to-tip fusion. Altogether, our work introduces a novel method for perturbing organelle-associated actin filaments and demonstrates a previously unknown role for actin in mitochondrial fusion.

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Mitochondrial complex I NDUFA12 as functional target of anticancer drug ertredin in human hepatoma cells.

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Many attempts have been made to develop new agents that target EGFR mutants or regulate downstream factors in various cancers. Cell-based screening showed that a natural small molecule, Ertredin, inhibited EGFRvIII mutant cancer cells. Previous studies have shown that Ertredin effectively inhibits anchorage-independent three-dimensional growth of sphere-forming cells transfected with EGFRvIII mutant cDNA. However, the underlying mechanism remains unclear. In this study, we investigated the target protein of Ertredin by combining drug affinity responsive target stability (DARTS) assays with liquid chromatography-mass spectrometry using label-free Ertredin as bait and HepG2 cell lysates as a proteome pool. NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12 (NDUFA12), a protein that is part of mitochondrial complex 1, was identified as the Ertredin-binding protein that was responsible for its biological activity. The interaction between NDUFA12 and Ertredin was validated by DARTS and cellular thermal shift assays. In addition, genetic knockdown of the identified target, NDUFA12, was shown to suppress cell proliferation. NDUFA12 was identified as a biologically relevant target protein of Ertredin that is responsible for its anti-tumor activity, and these results provide insight into the role of NDUFA12 as a downstream factor in EGFRvIII mutants.

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Mitochondrial components as adipokines: a novel fat-brain communication modality

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The brain integrates information from peripheral organs to fine-tune behaviors based on organismal needs. Within the brain, neuronal communication is largely governed by neurotransmitter release across synapses. Recently, a novel mode of neuronal communication involving mitochondrial exchange has received growing attention from colleagues. Indeed, the exchange of mitochondria between neurons in healthy brains can alter the metabolism of neurotransmitters, the strength of synaptic transmission, and, ultimately, the behavior of animals. Under stress, delivery of mitochondria from astrocytes to neurons or transfer of a subset of mitochondrial components from neural stem cells to neurons supports recovery from stroke or other brain disorders. These findings demonstrate the importance of mitochondrial exchange in neuronal communication. Hence, an intriguing possibility is that peripheral organs use organellar transfer to modulate neuronal metabolism and brain physiology. Nonetheless, whether other organs utilize these novel mitochondrial regulatory modules to communicate with the brain remains to be established. In the past year, we made the exciting discovery that specific mitochondrial components transferred from *Drosophila* adipocytes to glia and neurons. Unexpectedly, we find that these electron transport chain (ETC) subunits integrate with the brain mitochondria *in vivo* under normal physiological conditions. Notably, this transfer is specific and unidirectional, as it is observed only from adipocytes to the brain- akin to 'adipokines'. Distinct from other known mitochondrial transfer events from adipocytes, we find that only specific subunits of an ETC that catalyzes Oxidative Phosphorylation (OxPhos) is transferred but not whole or parts of mitochondria. We refer to this mode of adipocyte-to-brain communication as AMOC (Adipocyte mitochondrial OxPhos component) signaling. Strikingly, increased production of AMOC subunits in the fat tissue is dysregulated feeding behavior, specifically during obesogenic stress. Given that neurons heavily rely on energy from OxPhos, we propose

that AMOC are a means by which adipocytes regulate brain bioenergetics and neuronal properties to influence innate behavior in relation to fat store status. Obesity has been established as an independent risk factor for developing dementia, yet the mechanisms remain largely unknown. By uncovering a direct means by which adipocytes control brain mitochondria, we are poised to address this fundamental gap in knowledge. In this talk, provide insights into our current models on how the unidirectional fat-brain AMOC pathway works and I will discuss the broad implications of fat-brain AMOC signaling in healthy and diseased states.

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Mitochondrial DNA heterogeneity in Hematopoyesis

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Hematopoietic stem cells (HSCs) are recognized for their dual capacity for self-renewal and differentiation. Initially, HSCs relinquish their self-renewal ability, giving rise to diverse subsets of multipotent progenitors such as common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). These progenitors, influenced by mitochondrial OXPHOS adaptations, further develop into all the distinct mature blood cells. The equilibrium between reactive oxygen species (ROS) production, mitochondrial DNA (mtDNA) replication and expression, and mitochondrial quality control mechanisms make mitochondria major partners driving this differentiation process. However, there is limited knowledge regarding the factors that determine the fate of stem cells, the immune phenotype in the presence of rapid accumulation of mutations in the mitochondrial genome, and the resulting implications for the overall organismal physiology

Our project aims to investigate whether mtDNA mutations affects hematopoiesis and ultimately impacts the immune system and longevity by: (1) assessing mtDNA mutation load in HSC (2) understanding how mitochondria influence cell fate determination; and (3) exploring strategies to modulate mitochondrial performance to mitigate the development of premature ageing phenotype.

To investigate that we analyzed hematopoiesis in mice expressing a proofreading-deficient version of the mitochondrial DNA polymerase (PolgD275A) along with their wildtype littermates (PolgWT). PolgWT have fully functional polymerase activity but up to 80% of the mtDNA mutation load compared to PolgD275A mice. We are evaluating the impact of mito-nuclear crosstalk during hematopoiesis under conditions of normal vs high mitochondrial variability. Our study involves complete immune cells count via Flow Cytometry to study the cell composition in different organs, cytokine analysis and other biochemical parameters to comprehend the immune responses, enzymatic activity assays to determine the activity levels of various mitochondrial complexes and other enzymes associated with mitochondrial function, as well as ROS production measurement and respirometry and metabolic flux analysis.

Our results shows that PolgD275A mice exhibit imbalance immune populations during early life stages, coupled with a phenotype characterized by multi-organ dysfunction, with no observable pathology in PolgWT. These findings emphasize the specific role of mtDNA genome instability in the survival and differentiation of downstream progenitors. and shed light on how immune cells from this progeroid model contribute to inflammaging and age-related phenotype.

Mitochondrial dynamics regulates endothelial vascular function in response to increased flow-mediated dilation

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Endothelial dysfunction is an important predictor of future major adverse cardiac events and a direct contributor to coronary artery disease (CAD). Under physiological conditions, an increase in blood flow stimulates the release of endothelial-derived nitric oxide (NO) resulting in microvascular dilation. Under pathological conditions, such as CAD,

vasodilation is instead achieved by the release of mitochondria-derived hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS).

Mitochondrial dynamics is an important homeostatic process that regulates mitochondria's main functions: ATP production and ROS formation. DRP1 promotes mitochondrial fission, while MFN1 & 2 are pro-fusion factors. Mitochondrial fission is associated with elevated ROS production, while fusion results in improved ATP production. We hypothesize that endothelial mitochondrial fission/fusion is an important contributor to the phenotype switch from NO- to H_2O_2-mediated vasodilation in individuals with CAD.

Microvessels were obtained from surgical discard tissue from healthy individuals (those with ≤1 co-morbidities; non-CAD) and from individuals with a formal diagnosis of CAD. Expression of DRP1 and MFN2 were quantified by western blot and the mitochondrial network of the endothelium was assessed by confocal immunofluorescent microscopy. To assess the functional role of mitochondrial dynamics in the endothelium, microvessel diameter was measured in response to increased shear stress by video microscopy before and after genetic manipulation of DRP1/MFN1&2 (achieved by siRNA knockdown or AAV-mediated overexpression). Fluorescent probes were used to evaluate flow induced NO, H2O2 and ATP levels.

Our western blot results show that microvessels from individuals with CAD had higher levels of DRP1 (relative FC 110.3 to 44.9, $p < 0.05$), higher levels of p-DRP1 S616 (relative FC 101.7 to 49.2, $p=0.05$), and no changes in MFN2 levels when compared to non-CAD. Confocal images confirm that the endothelial mitochondria from individuals with CAD were more fragmented compared to non-CAD (assessed by mitochondrial fragmentation count: 3.34 to 0.98, $p<0.05$). Promoting mitochondrial fission (achieved by either upregulating DRP1 or downregulating MFN2) in microvessels from non-CAD promotes H_2O_2-mediated vasodilation in non-CAD vessels. Furthermore, downregulation of DRP1 or upregulation of MFN2 (pro-fusion) restored NO-mediated vasodilation in microvessels from individuals with CAD. Fluorescent images of microvessels exposed to maximal flow confirmed that ATP is increased in response to this stimulus in non-CAD but not in CAD (5.99 to 1.09 fluorescence units).

Our data suggests that the balance between mitochondrial fission/fusion may be an important contributor to H_2O_2-mediated vasodilation in the microvessels of individuals with CAD in response to increased flow. We hence argue that targeting mitochondrial

dynamics in the endothelium may be an important therapeutic tool that can improve CAD outcomes.

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Mitochondrial Dysfunction is crucially involved in the Pathogenesis of Facioscapulohumeral Muscular Dystrophy

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Facioscapulohumeral musculoskeletal dystrophy (FSHD) is an incurable hereditary disorder characterized by progressive muscle weakness and wasting. Genetically, FSHD has been linked to misexpression of the germline transcription factor double homeobox 4 (DUX4) which is usually silenced in somatic tissues. FSHD is associated with sensitivity to oxidative stress, especially detrimental to skeletal muscle with its high metabolic activity and energy demands. Mitochondrial dysfunction and perturbed hypoxia signaling are considered pathomechanisms in FSHD, however, the redox biology of FSHD is poorly understood.

Here we examined how altered mitochondrial reactive oxygen species (mitoROS) metabolism and impaired mitochondrial function cause metabolic stress in FSHD. RNAseq analyses of FSHD patient muscle biopsies revealed broad metabolic changes, including perturbed mitochondrial biogenesis, altered oxidative stress response, defective autophagy and downregulation of the mitochondrial transcriptome. Using patient-derived and DUX4-inducible human skeletal muscle models, we found that elevated mitoROS levels correlate with increases in steady-state mitochondrial membrane potential. DUX4 triggers mitochondrial membrane polarization prior to oxidative stress generation and apoptosis through mitoROS, suggesting mitochondrial dysfunction as early pro-apoptotic trigger. We identified complex I as the main target for DUX4-induced mitochondrial dysfunction, also changing cellular oxygenation and thus the molecular response to hypoxia, which further aggravates FSHD pathological

hallmarks under low oxygen tension. Importantly, mitochondria-targeted antioxidants rescue FSHD pathology more effectively than conventional antioxidants, highlighting involvement of disturbed mitoROS metabolism in the disease. To study the etiology of metabolic stress in FSHD in more detail, we have further performed a combined respirometric-transcriptomic-metabolomic analysis of FSHD patient-derived muscle cells. This analysis revealed a distinct metabolomic FSHD phenotype, with generally lower cellular ATP levels and an altered balance between glycolytic and oxidative metabolism. Importantly, these metabolic changes are consistent between several patient-derived FSHD muscle models.

In summary, our work provides a pathomechanistic model where changes in oxidative metabolism impair FSHD muscle function, amplified when metabolic adaptation to varying oxygen tension is required. Not only have we discovered novel aspects of FSHD pathology, but also identified potentially druggable intervention points for symptomatic treatment of this debilitating disease.

Mitochondrial dysfunction: The endocytic pathway connection

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Mitochondria physically and functionally interact with other organelles, including the endoplasmic reticulum and endosomes/lysosomes. These organelle contact sites are key controllers of cellular physiology, regulating not only the metabolic activity of mitochondria but also a range of cellular processes, from cell death to inflammation. We have previously shown that loss of mitochondrial function impairs lysosomal structure and activity. Here we demonstrate that mitochondrial activity also regulates endosomes,

the vesicular compartment that transports extracellular material from the plasma membrane to lysosomes for degradation.

Here, we used cells deficient for the mitochondrial fusion protein OPA1 and chemical inhibition of mitochondrial activity to probe the functional links between mitochondrial activity and endosome function. Our work reveals that mitochondrial dysfunction leads to perinuclear aggregation of early endosomes, disrupting cargo trafficking to lysosomes and recycling endosomes. We found that this is caused by oxidative stress-dependent changes in centrosomes and microtubules. Importantly, antioxidants that decrease the oxidative stress present in cells with mitochondrial dysfunction rescued this endosomal phenotype. Altogether, our work identified a new crosstalk between mitochondria and early endosomes that could play an important role in neurodegenerative diseases where mitochondria is affected.

Mitochondrial fragmentation promotes inflammation resolution responses in macrophages via histone lactylation

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Background: During the inflammatory response, macrophage phenotypes can be broadly classified as pro-inflammatory/classically activated ‘M1’, or pro-resolving/alternatively ‘M2’ macrophages. Although the classification of macrophages is general and assumes there are distinct phenotypes, in reality macrophages exist across a spectrum and must transform from a pro-inflammatory state to a pro-resolving state following an inflammatory insult. To adapt to changing metabolic needs of the cell, mitochondria undergo fusion and fission, which have important implications for cell fate and function.

Hypothesis: We hypothesized that mitochondrial fission and fusion directly contribute to macrophage function during the pro-inflammatory and pro-resolving phases.

Methods & Results: We find that mitochondrial length directly contributes to macrophage phenotype, primarily during the transition from a pro-inflammatory to a pro-resolving state. Phenocopying the elongated mitochondrial network (by disabling the fission machinery using siRNA) leads to a baseline reduction in the inflammatory marker IL-1b, but a normal inflammatory response to LPS, similar to control macrophages. In contrast, in macrophages with a phenocopied fragmented phenotype (by disabling the fusion machinery using siRNA) there is a heightened inflammatory response to LPS and increased signaling through the ATF4/c-Jun transcriptional axis compared to control macrophages. Importantly, macrophages with a fragmented mitochondrial phenotype show increased expression of pro-resolving mediator Arginase 1 and increased phagocytic capacity. Promoting mitochondrial fragmentation caused an increase in cellular lactate, and an increase in histone lactylation which caused an increase in Arginase 1 expression. *In vivo*, inhibition of Opa1 via MLYS22 in mice following zymosan-induced peritonitis induces mitochondrial fragmentation in peritoneal macrophages and promotes accelerated inflammatory resolution.

Conclusions: These studies demonstrate that a fragmented mitochondrial phenotype is critical for the pro-resolving response in macrophages and specifically drive epigenetic changes via lactylation of histones following an inflammatory insult.

Mitochondrial pearling as a putative microdomain management mechanism

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The dynamicity of the mitochondrial network is fundamental for its function as the metabolic and signaling hub of the cell, as well as for the maintenance of its essential genome (mtDNA). The intricate architecture of the inner membrane restricts the

movement of material within the mitochondrion, enabling its compartmentalization into microdomains around semi-regularly distributed mtDNA nucleoids, whose fitness depends on the integrity of the local nucleoid. Moreover, concentrated damaged material is ejected from peripheral regions.

However, the mechanisms by which mitochondria maintain nucleoid and microdomain organization, and segregate functional and damaged domains, remain largely unknown.

Using gentle high-throughput super-resolution imaging, we identified instances of reversible compartmentalization of the mitochondrial matrix, in semi-periodic patterns akin to nucleoid and microdomain positioning, and associated with large-scale rearrangement events (fission, branching). Employing neural network-driven real-time adaptive microscopy to overcome the trade-offs of traditional fluorescence microscopy, we report the structural and functional characterization of these “pearling” events, investigating their putative role in mitochondrial microdomain management, quality control, and nucleoid distribution.

Mitochondrial Precursor Overaccumulation Stress and Aging-associated Diseases

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The study of mitochondrial biology has been traditionally bioenergetics-centric, primarily focused on the mechanism of ATP synthesis and ROS production. We are interested in identifying mitochondria-induced non-bioenergetic processes that may affect cell fitness and survival. Mitochondrial biogenesis requires the import of >1,000 mitochondrial preproteins from the cytosol. Defect in mitochondrial protein import is expected to impair many mitochondria-associated functions, but the effect on extramitochondrial cellular pathways has been understudied. We previously found that many forms of mitochondrial damage can quantitatively affect protein import into mitochondria. This leads to the toxic accumulation of unimported mitochondrial precursor proteins in the cytosol, causing a unique form of proteostatic stress that we named mitochondrial

Precursor Overaccumulation Stress (mPOS) (Wang & Chen, *Nature* 524:481-4). We developed a knock-in mouse model expressing a clinically relevant mutant allele of *Ant1*, encoding the muscle/heart isoform of adenine nucleotide translocase. We found that the mutant Ant1 causes partial clogging of mitochondrial protein import. The "clogger" mice develop late-onset muscle pathology and a neurodegenerative phenotype, but without detectable bioenergetic defects (Coyne et al., *eLife* 12:e84330). In the current study, we introduced a "clogger" variant of Ant1 into an established mouse model of Parkinson's disease expressing the A53T mutated form of alpha-synuclein. We found that mitochondrial protein clogging increased the size of alpha-synuclein aggregates, co-aggregation of mitochondrial preproteins with alpha-synuclein, and worsened motor defect in the alpha-synuclein(A53T) genetic background. Importantly, we found no evidence of bioenergetic defects in any of the mutant mice, even with the added protein import stress. These data strongly suggest that mitochondrial protein import stress can contribute to neurodegeneration through cytosolic proteostatic stress and co-aggregation of mitochondrial and neuropathogenic proteins independent of bioenergetics. Given that protein import efficiency is affected by many types of mitochondrial stress, our findings may help the understanding of why the pathogenic mitochondrial dysfunction and cytosolic protein misfolding pathways often induce the same aging-associated degenerative diseases (e.g., Parkinson's disease). This study was funded by the National Institute of Health grants R01AG063499 (X.J.C) and F30AG-060702 (L.P.C), and the American Heart Association pre-doctoral fellowship # 906215 (A.R.).

Mitochondrial Protein Import Stress Induces a Cytosolic Signaling Response and improves Cardiac Systolic Function

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The integrated stress response (ISR) is a generalized cell response to diverse stressful stimuli including endoplasmic reticulum stressors. Recent investigations in cultured cells, model organisms, and human patients have found activation of the ISR in response to mitochondrial dysfunction. However, how ISR is activated upon mitochondrial

dysfunction and what role ISR activation may play in disease are unclear. Import of mitochondrial proteins is an intricate process that depends on the cooperation of various mitochondrial import machineries. It has recently been appreciated that defective import of mitochondrial proteins results in their cytosolic accumulation. The accumulation of these preproteins results in cytotoxicity independent of changes to the mitochondrial bioenergetic function. In this project we ask whether the accumulation of mitochondrial proteins within the cytosol of cardiac cells can activate ISR and affect the lifelong health of the heart. To answer this question, we modeled cytosolic mitochondrial precursor overaccumulation stress (mPOS) via over-expression of the inner mitochondrial membrane protein Adenine Nucleotide Translocase 1 (ANT1) in mice (Tg mice). The goals of this study are to determine: (1) Whether ANT1 overloading can induce mPOS prior to the onset of potential cardiac bioenergetic defects; (2) Whether over expression of ANT1 can induce the ISR; and (3) Whether chronic activation of the ISR is harmful to cardiac health. Using isolated cardiac mitochondria from 2 month old Tg mice, we detected moderate decrease in protein levels of Complex I, II ,and IV. Respirometry demonstrates only a mild reduction in complex II- but not complex I-based state 3 and 4 respiration, while ROS release from Tg mitochondria is significantly reduced. RNAseq of the heart at 2 and 6 months old demonstrates robust activation of the ISR, 1-carbon metabolism, the amino acid starvation response, and proteolysis. Microscopic examination indicates that Tg hearts have smaller myocytes and lack observable pathology. Using echocardiography we determined that Tg hearts have a surprising improvement in systolic function accompanied with mild asymmetric LV hypertrophy. Together these data raise the possibility that the ISR may act to mitigate the effects of mPOS within the heart. Ongoing work is aimed at understanding the effect of chronic ISR activation on Tg heart health in late life. This study was funded by the National Institutes of Health grants R01AG061204 (X.J.C) and the American Heart Association Predoctoral Fellowship # 906215 (A.R.).

Mitochondrial Protein Import Stress Triggers Proteostatic Signaling in the Cytosol and Causes Severe Muscle Atrophy

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Muscle atrophy is associated with morbidity and mortality in various diseases, as well as normative aging (i.e., sarcopenia). While it is known that mitochondrial dysfunction can cause muscle atrophy, the underlying mechanism remains elusive. Previous work has focused on the role of defective oxidative phosphorylation and elevated oxidative stress in the disruption of muscle mass homeostasis. We recently found that various forms of mitochondrial damage can perturb mitochondrial protein import. Consequently, this induces the toxic accumulation of unimported mitochondrial precursors in the cytosol and induces a cellular stress that we termed mitochondrial Precursor Over-accumulation Stress (mPOS) (Wang & Chen, *Nature* 524:481-4). To understand the physiological implications of mPOS, we generated transgenic mice overexpressing the inner mitochondrial membrane protein ANT1. We anticipated that ANT1 overloading may saturate the mitochondrial protein import machinery thereby causing mPOS. Interestingly, we found that these mice show severe muscle wasting with age (Wang et al., *iScience* 25(1):103715). Here, we found that mitochondrial respiration was only mildly reduced in 2-month-old transgenic muscle. Additionally, rates of reactive oxygen species production were reduced in these mice. Despite the lack of overt bioenergetic defects, *Ant1*-overexpression results in significantly reduced lean mass at 2-months. We found that transgenic quadricep muscles have increased expression of HSPB7, a proteotoxic stress marker. These mice show increased expression of pro-apoptotic markers at 2-months-old. The transcriptome of *Ant1*-transgenic muscle appears to be remodeled to mitigate proteostatic stress by activating the Integrated Stress Response (ISR), which is believed to repress protein synthesis. However, the rate of global protein synthesis is unaffected in vivo. We also found that *Ant1*-transgenic mice have increased protein ubiquitination, expression of lysosomal proteins and Cathepsin L activity. We propose that increased proteolytic activity may play a key role in protecting myofibers against mPOS. Consequently, this reduces protein content as a trade-off, which ultimately causes muscle atrophy. In conclusion, our studies reveal a novel mechanism of mitochondria-induced muscle wasting that is likely independent of bioenergetic defects. This study was funded by the National Institutes of Health grants R01AG061204 (X.J.C) and 1F30AG082400 (N.B.).

Mitochondrial STAT3 suppresses rheumatoid arthritis by regulating synovial fibroblast inflammatory cell death and Th17 cell activation with IL-17-

mediated autophagy dysfunction

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Th17 cells are activated by STAT3 factors in the nucleus, and these factors are correlated with the pathologic progression of rheumatoid arthritis (RA). Recent studies have demonstrated the presence of STAT3 in the mitochondria, but its function is unclear. We investigated the role of mitochondrial STAT3 (mitoSTAT3) in Th17 cells and fibroblast-like synoviocytes (FLSs) and analyzed the correlation of mitoSTAT3 with RA. We observed the accumulation of abnormal autophagosomes, increased inflammatory cell death signaling, and decreased mitoSTAT3 activity in both FLSs from patients with RA and IL-17 treated FLSs. We first discovered that IL-17 increased the accumulation of abnormal autophagosomes and expression of inflammatory cell death factors in synovial fibroblasts and decreased mitoSTAT3 activation. In a mouse model of collagen-induced arthritis, arthritis and joint inflammation were decreased by injection vectors that induced mitoSTAT3 overexpression. The abnormal accumulation of autophagosomes and expression of inflammatory cell death factors were also decreased in these mice. Zinc, a mitoSTAT3 enhancer, inhibited progression of RA and decreased production of proinflammatory cytokines and autophagosome accumulation. In mouse and human immune cells, zinc sulfate decreased the production of reactive oxygen species, the IL-17 concentration, and differentiation to Th17. However, mitoSTAT3 blockade accelerated the development of arthritis, inflammatory cell death, and abnormal autophagosome/autophagolysosome formation. Meanwhile, activation of mitoSTAT3 led to increased intact autophagosome/autophagolysosome formation and decreased inflammatory cell death by regulating mitochondrial function. Therefore, this study suggests a novel inhibitory mechanism of RA using mitoSTAT3 via regulation of autophagy, Th17 differentiation, and inflammatory cell death.

Mitochondrial transcription is required for metabolic adaptation and resistance to conventional chemotherapies in triple negative breast cancer

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The dynamic nature of mitochondrial structure and function, as well as their multi-faceted roles in energy production and cellular signaling, establishes them as key controllers of cellular adaptation. Indeed, the role of mitochondria, known for conferring adaptative survival advantages in response to exogenous stressors, is gaining prominence in numerous cancer types. Breast cancer remains a significant global health concern, with triple-negative breast cancer (TNBC) as an aggressive subtype with limited targeted therapy options. Neoadjuvant (pre-surgical) chemotherapy (NACT), the standard of care for TNBC, eradicates tumors in only half of patients, leaving those with substantial residual cancer burden facing dismal survival rates. To improve therapeutic outcomes, exploring innovative approaches to kill TNBC cells unresponsive to conventional treatments is imperative. Our previous studies uncovered elevated mitochondrial oxidative phosphorylation (OXPHOS) and mitochondrial fusion in residual TNBC cells, conferring a distinct therapeutic reliance of residual tumor cells post-NACT (PMID: 30996079 and 36813854). Seeking to understand mitochondrial mechanisms of chemoresistance in TNBC, we compared residual (i.e., the surviving population after treatment with doxorubicin, carboplatin, gemcitabine, or etoposide) and pre-treatment TNBC specimens (cultured human cell lines and orthotopic patient-derived xenograft, PDX, mouse models). Quantitative PCR revealed that residual TNBC cells exhibit an elevated level of mitochondrial DNA (mtDNA). Notably, this increase in mtDNA levels coincided with heightened expression of mitochondrial RNA polymerase, POLRMT. Kaplan-Meier survival analyses revealed that high *POLRMT* expression is significantly linked to a poorer prognosis in TNBC patients. Expanding on these insights, we treated TNBC cell lines with a novel inhibitor of POLRMT, LDC204857 (also known as IMT; PMID: 33328633). LDC204857 treatment reduced mtDNA-encoded electron transport protein levels, mitochondrial fusion, mtDNA content, and OXPHOS in TNBC cell lines. Furthermore, LDC204857 treatment sensitized TNBC cells to chemotherapy agents. Based on these promising results, we expanded our PDX testing to additional models in which we previously demonstrated resistance to additional conventional chemotherapeutic regimens. We found that LDC204857, when used as a single agent, significantly reduced tumor growth in three TNBC PDX models, BCM-5471, 2556 and

0002. Remarkably, treatment with LDC204857 achieved nearly complete tumor regression when combined with carboplatin in BCM-2665. The mice demonstrated tolerance to LDC204857 both as a single agent and when combined with chemotherapy during the course of treatment. Our study underscores the potentially crucial role of mtDNA and its transcription in driving chemotherapy resistance in TNBC and provides novel insights into mitochondria-targeted therapeutic approaches. We report a novel targeting option for TNBC and anticipate that these findings will stimulate further research into innovative combination therapies for TNBC patients, and may lead us to a viable inhibitor.

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Mitochondrial transplantation ameliorates rheumatoid arthritis by targeting abnormal cGAS-STING signaling activation, autophagosome accumulation, and necroptosis

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Objectives: Mitochondrial damage in fibroblast-like synoviocytes (FLSs) is a key factor involved in the development and progression of rheumatoid arthritis (RA). However, the underlying molecular mechanisms remain unclear. In this study, we investigated the role of mitochondrial dysfunction of FLSs in the pathogenesis of rheumatoid arthritis (RA).

Methods: We induced inflammation by stimulating FLSs with TNF-α and IL-17. Then, we transplanted fresh mitochondria into stimulated FLSs and evaluated the mitochondrial and lysosomal functions, autophagic activity, and STING-associated cell death pathway. Next, we transplanted mitochondria or gold nanoparticle-conjugated mitochondria (GNP-Mito) into collagen-induced arthritis (CIA) mice and evaluated their therapeutic effects *in vivo*.

Results: Mitochondrial and lysosomal activities were decreased and autophagosomes accumulated in the stimulated FLSs (inflammatory condition). Furthermore, the STING signaling pathway and STING-associated cell death were increased in the inflammatory condition. Mitochondrial transplantation into stimulated FLSs enhanced the mitochondrial and lysosomal activities and activated the autophagic activity, as

demonstrated by decreased numbers of autophagosomes and increased numbers of autolysosomes. Furthermore, the STING signaling pathway and STING-associated cell death pathway were decreased by mitochondrial transplantation and by treatment with STING inhibitor. *In vivo* mitochondrial or GNP-Mito transplantation ameliorated arthritis in a mouse model. Mitochondrial transplantation decreased and increased the Th17 and Treg populations, respectively. Mitochondrial function and autophagic activity were enhanced by mitochondrial transplantation.

Conclusions: Taken together, our results demonstrate that mitochondrial dysfunction in FLSs plays a pivotal role in the pathophysiology of RA and mitochondrial transplantation has therapeutic potential for RA development and progression.

Keywords: Rheumatoid arthritis (RA); mitochondria; lysosome; cGAS-STING; autophagy

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Mitochondrial-Derived Peptides: Are They Actually Important?

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NIH/NHLBI

Mitochondrial-derived peptides are encoded by mitochondrial DNA but have biological activity outside mitochondria. Eight of these are encoded by sequences within the mitochondrial 12S and 16S ribosomal genes: humanin, MOTS-c, and the six SHLP peptides, SHLP1-SHLP6. These peptides have various effects in cell culture and animal models, affecting neuroprotection, insulin sensitivity, and apoptosis, and some are secreted, potentially having extracellular signaling roles. However, except for humanin, their importance in normal cell function is unknown. To gauge their importance, their coding sequences in vertebrates have been analyzed for synonymous codon bias. Because they lie in RNA genes, such bias should only occur if their amino acids have been conserved to maintain biological function. Humanin and SHLP6 show strong synonymous codon bias and sequence conservation, providing compelling evidence of their importance in normal cell function. In contrast, SHLP1, SHLP2, SHLP3, and SHLP5 show no significant bias and are poorly conserved. MOTS-c and SHLP4 also lack significant bias, but contain highly conserved N-terminal regions, and their biological importance cannot be ruled out. An additional potential mitochondrial-derived peptide sequence was

discovered preceding SHLP2, named SHLP2b, which also contains a highly conserved N-terminal region with synonymous codon bias.

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Mitochondrial-Derived Vesicles in Cancer Cells

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Healthy mitochondria are essential to cancer cell survival through functions such as biomolecule synthesis and cell signaling. Damaged mitochondria are degraded in lysosomes via autophagy and the products may be recycled. Several cancer cell lines are dependent on autophagy for survival. However, some autophagy-dependent cells can develop resistance to autophagy inhibition and instead utilize mitochondrial-derived vesicles (MDVs).

MDVs are a newly characterized mitochondrial quality control mechanism that shuttle oxidized mitochondrial proteins to the lysosome independently of phagophores which characterize canonical autophagy. MDVs are significantly upregulated in cells resistant to autophagy-inhibition, demonstrating the dynamic nature of these structures and their strength in compensating for the loss of core metabolic pathways. Despite these important functions, much has yet to be elucidated about MDVs, including their mechanism of formation.

Our group confirms that the GTPase, DRP1, is necessary for the generation of one MDV subtype. In genetic models of DRP1 loss, we identified reduced mitochondrial respiration and metabolic activity, suggesting impaired de novo pyrimidine synthesis and a greater reliance on salvage pathways to supply pyrimidines. Indeed, cells which lack both MDVs and mitophagy demonstrate greater sensitivity to the pyrimidine analog gemcitabine, acquired by cells through salvage.

Ongoing studies aim to further characterize the functions and machinery of MDV subtypes as well as relevant in vivo applications.

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Mitolysosomes as New Pathway in Quality Control

Xingguo Liu

Mitochondrial quality control plays an important role in maintaining mitochondrial homeostasis and function. Disruption of mitochondrial quality control degrades brain function. We found that flunarizine (FNZ), a drug whose chronic use causes parkinsonism, led to a parkinsonism-like motor dysfunction in mice. FNZ induced mitochondrial dysfunction and decreased mitochondrial mass specifically in the brain. FNZ decreased mitochondrial content in both neurons and astrocytes, without affecting the number of nigral dopaminergic neurons. In human neural progenitor cells, FNZ also induced mitochondrial depletion. Mechanistically, independent of ATG5- or RAB9-mediated mitophagy, mitochondria were engulfed by lysosomes, followed by a vesicle-associated membrane protein 2– and syntaxin-4–dependent extracellular secretion. A genome-wide CRISPR knockout screen identified genes required for FNZ-induced mitochondrial elimination. We further found that following FNZ treatment, the expression of BAX was upregulated. BAX also localizes to both mitochondria and lysosomes. Knocking out BAX inhibited mitochondrial clearance and exocytosis, while overexpressing it accelerated the process. This suggests that BAX may serve as a critical link between mitochondria and lysosomes in mitolysosome formation. Future investigations will focus on the effects of ectopic BAX expression in mitochondria and lysosomes on mitolysosome formation. These results reveal not only a previously unidentified lysosome-associated exocytosis process of mitochondrial quality control that may participate in the FNZ-induced parkinsonism but also a drug-based method for generating mitochondria-depleted mammal cells.

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Mitophagy in neurons: Regulating PINK1's biology through insulin and AMPK signaling in concert with three organelles

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Neurons face exceptional challenges in maintaining mitochondria throughout neurites due to their highly extended and complex structures. The vast majority of mitochondrial proteins is encoded in the nucleus and synthesized in the cytosol before being imported into mitochondria. To ensure a constant supply of fresh mitochondrial proteins in every part of the neuron, short-lived proteins need to be locally translated in neurites since transport from the soma would exceed their lifetime. The short-lived protein PTEN-induced kinase 1 (PINK1) acts as a sensor for mitochondrial damage and is an important player in the removal of defective mitochondria via mitophagy. Evidence from our lab shows that *Pink1* mRNA is co-transported with mitochondria along neurites to ensure a constant supply of fresh PINK1 protein in distal parts of the neuron. The mitochondrial outer membrane protein Synaptojanin 2 binding protein (SYNJ2BP) and Synaptojanin 2a (SYNJ2a), which contains an RNA-binding domain, are involved in tethering *Pink1* mRNA to mitochondria. However, it still remains to be elucidated how mRNA transport as well as subsequent protein translation and function are regulated in response to local stimuli in neurons. In this study, we report that inhibition of the AMP-activated protein kinase (AMPK) by activation of the insulin signaling cascade prevents *Pink1* mRNA binding to mitochondria. Interestingly, loss of mitochondrial *Pink1* mRNA association upon AMPK inhibition increases PINK1 translation at mitochondria-endolysosomal-endoplasmic reticulum (ER) contact sites. While the endolysosomes supply the amino acids required for protein translation, the ER may support the PINK1 protein to reach its mitochondrial destination through the ER-SURF pathway. Mechanistically, AMPK phosphorylates the mitochondrial anchor protein SYNJ2BP, which is necessary for its interaction with the RNA-binding protein SYNJ2a. Consequently, insulin-mediated AMPK inhibition is required for PINK1 protein activation and its function as a ubiquitin kinase in the mitophagy pathway in neurons. Induction of insulin resistance *in vitro* using the key genetic Alzheimer's disease risk factor apolipoprotein E4 retains *Pink1* mRNA at mitochondria and prevents proper PINK1 function in neurons. Taken together, our findings reveal a metabolic switch controlling *Pink1* mRNA localization, PINK1 translation, and function via

insulin and AMPK signaling through the coordinated actions of three organelles. We furthermore propose a mechanistic connection between insulin resistance and mitochondrial dysfunction.

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Mito-Q supplementation reverses sarcopenic obesity in aged mice by restoring mitochondrial function and enhancing antioxidant response

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Sarcopenic obesity is a highly prevalent disease with poor survival and ineffective medical interventions. Mitochondrial dysfunction is purported to be central to the pathogenesis of sarcopenic obesity by impairing both organelle biogenesis and quality control. Obesity and aging increase mitochondria-derived reactive oxygen species (mtROS) that contribute to sarcopenic obesity, but the mechanisms underlying the development of sarcopenic obesity are incompletely understood. **Purpose**: To examine whether a mitochondria-specific antioxidant named mitoquinone mesylate (Mito-Q) improves mitochondrial function and restores muscle quality during sarcopenic obesity. **Methods**: 76-week-old male C57BL/6J mice were randomized by body weight to 12 weeks of low-fat (LFD) or high-fat diet (HFD) treatment. Following 4 weeks of diet, animals under HFD were further randomized based on body weight to Mito-Q (400 μM in tap water) or vehicle (400 μM of triphenylphosphonium cation (dTPP) in tap water) for 8 weeks. Body composition (NMR), food and water intake, and muscle function (grip strength) were determined at 0, 4, and 12 weeks. At 12 weeks, skeletal muscle was harvested and evaluated for mitochondrial function (high-resolution respirometry). **Results**: Mito-Q decreased body weight and fat mass in HFD mice (~27% and 39% reduction, respectively, vs. HFD + dTPP, $P<0.001$). Mito-Q attenuated the loss of lean mass in aged HFD mice (~70% increase vs. HFD + dTPP, $P<0.0001$). Conversely, Mito-Q restored grip strength (~91% increase vs. HFD + dTPP, $P<0.0001$) and attenuated the reduction in exercise capacity of aged HFD mice (~76% increase vs. HFD + dTPP, $P<0.0001$). Interestingly, Mito-Q reduced the frailty in aged HFD mice (~76% reduction vs. HFD + dTPP, $P<0.0001$).

Improvements in physical function and frailty in aged HFD + Mito-Q mice were mediated by restoration of NADH- and succinate-linked OXPHOS ($P<0.0001$ vs. HFD + dTPP) and greater reduced coenzyme Q9 (CoQ_9H_2) ($P<0.0001$ vs. HFD + dTPP). **Conclusions**: Mito-Q, a mitochondria-specific antioxidant, may mitigate the age-related decline in muscle mass and function by cellular bioenergetic adaptations that confer protection against sarcopenia through restoring defects in OXPHOS and antioxidant response.

Molecular architecture and dynamics of endogenous DRP1 revealed by super-resolution microscopy

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Mitochondrial fission is a highly regulated, dynamic process which is required to maintain the structural and functional integrity of the mitochondrial network. Its regulation involves mitochondrial membrane-associated adaptors, organelle contact sites, cytoskeletal rearrangements, and recruitment and oligomerization of the dynamin-like GTPase dynamin related protein 1 (DRP1). Although DRP1 is critical for the execution of mitochondrial fission, the regulation of its recruitment and the mechanism of the fission process itself are not well understood.

Here we apply super-resolution imaging of endogenous DRP1 to unravel its dynamics and structural rearrangements leading to mitochondrial fission. For this purpose, we successfully generated a U2OS cell line where DRP1 is endogenously tagged with mEGFP and HALO-tag using CRISPR/Cas9 technology. We utilize 2D and 3D stimulated emission depletion (STED) microscopy and stochastic optical reconstruction microscopy (STORM) to visualise the nanoscale structural organization of macromolecular DRP1 assemblies at mitochondria. Furthermore, we examine the dynamic reorganization of DRP1 oligomers at high spatial and temporal resolution by live-cell 2D/3D-structured illumination microscopy. We found evidence for a novel mechanism in which DRP1, besides its oligomerization at potential fission sites, is in dynamic equilibrium on mitochondria in a fission-independent manner.

We developed a novel analysis approach combining particle tracking analysis with a custom tracking algorithm that allows us to track the dynamics of DRP1 in relation to the mitochondrial network. For this, we associate single-particle tracking of DRP1 with a tubular model of the mitochondria to extract DRP1 trajectories corrected for the mitochondrial movement. Classifying the extracted trajectories, we found that DRP1 moves in different modes along the mitochondria, ranging from spatially confined oscillations to axial and longitudinal movement in what appears to be a directed manner.

These data, in combination with the nanoscale structural information of mitochondrial DRP1 complexes, will shed light on the role of the dynamic movement and the structural determinants modulating DRP1-mediated mitochondrial fission.

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mtDNA Encoded Non-coding RNA, mito-ncR-LDL805, Shapes an Adaptive Stress Response in Stem Cells of Lung Parenchyma

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Alveolar Epithelial Type 2 cells (AEC2) are lung parenchyma progenitor cells with multiple functions including progenitor maintenance of parenchyma homeostasis in normal and injured lungs. Molecular mechanisms that protect AEC2s from injury-associated death and enable them to repopulate damaged lung alveoli are poorly understood. We recently identified a novel cellular pathway that regulates mitochondrial function and energy production in these cells. The pathway involves the translocation of mito-ncR-LDL805 (mitochondrial non-coding RNA Light stand D-Loop 805), a small ncRNA transcribed by the mitochondrial genome, to the nucleus. Its presence in the nucleus correlates with an increase in expression of nuclear genes that encode regulators of mitochondrial function, and energy metabolism. Mito-ncR-LDL805 is highly conserved among mammals, suggesting an important function in the regulation of the expression of nuclear genes encoding regulators of mitochondrial function.

The pathway is specifically enhanced in AEC2 in response to the stress of cigarette smoke exposure, and confers protection against stress-induced death, while its depletion makes cells more susceptible to stress. Here we present data demonstrating that targeted nuclear enhancement of mito-ncR-LDL805 via a vector that mediates nuclear retention of this ncRNA (Sno-vector system) or via nucleus-targeted nanoparticles causes massive transcriptomic reprogramming. This reprogramming is characterized by the upregulation of multiple electron transport chain subunits, Krebs's cycle limiting step enzymes Idh1 and Idh2, upregulation of cholesterol and fatty acid metabolism, PiP3K, AKT1, and extracellular matrix remodeling transcripts. Transcriptomic changes are accompanied by an increase in corresponding metabolites and an increase in oxidative phosphorylation. We therefore posit that an increase in nuclear mito-ncR-LDL805 levels, is a prosurvival homeostatic mechanism, ensuring that mitochondrial energy metabolism is maintained in lung parenchyma stem cells to enable them to respond to injury and stress. Loss of AEC2 regeneration and exhaustion of their self-renewal capacity are factors that tip the balance toward the development of Chronic Obstructive Pulmonary Disease (COPD). Accordingly, we found that levels of mito-ncR-LDL805 are diminished in AEC2s in mice and in people who smoke with COPD. We propose that the failure to regenerate and thus exhaustion of these renewable cells is attributable to mitochondrial damage and energetic restriction.

Lastly, our findings show that nuclear delivery of mito-ncR-LDL805 *in vivo* is sufficient to induce mitochondrial bioenergetic responses. Thus, nuclear supplementation with mito-ncR-LDL805 is a potential strategy to enhance mitochondrial bioenergetics and delay AEC2 exhaustion, suggesting that the mitochondrial noncoding RNA can serve as a potential therapeutic target to restore mitochondrial function and halt the disease.

Multiomic profiling reveals a critical role for triacylgycerol mobilization in surmounting mitochondrial dysfunction

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Mitochondria are epicenters of metabolism with diverse activities that extend far beyond the generation of ATP. Given the positioning of mitochondria at the nexus of critical cellular processes, even moderate disruptions to their function can contribute to a variety of common pathologies. Cells have developed response pathways to stressors which reroute metabolism and buffer mitochondrial activity. Previous efforts to characterize response pathways to mitochondrial dysfunction have often focused on the response to insurmountable stressors. Furthermore, many studies have focused primarily on the role of protein effectors, marginalizing the contributions of metabolites and lipids to these pathways. Here, we leveraged the power of high-throughput mass spectrometry-based multiomic profiling to address these knowledge gaps. To this end, we developed a novel extraction method that allowed us to measure proteins, lipids, and metabolites from a single sample, thus minimizing technical variability and enhancing our ability to link protein function to fluctuations in lipid and metabolite abundances. Using this novel profiling method, we performed a screen in yeast using a variety of chemical and genetic perturbations causing moderate to complete mitochondrial dysfunction. We uncovered a unique molecular signature common to strains with moderate dysfunction, where yeast exhibit a widespread loss of mitochondrial proteins with a concomitant increase in the mobilization of stored triacylglycerols (TAGs). Over time, these strains adapted to mitochondrial dysfunction by increasing their mitochondrial content, thus resuming growth to near wild-type rates. This response was dependent on the mobilization of TAGs but independent of fatty acid oxidation, with TAG-derived donor acyl-groups shuttling into nascent cardiolipin biosynthesis. Correlation analysis linked the partially uncharacterized perilipin Pln1 to this TAG phenomenon. We observed that overexpression of Pln1 prevented the lipolysis of TAGs required for mitochondrial recovery. We found that this recovery mechanism was conserved in mammalian cells, and that the inhibition of TAG mobilization by knocking out the lipase *ATGL* resulted in increased sensitivity to mitochondrial stress. Collectively, our work suggests a model in which moderate mitochondrial dysfunction is surmounted by the biogenesis of new organelles, in part through the mobilization of TAG stores. This study expands our understanding of a fundamental biological stress response pathway, and potentially provides a novel approach for sensitizing cells to moderate mitochondrial dysfunction often observed in pathogenic conditions including cancer.

Nicotine exposure triggers mitochondrial damage in renal glomerular podocytes

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Introduction: Smoking and nicotine consumption through vaping are the leading causes of preventable death. Individuals with hypertension or kidney disease are more likely to exhibit renal pathologies associated with nicotine, such as glomerulopathy. In endothelial cells, nicotine was linked to mitochondrial oxidative stress and nitric oxide synthase (NOS) remodeling. However, there is a gap in knowledge regarding the effects of nicotine in glomerular epithelial cells, podocytes. We hypothesized that nicotine promotes nitrosative stress in the podocytes by producing peroxynitrite (ONOO-) and mitochondrial damage, thereby reducing glomerular function.

Methods: We performed confocal imaging on human cultured podocytes to detect $ONOO^-$ (HPF), Ca^{2+} (Fluo-8) and nitric oxide NO (DAF-FM) in response to nicotine. Seahorse assay (Agilent XFe24) was used to test mitochondrial respiration. Then, nicotine was chronically infused in Dahl SS rats (0.2 mg/kg/day s.c., 0.4% NaCl, 21 days), and blood pressure was recorded with telemetry. Glomerular damage was evaluated, and electron microscopy was employed to assess mitochondrial ultrastructure. Electron paramagnetic resonance (EPR) spectroscopy was used to access the NO levels in cultured podocytes and *in vivo*. OriginPro was used for statistical analysis.

Results: Acute appication of nicotine promoted intracellular Ca^{2+} and $ONOO^-$ transients in podocytes. The $ONOO^-$ response was blocked in the presence of SOD, indicating that $ONOO^-$ production requires superoxide. The application of specific α7 or α4β2, α2β4, α4β4, and α3β4 nicotinic acetylcholine receptor (nAChR) agonists elicited Ca^{2+} transients but did not produce $ONOO^-$, suggesting that nitrosative stress occurs independently from Ca^{2+} influx or nAChR activation. Incubation with nicotine (12 hrs) resulted in a decrease in podocytes' mitochondrial respiration (two-sample t-test, $p<0.05$). *In vivo*, nicotine infusion did not affect blood pressure but promoted mitochondrial damage in podocytes, which

exhibited swelling, loss of cristae, and mitophagy (t-test, $p<0.05$). Histopathological assessment showed higher glomerular damage in nicotine-exposed rats (t-test, $p<0.05$). Both EPR and confocal approaches demonstrated nicotine-mediated changes in NO bioavailability and an increase in NOS2 activity (t-test, $p<0.05$)

Conclusion: Nicotine elicits superoxide-mediated nitrosative stress and peroxynitrite formation in podocytes, promoting mitochondrial damage and glomerular dysfunction. Revealing the mechanisms of nicotine-mediated damage in the podocytes impacts our progress towards new treatments for smoking and vaping-associated renal pathologies.

O-GlcNAcTransferase (OGT) is a regulator of PINK1-dependent mitochondrial clearance

Kelly, Katie

UCL

Whilst the majority of PD cases are sporadic, much of our understanding of the pathophysiological basis of disease can be traced back to the study of rare, monogenic forms of disease. In the past decade, the availability of Genome-Wide Association Studies (GWAS) has facilitated a shift in focus, toward identifying common risk variants conferring increased risk of developing PD across the population. A recent mitophagy screening assay of GWAS candidates has functionally implicated the non-specific lethal (NSL) complex, a chromatin remodeler, in the regulation of PINK1-mitophagy. It has been suggested that the NSL complex may localise, at least partially, to the mitochondria. Indeed, our results from a weighted protein-protein interaction analysis (W-PPI-NA) have suggested that the NSL/PD relationship could be underpinned by both its nuclear and mitochondrial functionality. Here, we reveal O-GlcNAcTransferase (OGT), nutrient sensor and member of the NSL complex, to be the only member for which siRNA KD in the SH SY5Y (5Y) cell line increases pUb(Ser65) upon mitochondrial depolarization, a robust marker of mitophagy initiation, by both ICC and WB. RT-qPCR data suggests that OGT is a regulator of PINK1 mRNA expression in the 5Y cell line, which we suggest may underpin this regulation at the initiation stage. In addition, we have found OGT to regulate the latter stage of mitophagy, lysosomal- dependent clearance, harnessing two 5Y cell lines harboring pH-dependent fluorescent probes (Mt-Keima and Mt-SRAI). Experiments are

currently being carried out in a CRISPR-i3N-hiPSC cell model, to assess whether these phenotypes are maintained within a more relevant neuronal model.

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OPA1 mediates cardioprotection in a mouse model of pressure-overload-induced heart failure

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Background: Heart failure results from structural or functional impairment in cardiac function and its prevalence is increasing worldwide. Heart failure progression is characterized by alterations in mitochondrial function including irregular substrate metabolism, inefficient bioenergetics, reactive oxygen species overload and fragmented mitochondrial networks. Optic atrophy 1 (OPA1), an inner mitochondrial membrane protein that mediates mitochondrial fusion and supports oxidative metabolism, has been shown to have decreased expression in heart failure. Furthermore, impaired OPA1 function in cardiomyocytes leads to heart failure, while elevated OPA1 activity confers protection against heart failure.

Hypothesis: We hypothesized that OPA1 is cardioprotective during heart failure by maintaining oxidative metabolism, cristae structure and limiting ROS production in myocardial mitochondria.

Methods and Results: To elucidate how OPA1-mediated pathways may be cardioprotective, we employed a mouse model exhibiting a whole-body 1.5-fold increase in OPA1 (OPA1-OE). To induce heart failure, we used a modified, more physiological version of the transverse aortic constriction (TAC) surgery, whereby we constricted the descending aorta using a silk suture. This experimental model of left ventricular

hypertrophy and subsequent heart failure best resembles clinical heart failure progression. Both OPA1-OE and wildtype male and female TAC mice had significantly increased heart weights compared to their respective genotype sham mice. Cardiac function pre- and post-TAC was assessed using echocardiography. OPA1-overexpression protected against reduced cardiac function after TAC in female mice, as shown by sustained ejection fraction 12 weeks post-TAC at 43.37±8.60% in OPA1-OE mice compared to 34.62±4.90% in wildtype mice. Maximal respiration was assessed in the presence of fatty acids, complex I, and complex I+II substrates, where we observed no changes between sham or TAC mice in both genotypes, indicative of sustained oxidative function in our descending TAC model. Similarly, no changes in mitochondrial content were observed between WT or TAC mice in both genotypes, as measured by mitochondrial DNA content, citrate synthase activity and complex IV immunoblotting.

Conclusions: Overall, our data support the conclusion that OPA1 overexpression is protective against TAC-induced heart failure. Ongoing work aims to further characterize the functional protection observed using 4D echocardiography strain analysis. Additional molecular work will identify mechanisms by which OPA1 mediates this cardio protection including the maintenance of cristae structure and mitochondrial supercomplex assembly. Collectively, this work investigates the importance of mitochondrial function in the context of heart failure and whether targeting OPA1 or other mitochondria structural regulators is a relevant therapeutic avenue for treating heart failure.

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Outer Membrane Lipid Composition Directly Regulates Drp1-Dependent Mitochondrial Fission

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Mitochondria exist as dynamic networks that continuously undergo coordinated cycles of fusion and fission, which are critical for balancing essential cellular functions related to metabolism, signaling, and organellar quality control. While much is known about the protein assemblies that regulate mitochondrial fusion and fission events, the influence of specific membrane lipids on mitochondrial remodeling processes remain poorly understood. To address these shortcomings and more directly evaluate the effects of alterations to the local lipid composition on mitochondrial dynamics, we capitalized on newly-established methods that use induced-proximity of engineered lipid-modifying enzymes to selectively remodel the phospholipid composition of the outer mitochondrial membrane (OMM). Our approach used a modified bacterial phospholipase C (*Bacillus cereus* (*Bc*)PI-PLC) to initiate the rapid hydrolysis of resident phosphatidylinositol (PI) and locally generate diacylglycerol (DAG) within the cytosolic leaflet of the OMM. We complemented these unique live-cell approaches that directly alter the OMM lipid composition with a powerful *in vitro* reconstitution system consisting of supported membrane templates that are prepared to create distinctive topologies, including planar bilayer islands, variably curved membrane tubes, and even OMM-specific organellar mimics with membrane-anchored adaptor proteins. Our collective results demonstrate that acute production of DAG within the OMM causes dramatic fragmentation of the mitochondrial network, and the mechanistic basis for this phenomenon lies in the ability of DAG to directly influence the molecular activities of important membrane-shaping proteins that ultimately promote mitochondrial division. Specifically, we show that the presence of DAG can greatly potentiate the membrane remodeling functions of the essential GTPase, dynamin-related protein 1 (Drp1), by both enhancing the oligomeric assembly of Drp1 on cardiolipin-containing membranes and accelerating the mechanochemical processes that underly the Drp1-mediated membrane fission reaction. In parallel, production of DAG within the OMM also facilitates the local recruitment and self-assembly of other highly conserved families of curvature-sensitive effectors, including specific isoforms of Bin/Amphiphysin/Rvs (BAR) domain-containing proteins. We propose that the coordinate actions of these unique OMM-targeted membrane-shaping proteins, which can be regulated in concert by local increases to membrane DAG content, ultimately facilitate the rapid deformation of planar membranes into increasingly narrow constrictions that are permissive to the completion of ordered OMM fission events. Overall, these studies not only reveal a profound influence of DAG on Drp1-catalyzed mitochondrial fission, but also demonstrate the direct influence of OMM lipid composition on the dynamic regulation of mitochondrial morphology. Given that alterations to mitochondrial remodeling are associated with several pathological conditions in humans, understanding the contributions of OMM lipid metabolism and

transport may offer insights into novel therapeutic strategies that can selectively modulate mitochondrial dynamics.

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Oxidized LDL accelerates cartilage destruction and inflammatory chondrocyte death in osteoarthritis by disrupting the TFEB-regulated autophagy-lysosome pathway

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Osteoarthritis (OA) involves cartilage degeneration, thereby causing inflammation and pain. Cardiovascular diseases, such as dyslipidemia, are risk factors for OA; however, the mechanism is unclear. We investigated the effect of dyslipidemia on the development of OA. Treatment of cartilage cells with low-density lipoprotein (LDL) enhanced abnormal autophagy but suppressed normal autophagy and reduced the activity of TFEB, which is important for the function of lysosomes. Treatment of LDL-exposed chondrocytes with rapamycin, which activates TFEB, restored normal autophagy. Also, LDL enhanced the inflammatory death of chondrocytes, an effect reversed by rapamycin. In an animal

model of hyperlipidemia-associated OA, dyslipidemia accelerated the development of OA, an effect reversed by treatment with a statin, an anti-dyslipidemia drug, or rapamycin, which activates TFEB. Dyslipidemia reduced the autophagic flux and induced necroptosis in the synovial tissue of patients with OA. The levels of triglycerides, LDL, and total cholesterol were increased in patients with OA compared to those without OA. The C-reactive protein (CRP) level of patients with dyslipidemia was higher than that of those without dyslipidemia after total knee replacement arthroplasty (TKRA). In conclusion, oxLDL, an important risk factor of dyslipidemia, inhibited the activity of TFEB and reduced the autophagic flux, thereby inducing necroptosis in chondrocytes.

Keywords: Osteoarthritis, oxidized LDL, Dyslipidemia, Autophagy, Necroptosis

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Perm1 mechanisms and function in skeletal muscle mitochondrial oxidative metabolism

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Mitochondria are pivotal cellular organelles that are responsible for generating ATP, the energy currency of the cell; they also play critical roles in amino acid metabolism, lipid synthesis, and various signaling pathways. While present in all cells, mitochondria show remarkable cell type specialization, adapting to the specific needs and function of their host cell. These adaptations include changes in volume, shape, structure, proteome, subcellular localization, and interactions with other organelles. In skeletal muscle, mitochondria are specialized in multiple ways: they have an impressive dynamic range of respiratory rates; they show plasticity by increasing or decreasing their content in response to exercise or inactivity; and they localize at specific sites within the intricate structure of skeletal muscle. Subsarcolemmal (SS) mitochondria are globular and responsible for providing ATP needed for active transport. Intermyofibrillar (IMF) mitochondria are elongated and form an extensive reticular network organized along the Z line and sarcoplasmic reticulum. Past studies have elucidated signaling pathways (e.g., Ca^{2+} signaling) and transcriptional regulators (e.g., PGC-1α) that drive the plasticity seen in response to exercise. Interestingly, most of these pathways and regulators are present

in many cell types. Few muscle-specific regulators of mitochondrial oxidative function have been identified. PGC-1/ERR Regulator in skeletal Muscle 1 **(**Perm1) has been described by our lab to be a cardiac-and skeletal muscle-specific regulator that enhances oxidative capacity and contributes to mitochondrial adaptations in response to training. Strikingly, Perm1 expression is absent in many other tissues with high mitochondrial content (e.g., liver or kidney), suggesting a role specific to striated muscle. While past studies have defined the impact of Perm1 on mitochondrial biogenesis and respiratory capacity, the molecular mechanism by which Perm1 exerts such effects is largely unknown. To gain insights into the underlying mechanism we have performed proteomics to identify Perm1-interacting proteins and found two major types of interacting proteins: members of the SAM/MICOS complex and the adapter ankyrin proteins AnkR, AnkB and AnkG. As shown in a recent study by others, we also find Perm1 co-localizing and physically interacting with mitochondria. To define the role of Perm1 and Perm1 interactions in skeletal muscle function we have generated two mouse models: one lacking the entire Perm1 gene (KO) and another carrying a Perm1 variant that codes for a short Perm1 protein that lacks the ankyrin interaction domain ($Perm1^{Ank\Delta}$). Succinate dehydrogenase staining and transmission electron microscopy of muscle sections of Perm1 KO mice show defects in mitochondrial distribution, with decreases in the SS mitochondrial content. These structural defects are accompanied by decreased exercise fitness and altered substrate metabolism during exercise. Ongoing studies address the extent to which these defects rely on the ankyrin interaction domain of Perm1, and specifically test the hypothesis that Ank-Perm1-Samm50 interactions support skeletal muscle mitochondrial oxidative function by impacting proper spatial organization and/or mitochondrial interaction with other cellular structures.

Phosphoenolpyruvate controls oxidative phosphorylation through mitochondrial ADP Privation

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In pancreatic beta-cells glucose is metabolized to raise the ATP/ADP ratio to trigger proportionate insulin release. Since the discovery of the K_{ATP} channel, oxidative phosphorylation (OxPhos) has been assigned the task of raising ATP/ADP in response to changes in substrate supply. This is contrary to the known regulation of OxPhos in all other mitochondria by the ATP/ADP and not substrate availability. We rule out the canonical model with mitochondrial leak titrations in human islets by showing that substrate does not limit OxPhos. Furthermore, respiratory increases are driven by ATP hydrolysis and increases in mitochondrial leak but are not from pyruvate-driven OxPhos. Human genetics has suggested another possible explanation, namely that phospho*enol*pyruvate (PEP) controls the ATP/ADP ratio. Here we show that physiologic concentrations of PEP have more favorable bioenergetics that inhibits OxPhos through the process known as ADP privation. This is not a direct effect of PEP but requires hydrolysis by pyruvate kinase (PK) but not further pyruvate metabolism. ADP privation is independent of mitochondrial substrate/complex, requires ADP clearance, and is controlled by physiologic and pharmacologic regulators of PK. The PEP cycle works similar to a step-up transformer, supercharging PEP to amplify the ability of PK to raise the ATP/ADP. In beta-cells, extensive mitochondrial leak, cataplerotic efflux, glutamate anaplerosis, and the PEP cycle all contribute to enhancing PK flux at the expense of OxPhos. This PK-mediated mitochondrial ADP privation is generalizable to other primary tissues and cancer cell lines and can toggle mitochondria from regenerating ATP via OxPhos into a hyperpolarized organelle supporting higher energy requiring synthetic processes. ***Conclusion:*** Glucose stimulates the generation of the supercharged metabolite phospho*enol*pyruvate that (through pyruvate kinase) raises the ATP/ADP ratio that (through ADP privation) inhibits OxPhos and closes K_{ATP} to trigger insulin secretion.

Photodynamic Priming Modulates Cellular ATP Levels to Overcome P-glycoprotein-Mediated Chemoresistance in Triple Negative Breast Cancer

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P-glycoprotein (P-gp, ABCB1) is a well-researched ATP-binding cassette (ABC) drug efflux transporter linked to the development of multidrug resistance (MDR) in cancer. Despite extensive studies, therapies to inhibit P-gp in clinical settings have largely failed due to high toxicity and low selectivity, necessitating alternative strategies beyond conventional inhibitors or antibodies to reverse P-gp-mediated MDR. Previous studies have shown that mitochondrial ATP fuels ABC transporter-mediated drug efflux in chemoresistant cells. ATP is critical in the function of P-gp-mediated chemoresistance because ATP binding and hydrolysis cause a series of conformational changes in P-gp to allow for drug efflux. Photodynamic priming (PDP) provides an innovative strategy, harnessing sub-therapeutic photochemistry to induce sub-lethal effects in cancer cells. Utilizing the photosensitizer benzoporphyrin derivative (BPD) with photochemistry has demonstrated its capability to depolarize the mitochondrial membrane potential. We hypothesized that use of BPD-PDP will provide alternative approach to indirectly disrupt the function of P-gp by influencing ATP production. This study demonstrates that BPD-PDP can inhibit P-gp function by modulating cellular respiration and ATP levels. Using chemoresistant (VBL-MDA-MB-231) and chemosensitive (MDA-MB-231) triple negative breast cancer cell lines, we showed that PDP decreases mitochondrial membrane potential by 50.4% and reduces mitochondrial ATP production rates by ~95%. Flow cytometry study showed PDP can effectively improve the retention of fluorescent P-gp substrates (calcein-AM, rhodamine 123) by more than 2-fold in chemoresistant VBL-MDA-MB-231 without altering the cell surface expression of P-gp. These findings indicate that PDP can reduce cellular ATP that is required for the function of P-gp and improve intracellular substrate retention. These results make a strong case for further investigation of PDP's clinical utility as an alternative inhibition strategy for overcoming P-gp-mediated MDR.

PINK1 and Parkin regulate IP3R-mediated ER calcium release

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Although defects in intracellular calcium homeostasis are known to play a role in the pathogenesis of Parkinson's disease (PD), the underlying molecular mechanisms remain unclear. Here, we show that loss of PTEN-induced kinase 1 (PINK1) and Parkin leads to dysregulation of inositol 1,4,5-trisphosphate receptor (IP_3R) activity, robustly increasing ER calcium release. In addition, we identify that CDGSH iron sulfur domain 1 (CISD1, also known as mitoNEET) functions downstream of Parkin to directly control IP_3R. Both genetic and pharmacologic suppression of CISD1 and its *Drosophila* homolog CISD (also known as Dosmit) restore the increased ER calcium release in PINK1 and Parkin null mammalian cells and flies, respectively, demonstrating the evolutionarily conserved regulatory mechanism of intracellular calcium homeostasis by the PINK1-Parkin pathway. More importantly, suppression of CISD in PINK1 and Parkin null flies rescues PD-related phenotypes including defective locomotor activity and dopaminergic neuronal degeneration. Based on these data, we propose that the regulation of ER calcium release by PINK1 and Parkin through CISD1 and IP_3R is a feasible target for treating PD pathogenesis.

PPTC7 maintains mitochondrial protein content by suppressing receptor-mediated mitophagy

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PPTC7 is a resident mitochondrial phosphatase essential for maintaining proper mitochondrial content and function. Newborn mice lacking *Pptc7* exhibit aberrant mitochondrial protein phosphorylation, suffer from a range of metabolic defects, and fail to survive beyond one day after birth. Using an inducible knockout model, we reveal that loss of *Pptc7* in adult mice causes marked reduction in mitochondrial mass and metabolic rewiring with elevated hepatic triglyceride accumulation. *Pptc7* knockout animals exhibit increased expression of the mitophagy receptors BNIP3 and NIX, and *Pptc7*$^{-/-}$ mouse embryonic fibroblasts (MEFs) display a major increase in mitophagy that is reversed upon deletion of these receptors. Our phosphoproteomics analyses reveal a common set of elevated phosphosites between perinatal tissues, adult liver, and MEFs, including

multiple sites on BNIP3 and NIX. Follow up studies demonstrate that PPTC7 can directly interact with and dephosphorylate these mitophagy receptors, and that loss of PPTC7 significantly decreases BNIP3 and NIX turnover rates – likely by diminishing proteasomal degradation. These data suggest that *Pptc7* deletion causes mitochondrial dysfunction via dysregulation of several metabolic pathways and that PPTC7 may directly regulate mitophagy receptor stability. Overall, our work reveals a significant role for PPTC7 in the mitophagic response and furthers the growing notion that management of mitochondrial protein phosphorylation is essential for ensuring proper organelle content and function.

Quality control mechanisms in mitochondrial translation

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The process of protein synthesis is prone to errors, which can lead to the production of non-functional or even harmful proteins and the accumulation of stalled ribosomes. To prevent these, all organisms developed sophisticated quality control mechanisms that monitor and correct translation errors. Interestingly, while bacteria rely on trans-translation as their primary rescue pathway, this mechanism is notably absent in mitochondria. Yet, four members of the mitochondrial release factor family (RF1a, RF1, C12ORF62 and ICT1) have been identified, which not only show homology to the bacterial canonical release factor RF1 but also to the bacterial backup rescue system. It has been suggested that C12ORF62 is involved in the mitoribosome rescue (Desai et al., 2020), however, its mechanism of action is still largely unknown. We have recently shown that RF1a is a translation termination factor responsible for the recognition of non-canonical stop codons AGA and AGG in the mammalian mitochondria (Krüger et al., 2023). Through RNA sequencing-assisted ribosome profiling experiments, we have demonstrated that the absence of mtRF1 leads to both the accumulation of mitoribosomes upstream of non-canonical stop codons and partial readthrough of these affected codons. In the follow-up study, we focus on understanding the ribosome-associated rescue pathways that are activated upon ribosome stalling and readthrough. We have extended our analyses to cellular knockout models of both translation

termination factors, mtRF1 and mtRF1a, revealing remarkable transcript-specific differences in the fate of the affected mRNAs and downstream processes. Candidate genes that are responsible for the degradation of the stalled complexes are currently investigated and will be discussed.

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Quantitative and structural analysis of BAK apoptotic pore formation

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BAX and BAK are key apoptosis regulators that mediate the decisive step of mitochondrial outer membrane permeabilization (MOMP). During apoptosis, BAX and BAK oligomerize at the mitochondrial outer membrane to form discrete macromolecular assemblies termed apoptotic foci which leads to apoptotic pore formation and downstream cell death execution. However, the mechanism by which they assemble the apoptotic pore remains obscure.

Here, we present a novel approach combining protein stoichiometry quantification with super-resolution fluorescence microscopy and correlative light-electron microscopy to study the mechanism of apoptotic pore formation mediated by BAK. For this purpose, we generated a U2OS cell line with BAK endogenously tagged with HALO-tag using CRISPR/Cas9 technology. This allows us to investigate the mechanism of BAK-mediated apoptotic pore formation in native conditions with endogenous protein expression levels.

Using protein stoichiometry determination, we quantify the absolute number of BAK molecules present in individual apoptotic foci which we correlate with their nanoscale structural assembly visualized by stimulated emission depletion microscopy. In addition, we examine the integrity of the mitochondrial outer membrane and the rearrangement of the inner mitochondrial architecture using transmission electron microscopy.

This approach allows us to correlate BAK oligomerization with structural rearrangements of the mitochondrial membranes as well as with the downstream consequences for the mitochondrial integrity during apoptosis execution.

The results of this study will generate unprecedented insights into the molecular and biophysical mechanism of apoptotic pore formation at the nanoscale level and will contribute to solve the long-standing question of how MOMP is mediated during apoptosis.

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Reactive oxygen species from mitochondrial complex III are induced by NCLX to promote astrocytic STAT3-linked transcription and dementia-related pathology

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Mitochondrial reactive oxygen species (mROS), such as superoxide and hydrogen peroxide (H_2O_2), are implicated in diverse aging-associated neurological disorders. Mitochondrial complex III has the highest capacity for mROS production and generates mROS towards the cytosol, poising it to regulate intracellular signaling and disease mechanisms. However, the exact triggers of complex III-derived ROS (CIII-ROS) and its downstream molecular targets and functional roles remain unclear. Here, we investigated the drivers and consequences of CIII-ROS using site-selective mROS suppressors and genetic approaches together with live-cell imaging of sub-compartmental H_2O_2 dynamics, stoichiometric redox proteomics, transcriptomics, and mouse models of dementia-linked proteinopathy. We found that disease-related factors, including oligomeric amyloid-β and interleukin-1α, transiently increase astrocytic CIII-ROS levels and this effect is dependent on cation flux through NCLX, an astrocyte-enriched, mitochondrial sodium-calcium exchanger. CIII-ROS oxidized specific cysteines on astrocytic proteins and modulated gene expression linked to STAT3 and related immune pathways. In addition, CIII-ROS amplified STAT3 phosphorylation and nuclear translocation. Inhibition of other sites of ROS production, including mitochondrial

complex I or NADPH oxidase, had no effect on stimuli-induced astrocytic ROS or STAT3 signaling, demonstrating the specificity of CIII-ROS induction and modulation of STAT3 activities. Blockade of CIII-ROS in transgenic mouse models of dementia using a site-selective and brain-penetrant CIII-ROS suppressor reduced astrocytic alterations, neuropathology, and premature mortality. Together, our data suggest that disease-related stimuli induce mROS production in a cell type-selective and molecular site-specific manner, and that CIII-ROS promote precise post-translational redox modifications to amplify STAT3-linked signaling and gene expression. Our findings reveal CIII-ROS as an important node of mitochondrial-nuclear communication in pathogenic conditions, whereby mROS transients are converted into long-lasting changes in gene transcription and cell function. Therefore, CIII-ROS represents a novel therapeutic target for aging-associated neurological disorders.

Regulation of Mitochondrial Function by Orphan Protein Phosphatases

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Mitochondria are centers of metabolism and signaling whose function is essential to nearly all eukaryotic cells. Reversible phosphorylation is a widespread and underappreciated means of regulation within mitochondria, and its mismanagement could be an important underlying feature of mitochondrial pathophysiology. A handful of studies from our group and others have demonstrated that phosphorylation can alter the activities of proteins involved in OxPhos, the TCA cycle, mitochondrial protein import, heme biosynthesis, and ketogenesis, yet the vast majority remain uncharacterized.

Many mitochondrial proteins harbor dynamic phosphorylation sites that reproducibly change in abundance across biological states, yet appear unaffected by the loss of characterized phosphatases, suggesting they are managed by other phosphatases. To expand our investigations, we devised a large-scale screen to monitor the phosphorylation changes that accompany the independent depletion of 10 mitochondrial phosphatases using CRISPRi. Collectively, we identified 421 quantified

mitochondrial phosphosites on 206 proteins. Of these 421 sites, 262 change significantly across our dataset, and 125 of these can be linked to disruption of a single mitochondrial phosphatases.

The simultaneous analysis of many perturbations should enable the identification of "outliers" for a given perturbation (e.g., a phosphorylation site that is elevated only when a particular phosphatase is lost). This outlier analysis identified specific and dynamic phosphoregulation of the fatty oxidation enzymes ACADL and HADHA linked to a single matrix resident phosphatase.

The breadth of our phosphoproteomic profiling also allows for analyses based on the principle that correlated networks of phosphoproteins could be identified, thus generating new functional hypotheses into processes that are co-regulated by each phosphatase. A t-SNE analysis of all mitochondrial phosphosites identified a prominent cluster of 39 phosphosites from 20 individual proteins with similar regulatory profiles. Ontology analysis revealed a strong enrichment for proteins involved in cristae formation, which we discovered was linked to five members of the MItochondrial Contact site and cristae Organizing System (MICOS). Almost without exception, these sites were elevated following the silencing of a single inner mitochondrial membrane phosphatase. Preliminary results suggest that elevated MICOS phosphorylation correlated with increased mitochondrial branching and cristae density.

Through a comprehensive approach our work allows us to define connections between mitochondrial phosphatases and their substrates, establish a broad framework for understanding the role of this post translation modification in calibrating mitochondrial activities, and ultimately pave the way for a new therapeutic strategy to rectify mitochondrial dysfunction.

Rewiring of mitochondrial metabolism invokes subprograms of the interferon response to adapt host defenses

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Mitochondria have emerged as major players in intrinsic and adaptive immune defense against pathogens. Still, the mechanistic details of many of these mitochondrial activities including their regulation and how pathogens counteract these responses remain ill-defined. Recently, we identified viral-encoded micropeptides, which assemble into respiratory chain supercomplexes, that are derived from host proteins regulated by immune cues such as interferon. These data combined with newly discovered viral-encoded TCA cycle enzymes foreshadow unresolved roles for mitochondrial activities and immunometabolism in determining infection outcomes. To explore the impact of mitochondrial-dependent and mitochondrial-independent activities on viral replication, we infected cells primed with different interferons in the presence of macronutrients that favor either glycolysis (glucose) or OXPHOS (galactose). Using the model poxvirus - vaccinia - we find that human cells favoring glycolysis potently block viral replication by one hundred-fold when primed with interferon-gamma but not interferon-alpha or untreated. Notably, cells favoring OXPHOS showed no differences in poxvirus replication between untreated and interferon-primed. The glycolytic/interferon-gamma response is broad acting as it also markedly restricts the unrelated herpes simplex virus-1. We uncover that mitochondrial metabolic rewiring alters the combination of interferon-stimulated genes - in glucose and galactose conditions - at the protein but not RNA level in primary and transformed cells from different mammals. Mechanistically, macronutrients trigger selective protein degradation of some but not all induced interferon-stimulated gene products. Deletion of a single interferon-stimulated gene that is differentially stabilized in response to macronutrient cues is sufficient to rescue attenuated poxvirus replication. These data reveal unappreciated interferon-stimulated gene subprograms regulated by mitochondrial activities and conserved for nearly ~100 million years of evolution. In principle, these subprograms could rapidly adapt immune responses by sensing changing macronutrient levels consumed during viral replication and cell proliferation.

Role of MTCH2 in starvation-induced mitochondrial hyperfusion

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MTCH2 is a divergent member of the SLC25A family of mitochondrial solute carrier proteins localized in the mitochondrial outer membrane (MOM). Previous work has shown that MTCH2 is required for starvation-induced mitochondrial hyperfusion, a cytoprotective response to nutrient deprivation. Crosslinking immunoprecipitation analysis identified enzymes involved in *de novo* fatty acid synthesis, storage, and use as interactors of MTCH2. Pharmacological inhibition of enzymes phenocopied loss of MTCH2 in starvation induced mitochondrial hyperfusion, indicating a functional link between lipogenesis, MTCH2, and mitochondrial fusion. Consistently, lysophosphatidic acid stimulated mitochondrial fusion *in vitro* in a MTCH2-dependent manner. Alpha fold predictions of MTCH2 structure indicate that it possesses five transmembrane domains, in contrast to the six in SLC25A family members, which create a membrane-accessible groove. Molecular dynamics simulations from our group and others suggest that this groove in MTCH2 can facilitate lipid flippase activity and, recently, protein insertase activity for outer membrane alpha-helical transmembrane proteins. This raises the possibility that proteins that insert into the MTCH2 fenestration could regulate lipid flux in response to nutrient status. Proteomic analyses of MTCH2 KO cells indicate MTCH2 is required for the stability of ARMC1 and DNAJC11, two proteins associated with the MIB complex, containing mitochondria sorting and assembly machinery (SAM) complex and mitochondrial contact site and cristae organization system (MICOS). Crosslinking immuno-purification analysis of MTCH2 also identifies ARMC1 and DNAJC11, as well as components of MICOS, as interactors. Molecular modeling of ARMC1-DNAJC11-MTCH2 using AlphFold2 generated a high-confidence model in which the C-terminal tail of ARMC1 docks in the MTCH2 groove. Together, these data suggest a model in which under starvation ARMC1 interacts with MTCH2 to regulate lipid homeostasis to coordinate mitochondrial dynamics with nutrient stress.

Role of Plasmalogen Ether Phospholipids in Mitochondrial Membrane Dynamics and Aging

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Peroxisomes recently emerged as an important regulator of aging. Recent studies from our group and others reveal that peroxisomal protein import function is impaired in aged animals, leading to dysregulated lipid metabolism, elevated oxidative stress, increased inflammation, and altered mitochondrial morphology and function. To further understand how peroxisomes control aging, we focus on the interplay between peroxisomes and mitochondria, the two organelles known to work in coordination to regulate cellular and metabolic homeostasis. Through genetic screening, we find that among all peroxisomal functions, the plasmalogen ether phospholipid biosynthesis pathway plays a vital role in linking peroxisome to mitochondrial function and membrane dynamics. Lipidomic analysis reveals that plasmalogens are enriched in the mitochondria, and the levels of plasmalogens decrease with age. To further dissect the underlying mechanisms of plasmalogen-mediated inter-organelle communication, we have generated biotinylated plasmalogens and performed lipid-protein interaction analysis, through which we identify 307 novel plasmalogen-interacting proteins. About 33% of identified candidate proteins are localized to mitochondria, particularly those involved in mitochondrial respiration and membrane organization. Currently, we are carrying out functional analysis to investigate how plasmalogens interact with mitochondrial fission proteins (e.g., Drp1 and Mff) to control mitochondrial fission and why these interactions are dysregulated under aging. Taken together, our studies are expected to reveal an exciting role of peroxisome-derived ether phospholipids in peroxisome-mitochondria communication and mitochondrial dynamics during animal aging.

Role of the Mitochondrial Topoisomerase TOP1MT in Inflammation and Implications for Human Health

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Mitochondria are multifaceted organelles at the center of many essential cellular processes including ATP production, ROS signaling and inflammation. Mitochondria maintain their own genome (mtDNA), which encodes essential genes required for oxidative phosphorylation, and which can act as an immune stimulatory molecule.

TOP1MT is a mitochondrial topoisomerase that is important for regulating mtDNA. Though *Top1mt* $^{-/-}$ mice are viable, they are not healthy, and they have extreme cardiac sensitivity to the cancer drug doxorubicin. Recently, we found that loss of TOP1MT leads to release of mtDNA into the cytosol where it activates cGAS-STING innate immune signaling. Meanwhile, we also helped show that cGAS-STING inflammation contributes doxorubicin cardiotoxicity. These findings point to important physiological consequences of TOP1MT dysfunction in inflammation and doxorubicin cardiotoxicity that are relevant for human health. Indeed, we also identified a candidate pathogenic TOP1MT variant, P193L, in a family with autoimmune disease, which we linked functionally to mtDNA release and cGAS-STING activation. However, additional gene variants are needed to definitively link *TOP1MT* to autoimmune disease. To this end, we have identified multiple ultra rare *TOP1MT* variants among a small cohort of pediatric lupus patients that are candidates for functional analyses. We are generating stable cell lines expressing these variants, which will be characterized for mtDNA release and cGAS-STING activation. Functionally linking any of these TOP1MT variants to mtDNA/cGAS-STING activation will provide strong support that *TOP1MT* is novel autoimmune disease gene. Another important question is whether TOP1MT variants that activate cGAS-STING signaling also impact doxorubicin cardiotoxicity, a serious side effect that impacts ~1/3 of patients who take this drug. Intriguingly, we see that cardiomyocytes with reduced TOP1MT expression are more sensitive to doxorubicin. We have also developed cell assays to measure doxorubicin toxicity, which will allow us to test if TOP1MT variants that cause mtDNA release and cGAS-STING activation are more sensitive to doxorubicin. Determining a genetic basis for doxorubicin cardiotoxicity will lead to pharmacogenetic approaches for personalized medicine to prevent or reduce adverse effects of this commonly used and effective cancer drug. Collectively, functionally linking TOP1MT variants to mtDNA release and cGAS-STING innate immune signaling will have important health implications by aiding with the genetic diagnosis of autoimmune diseases and predicting the susceptibility to doxorubicin cardiotoxicity.

Sepsis induces long-term metabolic alterations and dysfunction in CD4 T cells

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Advances in critical care medicine have substantially increased the survival rate of patients diagnosed with sepsis. However, sepsis survivors are at high risk of infection-related rehospitalization and mortality for years following the acute septic event. This infection risk has been attributed to post-septic immunosuppression or immune dysfunction, which is observed both in human patients and animal models of sepsis. Multiple immune cell types demonstrate altered responses following sepsis, but our group focuses on T cells given their importance in the development of long-lasting protective immunity. The mechanisms that cause persistent changes in T cell immunity following sepsis are currently unknown. To study sepsis-induced long-term effects, we used a murine model of sepsis with ICU-like interventions that allow for the study of survivors. CD4 T cells were isolated from non-septic and post-sepsis (3-weeks) mice, metabolism and function were accessed in vitro and in vivo. In sepsis survivors. CD4 T cells present persistent alterations in function, such as, reduced proliferation, and are prone to produce IL-17 regardless of stimuli milieu. In the context of post-sepsis secondary infection, in response to Klebsiella pneumonia (Kp) infection, lung CD4 T cells from post-septic mice also produce higher levels of IL-17. Interestingly, these post-sepsis CD4 T cells fail to induce protective immune responses when transferred to Kp infected RAG2-/- mice, showing that sepsis induces cytokine dysregulation that can lead to profound functional defects. Moreover, after sepsis, CD4 T cells present persistent mitochondrial dysfunction which shift the metabolic program towards glycolysis, that become the main source of ATP. Electron microscopy showed striking morphological alterations (aberrant and empty mitochondria) and reduced mitochondrial numbers in sepsis survivors. In addition, there is a significant decrease in mitochondrial membrane potential (DYm) and increase in proton-leak, which indicates defects in the electron transportation chain (ETC) and ATP synthesis. Notably, dysregulated IL-17 production by post-septic CD4 T cells is dependent on increased glycolytic metabolism. Blocking glycolysis with 2-DG results in a significant reduction in the levels of IL-17. It has recently been demonstrated that the nonspecific lethal (NSL) complex regulates mitochondrial metabolism by controlling mitochondrial and genomic DNA expression. The catalytic subunit of the NSL complex is the chromatin-modifying acetyltransferase MOF (Males absent On the First), which acts by altering chromatin structure to increase gene transcription. We have observed reduced expression of several NSL family members in post-septic CD4 T cells, suggesting a link between epigenetics, mitochondrial metabolism and CD4 T cell function. Taken together, these

results show that sepsis induces long-lasting hyper glycolytic state in CD4+ T cells which supports dysregulated IL-17 production and dysfunctional response to secondary infections. Further studies will be carried to investingate if sepsis-induced NSL/MOF downregulation is linked to mitochondrial dysfunction and metabolic alterations.

Sex hormones-mediated differences in renal mitochondrial bioenergetics and metabolism

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Introduction. Females are generally protected from renal diseases until menopause, which could be partially explained by the effects of female sex hormones. Mitochondrial bioenergetics is crucially important for renal function, it is affected by sex hormones, and has been proposed among factors contributing to sexual dimorphisms in renal disease. Our previous studies identified sex differences in mitochondrial function in renal tissues; however, there remains a gap in knowledge regarding the impact of sex hormones on these dimorphisms. We hypothesize here that males and females have divergent renal mitochondrial bioenergetics and metabolic signatures, which are defined by sex hormones.

Methods. Male and female Sprague Dawley (SD) rats underwent a gonadectomy or a sham surgery before 4 weeks of age. At 6 months of age, kidney flush was performed, tissues were removed, and renal cortex was isolated and used for immediate ex vivo experiments, or snap frozen for future analyses. Mitochondria were isolated, and spectrofluorimetry and Seahorse assay were used to measure mitochondrial OCR, membrane potential and H_2O_2 production. Metabolic profiles of renal cortices were generated using UHPLC-HRMS, and metabolites were identified by retention time exact mass using MAVEN and MetaboAnalyst software. IPA was used to identify pathways of interest. OriginPro was used for statistical analysis.

Results. Male renal OCR parameters (basal, ADP-linked, maximal and spare capacity) were lower than OCR observed in females ($p<0.05$). Orchiectomy resulted in an increase in male OCR parameters ($p<0.05$); ovariectomy did not affect renal cortical OCR

(p>0.05). Spectrofluorimetry revealed that male gonadectomy increased ΔΨm up to the level of female values (p=0.4, sham females vs gonadectomized males), while ovariectomy had no effect on ΔΨm in females (p=0.3). Female cortical H_2O_2 production was higher than in males (p<0.001), an ovariectomy lowered H_2O_2 (p<0.001). In metabolomics, 174 mitochondria-related metabolites were identified. When male and female animals were compared, the sham group had differentially abundant 55 metabolites, with only 4 in gonadectomized animals. The top differential metabolite in sham animals was 5'methylthioadenosine (MTA), an intermediate in the methionine salvage pathway and a byproduct of polyamine metabolism. Males exhibited a 41.9 fold increase in renal MTA levels compared to females. After gonadectomy, MTA levels were found to be similar between males and females; importantly, major metabolic pathways that were altered in sham rats (the urea and methionine cycles and salvage, and polyamine biosynthesis) were similar in gonadectomized animals.

Conclusions. We report distinct mitochondrial bioenergetic and metabolic profiles in healthy SD male and female kidneys, which are dependent on sex hormones. The observed sex-related dissimilarities indicate that male and female renal mitochondria have unique metabolic challenges, and focus on different pathways to resolve them (such as polyamine metabolism in males), which potentially affects disease susceptibility later in life. Our data established the impact of sex hormones on mitochondrial bioenergetics and will help elucidate targets that can mitigate the sex-related differences in renal disease.

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Shared Structural Features of Miro Binding Control Mitochondrial Homeostasis

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Miro proteins are universally conserved mitochondrial calcium-binding GTPases that regulate a multitude of mitochondrial processes, including transport, clearance and lipid trafficking. The exact role of Miro in these functions is unclear but involves direct binding to a variety of client proteins. How this binding is operated at the molecular level and whether and how it is important for mitochondrial health, however, remains unknown. Here, using structural predictions alongside yeast two-hybrid and mitochondrial recruitment assays, we show that known Miro clients - namely, CENPF, Trak and MYO19 - all use a similar short motif to bind the same structural element: a highly conserved hydrophobic pocket in the first calcium-binding domain of Miro. Using these Miro-binding motifs, we identified direct interactors de novo, including MTFR1/2/1L, the lipid transporters Mdm34 and VPS13D, and the ubiquitin E3-ligase Parkin. Given the shared, and therefore competitive, binding mechanism of these functionally diverse clients and its conservation across eukaryotes, we propose that Miro is a universal mitochondrial adaptor coordinating mitochondrial health.

Silencing mitochondrial gene expression in living cells

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Mitochondrial proteins are predominantly encoded in the nucleus and post-translationally imported into the organelle through the TOM and TIM machineries. Yet, a small subset of the mitochondrial proteome is encoded by mitochondrial DNA. These 13 proteins are co-translationally exported across the inner membrane by OXA1L and assemble with newly imported proteins into membrane protein complexes of the oxidative phosphorylation system. In order to maintain mitochondrial function, the assembly of the oxidative phosphorylation system complexes from imported and

mitochondria-encoded subunits has to be tightly regulated to adapted to cellular requirements. Yet, our understanding of mitochondrial gene expression and proteostasis are limited due to the lack of appropriate techniques to modulate and interfere with gene expression in mitochondria. Our recent analyses has provide a new strategy to interfere with translation in purified mitochondria upon import of protein-morpholino chimera that target individual mRNA molecules. Here we report on the development of a new approach that enables us to block translation of specific mitochondrial mRNAs in living cells. This technique will enable us to address so far unresolved questions of mitochondrial biology.

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SIM2s Regulation of STING-Mediated Immunity During Mammary Epithelial Cell Differentiation

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There is increasing evidence that mitochondria have an underrecognized function as activators of innate immune pathways. Activation of the innate immune system necessitates an increase in cellular energy output as well as significant metabolic reprogramming, and thus, requires communication between mitochondria and innate immune pathways; however, the key mechanisms involved in the dialogue between mitochondria and the innate immune response during normal mammary gland development have yet to be elucidated The goal of this research is to advance understanding of the mechanism by which innate immune pathways functionally contribute to lactation. Single-minded 2s, a transcription factor of the bHLH-PAS family, has been shown to possess significant roles in the development of the mammary gland and is also proposed to be a funcdamental component in mediating stress responses during mammary epithelial cell (MEC) differentiation. Activation of specific innate immune pathways, such as the cGAS-STING pathway, is known to result in downstream production of type I interferons and proinflammatory cytokines. Due to high energetic demand, we hypothesize that lactation serves as a prime environment for a mitochondrial-dependent immune response. Using CIT3 mouse MECs as a model, we

have uncovered cGAS-independent STING expression throughout MEC differentiation, accompanied by increased gene expression of type I interferons and proinflammatory cytokines. This data was mirrored in vivo at lactation day 6 via IHC staining, as we observed STING expression in cGAS-/- mammary glands comparable to that of wildtype controls. Moreover, we observed a dramatic increase in STING expression at the onset of lactation, suggesting its importance during this highly metabolic time. Furthermore, we have demonstrated that STING is present at the mitochondria/mitochondria-associated membrane in CIT3 MECs at 24 hours of differentiation (hD), further highlighting the relevance of the mitochondria in modulating innate immunity during differentiation. We have also observed a decrease in STING expression in CIT3 SIM2-/- MECs at 24hD. This data was recapitulated in mammary gland-specific SIM2sfl/fl mice at lactation day 10. MEchanistically, we have evidence demonstrating that STING expression during MEC differentiation is in part due to mitochondrial-specific ROS (mtROS) as we observed a decrease in STING expression following treatment with the mitochondrial-specific ROS scavenger, Mitoquinol. These data are indicatie of an interesting dynamic between SIM2, STING and mtROS. Of significance, we have recently revealed that STINGgt/gt mice exhibit aberrant structural mammary development as observed via H&E staining at lactation day 10, suggesting a role of STING in normal mammary gland development. Together, out preliminary data suggests that SIM2s influences STING acitivity in differentiation of the mammary gland. We anticipate that continued efforts to elucidate the mechanism of the dialogue between SIM2, STING and the mitochondria will facilitate discover of novel biomarkers to mediate innate immunity and inflammation during lactation. Overall, this research is significant becasue it enhances knowledge of how innate immune pathways within MECs functionally contribute to normal mammary gland development.

Skeletal Muscle Mitophagy is Impaired in a Mouse Model of Diet-Induced Obesity

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Obesity and type 2 diabetes are associated with declines in skeletal muscle mitochondrial content and impaired bioenergetic function. Decreases in mitochondrial biogenesis and impaired cellular redox contribute to skeletal muscle mitochondrial dysfunction in obesity. Here, we investigated the role of mitophagy in maintaining skeletal muscle mitochondrial content and quality in obesity and type 2 diabetes using mito-QC reporter mice. The mito-QC reporter contains a mCherry-GFP tandem tag with an OMM targeting sequence of Fis1, which can be used to monitor mitophagy. Mito-QC mice were randomly allocated to receive either a standard chow diet (CD) or a high-fat diet (HFD, 45% kcal from fat) for 12 weeks. After 8 weeks of consuming the experimental diets, a subset of mice receiving the HFD were injected interperitoneally with streptozotocin to reduce functional β-cell mass (HFD-Stz), and intraperitoneal citrate injections were used as a control. Confocal imaging of *extensor digitorum longus* (EDL) single-fibres and semi-automated analyses of the mito-QC reporter demonstrated that mitophagy was markedly reduced in both HFD and HFD-Stz mice. Immunoblot analysis of autophagic flux using colchicine showed a trend for increased LC3II/I in HFD and HFD-STZ mice suggesting that autophagy may be elevated in skeletal muscle. High resolution respirometry conducted on saponin-permeabilized *tibialis anterior* fibres revealed that complex I + II OXPHOS was lower in both HF and HF-Stz mice, whereas FCCP-induced maximal respiration was only lower in HFD-Stz mice. Together, these results show impaired skeletal muscle mitophagy in diet-induced obesity and type 2 diabetes, which may contribute to mitochondrial dysfunction through compromised mitochondrial quality control mechanisms.

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Small heterodimer partner interacting leucine zipper protein (SMILE) ameliorates autoimmune arthritis by controlling autophagy via AMPK signaling pathway and the of B cell regulation

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Rheumatoid arthritis (RA) is an autoimmune disease that causes joint swelling and infammation and can involve the entire body. RA is characterized by the increase of pro-infammatory cytokines such as interleukin (IL) and tumor necrosis factor, and the over-activation of T lymphocytes and B lymphocytes, which may lead to severe chronic infammation of joints. However, despite numerous studies the pathogenesis and treatment of RA remain unresolved. This study investigated the use of small heterodimer partner-interacting leucine zipper protein (SMILE) overexpression to treat a mouse model of RA. SMILE is an insulin-inducible corepressor through adenosine monophosphate activated kinase (AMPK) signaling pathway. The injection of a SMILE overexpression vector to mice with collagen induced-arthritis resulted in a milder clinical pathology and a reduced incidence of arthritis, less joint tissue damage, and lower levels of Th17 cells and plasma B cells in the spleen. Immunohistochemistry of the joint tissue showed that SMILE decreased B-cell activating factor (BAFF) receptor (BAFF-R), mTOR, and STAT3 expression but increased AMPK expression. In SMILE-overexpressing transgenic mice with collagen antibody-induced arthritis (CAIA), a decrease in the arthritis score and reductions in tissue damage, the number of B cells, and antibody production were observed. The treatment of immune cells in vitro with curcumin, a known SMILE-inducing agent, led to decreases in plasma B cells, germinal center B cells, IL-17-producing B cells, and BAFF-R-positive B cells. Taken together, our findings demonstrate the therapeutic

potential of SMILE in RA, based on its inhibition of B cell activation mediated by the AMPK/ mTOR and STAT3 signaling pathway and BAFF-R expression.

Keywords : Small heterodimer partner-interacting leucine zipper protein (SMILE), Rheumatoid arthritis (RA), B cell, BAFF receptor, AMPK/mTOR.

SNX10 Regulates Mitochondria Homeostasis

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SNX10 is a member of the sorting nexin (SNX) protein family, having a PX domain that interacts with PI(3)P. SNX10 is linked to autosomal recessive osteopetrosis—a genetic disorder marked by heightened bone density and impaired osteoclast function. In the current study, we demonstrate that SNX10 localizes to early and late endocytic compartments in a PI(3)P-dependent manner and that it regulates endocytic trafficking. Intriguingly, we find that SNX10 positive vesicles interact dynamically with the mitochondrial network and that SNX10 interacts with mitochondrial proteins. Notably, these vesicles contain mitochondrial material, including COXIV and SAMM50, proteins essential for mitochondrial respiratory chain assembly. Depletion of SNX10 leads to reduced COX4 and SAMM50 levels in vitro and hampers mitochondrial respiration and reduces citrate synthase activity, indicating a role for SNX10 in piece-meal mitophagy for sustaining mitochondrial bioenergetics. Importantly, knockout of Snx10 homologues in zebrafish results in reduced Cox4, elevated cell death, and ROS levels, highlighting the relevance of SNX10 in mitochondrial homeostasis in vivo.

Specific OPA1 inhibitors enhance the apoptotic release of cytochrome c and cell death

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The GTPase activity of the dynamin-related mitochondrial protein Optic Atrophy 1 (OPA1) controls cristae remodeling, cytochrome c release and apoptosis. OPA1 is hence being increasingly identified as a vulnerability of cancer cells. From a high throughput screening of 10,000 drug-like compounds for OPA1 GTPase inhibition, we identified MYLS22 as the most promising hit. This compound is not mitochondriotoxic but increases cytochrome c release in response to proapoptotic stimuli. MYLS22 causes mitochondrial fragmentation, and cristae remodeling and it sensitises a panel of cancer cells to drug treatment. In vitro, MYLS22 binds recombinant OPA1 GTPase and did not inhibit recombinant Dynamin 1 GTPase activity. It does not display any additional effect over OPA1 deletion in cells, further substantiating its specificity. Structural activity relationship (SAR) analysis of the OPA1 inhibitor turned out into a series of MYLS22 derivatives with improved water solubility. Furthermore, three of these compounds exhibit significantly enhanced inhibitory effects on OPA1 GTPase activity *in vitro*, as well as mitochondrial fragmentation ability. In conclusion, MYLS22 and its derivatives are the first-in-kind specific OPA1 inhibitors that exert anti-cancer properties.

Structure and function of intramitochondrial junctions in primary human T cells

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Various heterotypic organelle-mitochondria membrane contacts sites have been described and investigated intensively to date. Homotypic mitochondria membrane contact sites are more rarely observed and have been described e.g., in muscle cells where they contribute to cristae alignment across contacting mitochondria. Early reports of mitochondria-mitochondria contact sites indicate a regular lattice-like birding pattern between neighboring mitochondria, so called intramitochondrial junctions (IMJs). IMJs have been described in various cell types under a variety of metabolic conditions. Despite being anecdotally observed over the past 70 years the identity and function of IMJs remains enigmatic. In primary human T cells such IMJs have been found to naturally occur in a subpopulation of T Cells where they show a length of around 8 nm and a periodicity of around 15 nm. T cell fate including memory cell formation is known be dependent on metabolic programming controlled by mitochondrial activity and dynamics. Here we use cryo-electron tomography (cryo-ET) and correlative light and electron microscopy (CLEM) to characterize the structure and function of IMJs directly within primary human T cells. We use subtomogram averaging to identify the individual constituents of IMJs and unveil their relative structural arrangement. We further apply Fluorescence-activated cell (FACS-) sorting and CLEM approaches to characterize IMJ formation across well-defined T cell memory populations.

Subcellular molecular profiling of dopaminergic mitochondria reveals proteomic adaptations to the axonal microenvironment

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Axons are complex neuronal compartments that perform energetically demanding presynaptic functions and often dwarf the size of the cell body they originate from. These features present formidable spatial and bioenergetic challenges that neurons must overcome. While recent findings indicate that axonal mitochondria exhibit morphological and functional specialization to meet these demands, the extent to which axonal mitochondria diverge from their somatodendritic counterparts at the protein level has remain largely uncharacterized. To address this question, we expressed a mitochondria-localized epitope tag (Mitotag) within ventral midbrain dopamine (DA) neurons. We then leveraged the anatomic separation of the axons from the cell body to immunopurify axonal and somatodendritic mitochondria and evaluate their proteomic differences. We find that axonal mitochondria exhibit a reduction in critical pathways for the synthesis of mitochondrial DNA (mtDNA)-encoded proteins, and an enrichment for proteins involved in DA metabolism, reactive oxygen species (ROS) detoxification, mitochondrial fission/fusion dynamics, and proteins linked to monogenic Parkinson's disease (PD). Consistent with our observations that axonal mitochondria may be less capable of new protein synthesis, we find that axonal mitochondria are strongly enriched for mitochondrial long-lived proteins (mtLLPs) and show reduced abundance of more labile non-mtLLPs. Taken together, the diminished protein synthesis and increased exposure to the stressors of the axonal environment may render DA axonal mitochondria vulnerable to progressive damage and defects in mitochondrial trafficking and quality control, implicating them as a nexus of early vulnerability in aging and neurodegeneration.

Targeting mitophagy to maximize the immunostimulatory effects of radiation therapy in ER+ breast cancer

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Radiation therapy (RT) has attracted attention as a tool to convert immunologically "cold" estrogen receptor-positive (ER+) breast cancer (BC) into "hot" lesions, as a consequence of apoptotic, BCL2 associated X, apoptosis regulator (BAX) and BCL2 antagonist/killer 1 (BAK1)-dependent mitochondrial outer membrane permeabilization (MOMP), mitochondrial DNA (mtDNA) release in the cytosol, and type I interferon (IFN) signaling, as demonstrated by the laboratory (Yamazaki et al., 2020). However, cytoprotective mechanism limits type I IFN secretion, for instance, inhibition of autophagy in mouse ER+ BC cells improves the type I IFN-dependent activation of antitumor immunity by RT *in vivo* (Yamazaki et al., 2020). Because autophagy is also essential for cancer cells undergoing immunogenic cell death to secrete ATP in support of immunostimulation, preferentially inhibiting mitophagy, a specific variant of autophagy that eliminates damaged mitochondria, instead of general autophagy should provide superior benefits on RT-driven antitumor responses. Using mouse mammary carcinoma cell lines EO771 and TS/A, we observed *in vitro* that irradiation drives mitochondrial depolarization and mitochondrial reactive oxygen species production, as well as the ability of irradiation to induce the removal of damaged mitochondria by mitophagy in a time and RT dose-dependent manner, as observed by flow cytometry and cells stably expressing the mitochondria-targeted pH sensitive mKeima protein. In addition, RT-induced mitophagy was limited by genetic deletion of essential autophagy genes *Atg5* or *Atg7*. We also observed that inducing MOMP with the anti-apoptotic protein BCL2 inhibitor Venetoclax (V) enhanced RT-driven mitochondrial depolarization, which consequently drastically increased type I IFN production and secretion. However, RT combined with V also increased degradation of permeabilized, damaged mitochondria via mitophagy, whereas BAX/BAK1^{DKO} clones failed to induce mitophagy after irradiation and did not respond to V anymore. Our observations suggest a key role of BAX/BAK1-

dependent MOMP in inducing type I IFN production after RT, but also in activating mitophagy, which ultimately reduces the number of damaged, mtDNA-releasing mitochondria, meaning that blocking mitophagy should increase RT-driven type I IFN production and secretion. We are currently investigating whether the key mitophagy regulators, PINK1 and PARKIN, are involved in RT-mediated mitophagy, in order next to generate mitophagy-deficient BC cells, and to evaluate their impact on tumor growth and immunological response to RT *in vivo*. To conclude, we are trying to elucidate for the first time the role for mitophagy as an immune checkpoint that suppresses anticancer immune responses driven by RT, potentially paving the way to the identification of novel approaches to break through therapy resistance in ER+ BC.

T-cell-restricted intracellular antigen-1 directs cellular senescence by regulating mitochondrial dynamics

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Mitochondrial homeostasis is critical for various cellular processes and mitochondrial dysfunction is involved in the pathophysiology of cells. Senescent cells exhibit a diverse spectrum of changes in their morphology, proliferative capacity, senescence-associated secretory phenotype (SASP) production, and mitochondrial homeostasis. These cells often manifest with elongated mitochondria, a hallmark of cellular senescence. However, the precise regulatory mechanisms orchestrating this phenomenon remain predominantly unexplored. In this study, we provide compelling evidence for decreases in T-cell-restricted intracellular antigent-1 (TIA-1), a pivotal regulator of mitochondrial dynamics, in models of both replicative senescence and ionizing radiation (IR)-induced senescence. The downregulation of TIA-1 was determined to trigger mitochondrial elongation and enhance the expression of senescence-associated β-galactosidase, a marker of cellular senescence, in human fibroblasts and keratinocytes. Conversely, the overexpression of TIA-1 mitigated IR-induced cellular senescence. Taken together, our findings underscore the significance of TIA-1 in governing mitochondrial dynamics and cellular senescence.

Tetrahydrobiopterin metabolism is impaired in chemically-induced, idiopathic and genetic mitochondrial deficiencies

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Tetrahydrobiopterin (BH4) metabolism has recently emerged as a promising metabolic pathway that can be positively modulated to inhibit deleterious signaling that may result in cell death. The objective of this work was to investigate BH4 metabolism and mitochondrial activity in different tissues and conditions linked to mitochondrial dysfunction. BH4 synthesis was investigated in (*i*) the brain, liver, and muscle of NDUFS4 (an accessory subunit essential for complex I activity) null mice; (*ii*) the brain, muscle, and fluids of mice submitted to reserpine-induced complex I deficiency to model experimental Parkinson's disease (PD); (*iii*) fluids and platelets of idiopathic PD individuals; and (*iv*) cell lines of individuals affected by primary mitochondrial disorders (PMD). Differential expression analysis of BH4-linked biosynthetic genes was also performed in PMD cell lines. Marked reduction of BH4 levels, expression of *Gch1* (a key gene involved in BH4 biosynthesis), and complex I activity were found in the hippocampus, cerebellum, striatum, liver and muscle of NDUFS4 mice. Reserpine-receiving mice showed complex I inhibition and increased fragmented mitochondria in the brain and skeletal muscle, in association with reduced levels of BH4 in the brain, serum and urine. Individuals affected by idiopathic PD showed impaired BH4 metabolism in the plasma and urine, with reduction of complex I activity in platelet-isolated mitochondria. Fibroblasts from the PMD DARS2 deficiency showed impaired activation of BH4 biosynthesis, complex I deficiency, reduced antioxidant defenses and mitochondrial

fragmentation. Furthermore, DARS2 deficient cells, when exposed to sepiapterin, a precursor of BH4, showed increased BH4 levels, cellular oxygen consumption, efficiency to synthesize ATP, and increased levels of glutathione. The transcriptome analysis of several PMD cell lines showed downregulation of genes involved in BH4 production and upregulation of genes linked to the activation of the innate immune system and to the response to hypoxia. The information presented shows a novel and intimate association of impaired mitochondrial physiology and dynamics with BH4 deficiency in tissues with high energy demand. The administration of BH4 may result in a new approach for treating primary and acquired mitochondrial disorders.

Key words: antioxidant, BH4 metabolism, mitochondrial dysfunction.

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The associations of pain/stress, mitochondrial dysfunction with neurodevelopmental outcomes in preterm infants during NICU Hospitalization

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Background and Purpose. Preterm infants experience tremendous early life pain/stress during their neonatal intensive care unit (NICU) hospitalization which impacts their neurodevelopmental outcomes. Mitochondrial dysfunction may interface between perinatal stress and neurodevelopment. This study aims to investigate the associations between pain/stress, mitochondrial function-related proteins, and mitochondrial DNA copy number (mtDNAcn), and neurobehavioral responses in preterm infants.

Methods. A prospective cohort study was conducted. Daily pain/stress experience during NICU stay was documented. Neurobehavioral outcomes were evaluated using the NICU Network Neurobehavioral Scale (NNNS), buccal swabs and blood were collected at 36-38 weeks of postmenstrual age (PMA). Proteomic mass spectrometry was conducted to assess the buccal proteins and ddPCR was conducted measuring blood mtDNAcn. Lasso statistical methods were conducted to study the association between protein abundance and infants' NNNS summary scores. Gene Ontology (GO) enrichment analyses were performed to examine the underlying molecular pathways. A linear regression analysis was conducted to explore the association between the clinical characteristics, neurobehavioral outcomes with protein levels/functions and mtDNAcn respectively.

Results. Early life adverse experiences were associated with poor neurobehavioral outcomes. Protein functions, such as glutathione disulfide oxidoreductase activity, response to oxidative stress, as well as those related to lipid metabolism, phosphate and proton transmembrane transporter activity, were found to be linked to poorer neurobehavioral outcomes. Conversely, functions related to cytoskeletal regulation, epithelial barrier maintenance were associated with more favorable neurodevelopmental outcomes. Furthermore, proteins associated with mitochondrial function, including SPRR2A, PAIP1, S100A3, MT-CO2, PiC, GLRX, PHB2, and BNIPL-2, demonstrated a positive correlation with positive neurodevelopmental outcomes. On the other hand, proteins like ABLIM1, UNC45A, Keratins, MUC1, and CYB5B were found to be positively associated with adverse neurodevelopmental outcomes. Increased mtDNAcn was found to be positively associated with adverse early life experiences and poor neurodevelopmental outcomes.

Conclusions. The relationship between early life pain/stress and neurodevelopmental outcomes in infants can be mediated by mitochondrial dysfunction-related proteins and their respective biological functions. Additionally, elevated mtDNAcn may serve as a substantial predictor of adverse early life neurodevelopmental outcomes.

Implications. The levels of buccal proteins and blood mtDNAcn could serve as valuable indicators for predicting potential neurobehavioral outcomes in preterm infants. It is advisable to implement early pain alleviation measures and provide personalized skin integrity protection to these infants during their stay in the NICU.

Limitation. Limited sample size and the results should be interpreted with caution. Future genetic, deep proteome, and PTM studies would greatly expand our preliminary findings and compose a more accurate and holistic picture of the infant's response to pain and stress in early development.

Key words

Preterm infants, Mass spectrometry, Mitochondrial DNA copy number, Pain/stress, Mitochondrial dysfunction, Neurodevelopmental outcomes

The extracellular matrix integrates mitochondrial homeostasis to promote immunity

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Cellular homeostasis is intricately influenced by stimuli from the microenvironment, including signaling molecules, metabolites, and pathogens. Functioning as a signaling hub within the cell, mitochondria integrate information from various intracellular compartments to regulate cellular signaling and metabolism. However, it remains elusive how changes in the extracellular microenvironment, particularly the extracellular matrix (ECM), can impact mitochondrial homeostasis.

In mammals, ECM remodeling occurs during aging and in multiple diseases, including infections, cancers, and neurodegeneration. Remodeled ECM may liberate bioactive fragments that promote tissue damage-related responses, such as wound healing and inflammation. We find that ECM remodeling induces remarkable changes of mitochondrial form and function in an evolutionarily conserved manner. Mechanistically, ECM remodeling triggers a TGF-β response to induce changes in mitochondrial dynamics. At the organismal level, ECM remodeling promotes immunity of animals against pathogens through the enhanced mitochondrial stress responses. We postulate that this ECM-mitochondria crosstalk represents an ancient immune pathway, which detects

infection- or mechanical stress-induced ECM damage, thereby initiating adaptive mitochondria-based immune and metabolic responses.

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The germline coordinates mitokine signaling

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An organism's ability to coordinate stress responses across tissues is imperative for its survival. One way this occurs is through mitochondria, where mitochondria experiencing stress in one tissue can active a protective stress response in another. In *C. elegans,* neuronal mitochondria experiencing stress activate the mitochondrial unfolded protein response (UPR^{MT}) in intestinal cells to protect organismal health and lifespan through the release of mitokine signals, such as serotonin or the WNT ligand EGL-20. In intestinal receiving cells, canonical WNT signaling is required to induce the UPR^{MT}. However, a conundrum exists because this WNT mitokine signaling does not induce canonical WNT developmental pathways, suggesting that there is an additional regulatory step in mitokine signaling. This regulatory step may lie in germline mitochondria, which we discovered play a surprising role in mediating neuron-to-intestine UPR^{MT} signaling downstream of WNT. Through a classic genetics screen in *C. elegans*, we identified a germline-localized mitochondrial complex III subunit *ucr-2.3* (UQCRC2 in humans) that is required for neuronal mitokine signaling. We find that germline mitochondria signal downstream of previously discovered neuronal signals like the WNT/EGL-20 mitokine and serotonin, and upstream of lipid metabolic pathways in the intestine to regulate UPR^{MT} activation. We also find that this was not specific to *ucr-2.3*, as other germline-specific mitochondrial perturbations also disrupt neuron-to-intestine mitokine signaling. Most surprisingly, we find that loss of the germline tissue itself also abrogates mitochondrial stress signaling, redefining the role of the germline in organismal health and stress

resistance. Overall, this work re-categorizes the role of the germline in inter-tissue signaling and resolves many long-standing questions regarding how organisms divide resources between the soma and the germline, especially under conditions of stress. That germline mitochondria can sense and respond to stress in somatic tissues may explain how stress conditions, such as aging or metabolic disorders, impact germline mitochondrial quality and reproductive outcomes.

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The Ketogenic Diet Metabolite β-Hydroxybutyrate Promotes Mitochondrial Elongation via Deacetylation and Improves Locomotor Behaviour in a Zebrafish Model of Autism

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The ketogenic diet (KD) is beneficial clinically and has therapeutic potential across a growing list of neurological disorders, including autism spectrum disorder (ASD). However, the underlying mechanisms mediating the benefits of the KD, which can also have undesirable side effects, remain undefined. To this end, improvements in mitochondrial morphology and function correlate with improved ASD behaviours in response to the KD, though how the KD influences mitochondrial morphology, and whether this is sufficient to improve behaviour remains unknown. Here, we investigated how beta-hydroxybutyrate (BHB), a key metabolite produced by the KD regulates mitochondrial morphology, and whether this pathway can be exploited to improve ASD behaviours. We found that β-oxidation of BHB is necessary to promote mitochondrial elongation by increasing NAD^+ levels, which in turn activates SIRT enzymes that deacetylate key regulators of both mitochondrial fusion (OPA1) and fission (DRP1). We also observed that BHB increased levels of total OPA1 protein and reduced the pro-fission $pDRP1^{616}$ post-translational modification. Our data also show that increasing NAD^+ levels by supplementation with the NAD^+ precursor nicotinamide nucleotide (NMN), recapitulates changes in acetylation and $pDRP1^{616}$, and is sufficient to promote hyperfused mitochondrial networks. Finally, we found that both BHB and NMN improve locomotor behaviour in the *shank3*$^{+/-}$ zebrafish model of ASD. Together, our findings elucidate a mechanism by which the ketogenic diet promotes mitochondrial elongation.

Moreover, manipulation of this pathway may provide a novel avenue for the treatment of neurological disorders such as ASD, one which may avoid undesirable complications of the KD.

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The MFN2 Q367H variant from a patient with late-onset distal myopathy reveals a novel pathomechanism connected to mtDNA-TLR9 mediated inflammation

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We describe a novel heterozygous MFN2 variant, MFN2 Q367H, found in a patient diagnosed with late-onset distal myopathy, but lacking the typical MFN2-associated pathology of peripheral neuropathy. Indicative of MFN2 dysfunction, characterization of patient fibroblasts revealed mitochondrial fragmentation, reduced mitochondrial ER contacts, and mtDNA alterations, but only when cultured in galactose media, where mitochondrial function is required for energy production. Meanwhile, under 'normal' glucose media cell culture conditions, we observed alterations in mitochondrial respiration, as well as an increase in abundance of cellular lipid droplets, perhaps connected to changes in mito-lipid droplet tethering (a key MFN2 function), suggesting these MFN2-related phenotypes were more adversely impacted by the Q367H variant. Given the myopathy presentation of the patient, we hypothesized that there could be

activation of the TLR9-NFκB inflammatory pathway, which is reported to be activated upon disrupted mitochondrial dynamics and lead to myopathy. Excitingly, we discovered an increased expression of several inflammation-related genes connected to the TLR9-NFκB signalling (e.g., ASC, IL6, NLRP3, S100A9, TNF-α), and observed mtDNA co-localizing with early endosomes. Taken together, these observations are consistent with mtDNA activation of the TLR9-NFκB pathway. To gain tissue-specific insight, we transdifferentiated Q367H fibroblasts into myoblasts. Strikingly, we observed a reduction of the mito-ER contacts in the Q367H myoblasts grown in 'normal' glucose media. We also observed elevated expression of inflammation genes in Q367H myoblasts, which was notably 10-fold higher than in fibroblasts. Collectively, the elevated mtDNA/TLR9 inflammatory signal and aggravated myoblast phenotypes provide a possible mechanistic explanation for the myopathy in the patient. Overall, this work highlights myopathy in the absence of peripheral neuropathy as a novel disease phenotype associated with MFN2 dysfunction, and links mtDNA activation of TLR9 signalling mediated inflammation as a pathomechanism of MFN2 dysfunction.

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The mitochondrial phosphatase PGAM5 is a novel interactor of Amyloid Precursor Protein

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Deposition of Amyloid-ß (Aß) plaques in the brain is a pathological hallmark of Alzheimer's disease (AD). Aß peptides are cleavage products of the amyloid precursor protein (APP). Ongoing clinical trials are using APP siRNA to decrease Aß levels as a potential treatment for AD. Along with Aß, this treatment reportedly decreased the levels of full-length APP. Thus, it is crucial to understand the physiological roles of APP and its fragments. Previously, Rice et al. identified phosphoglycerate mutase family member 5 (PGAM5) as a top candidate interactor of APP in rat brain synaptosomes using a proteomics screen. PGAM5 is a mitochondrial phosphatase with critical roles in mitochondrial functions and dynamics. A study has shown that APP affects mitochondrial morphology and function, suggesting its biological role in mitochondria. Here, we aimed to further investigate the interaction between PGAM5 and APP. Using in vitro binding assays, we narrowed down the interacting domains of both APP and PGAM5 sufficient for this interaction. Moreover, our proximity ligation assays show the endogenous interaction of these proteins in wild-type mouse brain tissue and primary astrocytes under basal conditions. Subcellular fractionation of HeLa cells shows the presence of PGAM5 and APP-FL in mitochondria-associated ER membranes (MAMs) while APP-CTF was present in mitochondria with PGAM5. Our data indicates that PGAM5 and APP interact under basal conditions, potentially in the MAMs. Our current efforts are directed towards understanding whether this interaction is modulated in AD conditions as well as the implications of this interaction on mitochondrial function and dynamics. Precise understanding of the interaction of APP with other proteins, like PGAM5 is critical to appreciate the impact of APP knockdown on neurobiology and to ensure the long-term safety of potential treatments for AD patients.

The role of HtrA2 in mitochondrial ROS regulation in neurodegenerative diseases

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Oxidative stress in neurons may result from an excessive production of reactive oxygen species (ROS) in the mitochondria or a decrease in antioxidant defenses. This can cause mitochondrial dysfunction and ultimately lead to cellular death. Disruption of the mitochondrial redox balance has been implicated in several neurodegenerative pathologies. The degeneration of striatal neurons is a major feature of HtrA2-inactivated motor neuron degeneration 2 (mnd2) mice. However, the molecular mechanisms leading to the pathophysiological consequences of HtrA2 activity and its association with mitochondrial dysfunction in neurodegenerative conditions remain unclear. We discovered that HtrA2 inhibits the generation of ROS in mitochondria by initiating the proteolysis of α-Syn, thereby preventing the accumulation of α-Syn in mitochondria. Additionally, we observed that the reduction of HtrA2 stimulates microglia by promoting mitochondrial ROS production mediated by α-Syn. Furthermore, we show that an increase in the mitochondrial insoluble SOD2 within the brain tissue of mnd2 mice, indicating a reduction in mitochondrial antioxidant capacity. Overall, these data indicate that HtrA2 plays a crucial role in maintaining cellular homeostasis by suppressing the accumulation of mitochondrial reactive oxygen species through the eliminating abnormal mitochondrial α-Syn and SOD2 within mitochondria. This research highlights the potential importance of HtrA2 in the pathogenesis of neurodegenerative diseases and provides new insights into the common pathological processes of neurodegenerative diseases and effective therapeutic strategies.

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The Role of Mitochondrial Inheritance in the Early Rise of Asymmetric T Cell Fates

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T cell immunity is impaired during ageing, which impinges immune responses reliant on diversity, including those necessary for efficient vaccinations. Autophagy and asymmetric cell division (ACD) are amongst the cell biological mechanisms that contribute to memory formation but undergo deterioration upon ageing. Thus, we aimed to decipher whether autophagy regulates the early-rise of asymmetric T cell fates. Firstly, we characterized the proteome of (a) young wild type, (b) autophagy-deficient and (c) aged first-daughter $CD8^+$ T cells following TCR-triggered activation, which enabled us to identify novel cargoes that potentially play a role in cell fate determination and are autophagy-targets. Confocal and electron microscopy imaging of first-daughter-cells validated proteomics results by revealing a correlation between autophagy-sufficiency and asymmetric inheritance of a variety of cell cargoes that can control T cell fate. Amongst the asymmetrically inherited fate determinants identified, which were impacted by autophagy impairment (ageing) or loss, was damaged mitochondrion. Hence, to functionally access the impact of mitochondrial inheritance on T cell fate, we took advantage of a pioneer murine model that allows sequential tagging of aged vs. young mitochondria (SnapTag system). This enabled us to isolate first-daughter cells based on a fate-determinant (aged/damaged mitochondria) regulated by autophagy (mitophagy) and to perform adoptive transfer experiments to functionally compare the immune responses built by these two cell types. We observed that cells that mostly inherit aged mitochondria are more glycolytic and show poorer memory potential, measured by survival and re-expansion upon cognate-antigen challenge by an acute infection. Proteomics analysis of these distinct populations ($CD8^+$ T cells inheriting or not aged mitochondria) revealed further differences in the inheritance of proteins involved in metabolism and mitochondrial function and quality control, but also those involved in regulating organelle transport. Finally, to determine whether autophagy is required for the asymmetric partitioning of aged mitochondria, we created autophagy-deficient SnapTag cells, which exhibited increased mitochondrial mass and distinct network organization in comparison to autophagy-sufficient cells. Furthermore, we could observe that loss of autophagy led to higher co-localization rates between aged and young mitochondrial structures and resulted in symmetric inheritance of aged mitochondria during mitosis. We anticipate that these findings will be relevant to better understanding on how ACD is coordinated and on how T cell diversity is early-imprinted. Furthermore, as mitophagy/autophagy can be pharmacologically modulated, these results can potentially lead to the development of more efficient vaccination schemes and to therapeutic strategies in the context of regenerative medicine, both particularly important in the context of ageing.

The role of the mitoribosome accessory protein OXA1L in chemotherapy-induced metabolic adaptation of breast cancer

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Mitochondria are dynamic organelles, responsible for energy production, reduction-oxidation reactions, and signaling to ensure cellular homeostasis and survival. Our group previously demonstrated that in an aggressive subtype of breast cancer, termed ‘triple negative’ (TNBC), cells rely on mitochondrial structure and function adaptation to survive the stressful assault of chemotherapeutic treatment. Specifically, chemo-refractory cells exhibited heightened mitochondrial fusion, mtDNA content, and oxidative phosphorylation (oxphos) (PMID: 36813854). Thus, we set about to mechanistically dissect mitochondria-mediated mechanisms of chemotherapeutic resistance in TNBC.

To start, we inspected tandem mass tag mass spectrometry proteomic data generated from biopsies of TNBC patients whose tumors went on to be responsive or resistant to conventional chemotherapy treatments (PMID: 36001024). This revealed several core mitoribosome components, as well as the accessory protein Oxidase (Cytochrome C) Assembly 1-Like (OXA1L), were significantly higher in chemoresistant tumors. OXA1L plays two key roles in the mitochondria: 1) it promotes translation termination of the 13 mtDNA-encoded electron transport chain (ETC) components, and 2) it aids in inner mitochondrial membrane insertion of both mtDNA- and nDNA-encoded ETC

components. Therefore, we hypothesize enhanced mitochondrial translation via OXA1L is critical for the optimization of mitochondrial function in chemoresistant TNBC.

Transient siRNA-mediated knock down (KD) of OXA1L in human TNBC cell lines reduced levels of ETC components, including complex II SDHB, complex III UQCRC2, complex IV mt-COX2, complex V APT5A and mt-ATP8, and diminished oxygen consumption rate (OCR). Further, we show the chemotherapy-induced oxphos boost we observe to be characteristic of 'residual' survivor cells exposed to chemotherapies, was significantly disrupted by OXA1L KD. Concomitantly, OXA1L KD cells exhibited enhanced chemotherapeutic sensitivity relative to control KD cells.

To complement these studies, we are repurposing the FDA-approved conventional antibiotic tigecycline as an anticancer drug based on its mitoribosome inhibitory function (PMID: 25625193). Indeed, we observe minimal toxicity to TNBC cells as a single agent but enhanced efficacy when combined with conventional chemotherapeutic agents. In summary, our data provide evidence that targeting mitochondrial translation may be a promising approach to overcome pro-survival metabolic adaptations in residual TNBC cells refractory to conventional chemotherapies.

Therapy-resistant prostate cancer cells alter mitochondrial morphology and metabolism to survive therapeutic stress

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Mitochondria are essential organelles that have multi-faceted roles in the cellular stress response. These organelles are known to be dysregulated in many cancers. Recent studies have shown that mitochondrial adaptations contribute to drug resistance in cancer, where cancer cells alter mitochondrial morphology, metabolism, and signaling to in response to a wide range of chemotherapies.

Therapy resistance is responsible for 90% of cancer-related deaths. We identified a novel mechanism of therapy resistance in which cancer cells enter a non-proliferative endocycle, repeatedly undergoing repeated S (DNA synthesis)- and G (growth)-phases. As a result, resistant endocycling cells are 40 times larger than mitotic cancer cell controls. We found that this non-proliferative state is reversible, and endocycling cells eventually give rise to a proliferative population weeks after chemotherapy is removed, modeling cancer recurrence. We hypothesize that this endocycling cell state is a key player in therapy resistance and cancer lethality.

We evaluated oxidative stress in endocycling cells that survived the days and weeks following chemotherapy treatment using DCF-DA staining. We found increased levels of reactive oxygen species (ROS) normalized to cell size in endocycling prostate cancer cells that survived chemotherapy compared to non-treated mitotic cells. Given that mitochondria are major producers and regulators of ROS, we hypothesize that cells surviving in this resistant state alter mitochondrial structure and function in response to increased ROS in the cell.

We performed immunofluorescence and confocal microscopy on mitochondria in untreated cells and cells following chemotherapy treatment to observe their morphology. Surviving endocycling cells showed more mitochondrial fragmentation when compared to the untreated group, a result that was further supported by reduced expression of phospho-DRP1 Ser637 via western blot. This phenotype is often caused by increased levels of ROS and is associated with reduced mitochondrial metabolism.

To assess changes in glucose metabolism, we then performed isotope tracing-based metabolomics with [U-^{13}C]glucose on untreated and surviving endocycling cells following chemotherapy treatment. Endocycling cells had decreased labeling of ^{13}C TCA cycle metabolites when compared to untreated cells, suggesting a decrease in mitochondrial function. Ongoing work involves further studying metabolic reprogramming as cells enter this resistant state with parallel labeling experiments using [1,2-^{13}C]glucose and [U-^{13}C]glutamine to perform mathematical modeling-based ^{13}C-metabolic flux analysis. In addition, we engineered a cell line to track mitochondria in real-time; time-lapse imaging will enable quantification of mitochondrial dynamics underlying the morphology observed in untreated and surviving cells.

Our goal is to characterize how this therapy-resistant cell state alters its mitochondrial structure and function to survive therapeutic stress. Identifying vulnerabilities of this phenotype will enable new approaches for eliminating drug resistance in cancer.

Uncovering biogenesis pathways for alpha-helical mitochondrial outer membrane proteins using genome-wide approaches

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Mitochondrial function and their ability to communicate with the cytosol and other organelles depend on proteins embedded in the outer mitochondrial membrane. In mammals, the mitochondrial outer membrane contains ~150 different proteins which are encoded in the nuclear genome and must be targeted and inserted into the membrane. For beta-barrel proteins, this is a two-step process requiring translocation into the intermembrane space via the translocase of the outer membrane (TOM) and subsequent insertion by the sorting and assembly machinery (SAM) complex. However, the factors involved in the biogenesis of alpha-helical proteins in the outer membrane, which are critical for regulating mitochondrial dynamics and function, were unknown. Recently, our lab used a genome-wide CRISPRi screen with an outer membrane tail anchored protein as a reporter to identify a novel insertase, MTCH2, which was shown to be necessary and sufficient for the integration of biophysically diverse alpha-helical proteins into the outer membrane. However, the broader targeting and biogenesis pathways for alpha-helical proteins with diverse topologies had not been systemically explored. Critically, our understanding of cytosolic factors involved in chaperoning and targeting different types of alpha-helical proteins to the outer mitochondrial membrane remained incomplete.

To explore this, we conducted a series of CRISPRi screens with a biophysically and functionally diverse set of outer membrane proteins (signal-anchored and polytopic proteins of varying topologies). Using this approach, we discovered factors involved in all stages of outer membrane biogenesis, targeting, insertion and quality control, and organized these factors into distinct pathways. We defined a set of rules linking substrates to their biogenesis pathways and preventing mislocalization, largely determined by their topologies (this separates signal-anchored from tail-anchored proteins, and also polytopic proteins based on their topologies). Specifically, we showed

a new role for the ribosome-bound chaperone NAC in targeting polytopic proteins to the outer membrane. We also identified a novel putative cytosolic chaperone, TTC1, required for the biogenesis and targeting of signal-anchored proteins to the outer membrane. We showed a range of mitochondrial dysfunction phenotypes associated with loss of TTC1, including mitochondrial fragmentation, lowered oxygen consumption rate, and membrane depolarization. Mutational and biochemical studies indicate that an evolutionarily well-conserved hydrophobic groove at the C-terminus is critical for its function. Cumulatively, our work sheds light on the complexity of pathways involved in outer mitochondrial membrane protein biogenesis and their connection to broader mitochondrial dynamics and quality control, identifies novel factors involved in different stages of biogenesis and lays the foundation for ongoing mechanistic studies. The existence of multiple distinct biogenesis and quality control factors with a high degree of substrate specificity could allow individual mitochondria to regulate their outer membrane composition and thereby communication with the cytosol and other organelles under various physiological conditions and disease states. We anticipate that these discoveries could be adapted to tune the outer mitochondrial membrane proteome at high precision, which in turn could inform efforts to mitigate outer membrane related diseases such as Alzheimer's, Parkinson's and a variety of cancers.

Understanding C9orf72 Hexanucleotide Repeat Linked to Mitochondrial Dysfunction

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FTD and ALS are neurodegenerative diseases, with the former characterised by declining cognitive functions and the latter affecting motor neurons. Even though these diseases present with distinct sets of symptoms, FTD and ALS are two extremes of the same disease spectrum, and they show considerable overlap in genetic, clinical, and

neuropathological features. Among these overlapping features, mitochondrial dysfunction is associated with both diseases. Recent studies have shown that cells derived from patient iPSCs display mitochondrial abnormalities, and similar abnormalities have been observed in several animal disease models. A hexanucleotide repeat expansion in the C9orf72 gene is the most common genetic cause of both FTD and ALS. C9orf72 (C9) gene has been linked to mitochondrial abnormalities. We have investigated mitochondrial dysfunction in a Drosophila C9 model and characterised how mitochondrial genes are modulated by C9 toxicity in the Drosophila brain. Our RNA sequencing results show that differentially expressed genes involved in mitochondrial-related gene functions were downregulated. We have measured the expression level of electron transport chain components and mitochondrial copy number by qPCR assays over time. We have also measured mitochondrial respiration rates with Oroboros. We are currently characterising mitochondrial function and morphology via immunofluorescence staining. All mitochondrial subunits were significantly downregulated upon C9 induction and we found mitochondrial DNA copy number decreased in C9 expressing Drosophila brains. However, we observed only minimal effects on mitochondrial respiration rates. We conclude that the expression of mitochondrial and nuclear-encoded subunits and mitochondrial DNA copy number are reduced in C9 expressing Drosophila brains.

USP30 inhibiting molecules enhance mitophagy with potential to stop disease progression in multiple energetically demanding tissues

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Mitophagy declines with age resulting in the accumulation of dysfunctional mitochondria. This in turn contributes to several age-related diseases, particularly in energetically demanding tissues such as the brain and kidneys. For several of these diseases, including Parkinson's disease (PD) and acute kidney injury (AKI), disease modifying therapeutics do not currently exist, thereby presenting an unmet need that may be addressed with small molecules that enhance mitophagy or mitochondrial repair. USP30 has recently emerged as a key regulator of mitochondrial clearance by counteracting the ubiquitination activity of the E3 ligase parkin, with USP30 inhibition having been demonstrated to increase ubiquitination and clearance of depolarized mitochondria. Our team has developed proprietary compounds with low nanomolar (nM) *in vitro* potency for USP30 inhibition as measured by two orthogonal methods. The compounds are cell-penetrant and enhance mitophagy in the presence of mitochondrial toxins in human neural cells expressing endogenous USP30, parkin, and substrates. Importantly, these compounds do not damage or depolarize healthy mitochondria, as evidenced by a TMRE-based mitochondrial function assay. Candidate compounds are highly selective when tested against a panel of over 40 deubiquitinating enzymes in two orthogonal assays. We have profiled compounds for multiple ADME properties and successfully optimized properties including solubility, permeability, microsomal stability, and plasma protein binding. Our lead compound has an excellent PK profile in both rat and mouse, penetrating the brain and kidney at levels well above 2x the compound's low nM IC_{50}. In sum, our proprietary USP30 inhibitors, which demonstrate strong *in vitro* efficacy and favorable pharmacokinetics, show promise as a new therapeutic avenue for age-related diseases by promoting the clearance of dysfunctional mitochondria in vital organs including the brain and kidneys.

Why Kiss and Run is good: N-terminus of V-ATPase "a"subunits induce mitochondrial catastrophe

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Mitochondrial homeostasis depends on proper tethering to other organelles such as the ER, Golgi and the plasma membrane (PM). These interactions are regulated by small GTPases of the Rab family along with proper pH regulation of organelles by the proton pumping V-ATPase. Proper Rab7a activity is necessary for mitochondrial tethering and mitophagy, while localization of the proton pump is determined by 4 isoforms of the V-ATPase "a' subunit (a1-a4). While characterizing the interaction of Rab7a with the lysosomaly localised V-ATPase a3 subunit we employed the knock-sideways approach to localize the cytoplasmic a3 N-Termini (NTa3) onto the mitochondria using the outer mitochondrial membrane localized Mavs TM domain to see if Rab7a would follow. We created FLAG-mCherry-Mavs (FCMavs) and a GFP version (FGMavs). When these were expressed in HeLa, HEK293 or HEK293T cells, we observed a well-defined mitochondrial network. The NTa3-FCMavs construct caused severe mitochondrial aggregation in HEK293/T cells such that 50% of the cells had an apparent single mitochondrion. This phenotype was less pronounced in HeLa cells, however the mitochondrial network was still abnormal. This phenotype is not restricted to NTa3 as NTa1 (secretory vesicles/PM), NTa2 (Golgi) or NTa4 (PM) show a similar phenotype. The NTa2-FCMavs shows the least severe phenotype, however this is most likely due to lower expression or possibly degradation of the fusion protein. Preliminary results so far suggest the involvement of Rab7a, but we can't exclude the possibility that the NTa's perturb novel activities or interactions. We demonstrate that the large mitochondrial aggregate is composed of tiny, individual mitochondria suggesting activation of the fission process. Normally mitochondria make contacts with the ER and the lysosome where the a3 subunit normally resides. It was recently shown that lysosomal GTP-Rab7a induces fission while the Fis1-TCB1D15/17 complex dampens this by converting Rab7a to the GDP form. We propose that by permanently placing NTa's onto the mitochondria we are continuously activating fission possibly through Fis1 which then could also inhibit the function of fusion proteins MFN1/2 by not releasing the individual mitochondria.

Yme1 is Required for Mitochondrial Remodeling via the MDC Pathway

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Mitochondria are dynamic hubs at the nexus of energy production and signaling. Mitochondrial functions are preserved through coordinated mechanisms to ensure cellular homeostasis. Our lab discovered one such homeostasis mechanism termed the Mitochondrial Derived Compartment (MDC) pathway. Initially observed in old-aged yeast cells, MDCs are micron-sized, multi-lamellar structures that form from the mitochondrial outer membrane at ER-mitochondria contact sites. Recent studies indicate that a myriad of acute stressors that alter amino acid homeostasis, and/or lipid regulation induce MDC formation. Upon formation, MDCs sequester a selective portion of the mitochondrial outer membrane proteome, and then are subsequently degraded in the vacuole through an autophagy-dependent mechanism. Our current model is that the MDC pathway facilitates mitochondrial remodeling to alter mitochondrial form and function in response to stress and/or changing metabolic conditions.

To gain mechanistic insight into the MDC pathway, we performed a microscopy-based screen of the yeast non-essential gene deletion collection to identify genes that regulate the formation of MDCs. Through this screen, we found that the conserved mitochondrial protease, Yme1, is required for MDC formation. Deletion of Yme1 prevented MDC formation in response to metabolic stress, and we found that this defect was not linked to the loss of mitochondrial DNA or caused by a significant change in mitochondrial morphology. Instead, we found that the catalytic activity of Yme1 is required for MDC formation. Moreover, overexpression of Yme1 was sufficient to induce MDC formation in the absence of any additional stressors. These results strongly indicate a role for Yme1 proteolysis in MDC biogenesis. Our current focus is to identify the target(s) of Yme1 in this pathway, and determine whether Yme1 functions as an integrator of MDC biogenesis signaling cues. Along these lines, we previously found that the level of the mitochondrial phospholipid phosphatidylethanolamine (PE) is an important regulator of MDC biogenesis. Given the well-known relationship between Yme1, PE, and proteins involved in PE synthesis, we are currently exploring the relationship between Yme1, PE, and MDC biogenesis. Results from this study will enhance our understanding of how cells generate MDCs to promote mitochondrial remodeling, and provide new information regarding the expanding role of mitochondrial proteases in facilitating mitochondrial adaptation in response to stress.