Mitochondrial Triggers of the Integrated Stress Response: Disentangling the Bioenergetic Rubik's Cube

Mitochondrial Triggers of the Integrated Stress Response: Disentangling the Bioenergetic Rubik's Cube

A dissertation presented

by

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to

The Committee on Higher Degrees in Systems Biology

in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
in the subject of
Systems Biology

Harvard University
Cambridge, Massachusetts

October 2018

Dissertation advisor: Vamsi Mootha Eran Mick

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ABSTRACT

Mitochondria are hubs of metabolism and signaling in eukaryotic cells whose dysfunction underlies a class of devastating genetic disorders and is also frequently associated with common conditions, such as neurodegeneration, diabetes, cancer and the ageing process. Mitochondrial dysfunction, particularly breakdown of the electron transport chain (ETC) and oxidative phosphorylation, yields a perplexingly variable spectrum of consequences at the cellular, tissue and whole-organism level. Deciphering the context-dependent pathophysiology of mitochondrial dysfunction is thus a major challenge in both basic and translational biomedical research.

Studies over the past decade have revealed that a prominent molecular signature of mitochondrial dysfunction *in vivo* is activation of the integrated stress response (ISR), a gene expression program eukaryotic cells engage upon different types of insults. How mitochondrial dysfunction is sensed to trigger the ISR and whether the response serves a protective role or contributes to pathology remain far from understood.

The work in this thesis sought to delineate functional parameters tied to the ETC, such as ATP synthesis or NADH oxidation, that can lead to ISR activation. We used chemical and genetic tools to perturb ETC functions in mouse muscle cells while specifically compensating for some of the resulting metabolic effects. We then monitored the impact of these interventions on ISR-dependent gene expression by RNA sequencing.

Our results revealed that in proliferating cells, the increase in the cytosolic [NADH]/[NAD^+] ratio during complex I dysfunction potently triggered the ISR, mostly by sharply depressing aspartate levels and activating the amino acid sensitive eIF2α kinase GCN2. Strikingly, this route to ISR activation became inoperative in terminally-differentiated myotubes where only ATP synthase inhibition elicited a significant response. The path to ISR activation in the latter case was dependent on residual ETC activity and could be abolished by co-inhibition of complex I, mild uncoupling or mild hypoxic preconditioning. Finally, our data suggests dysfunction of mitochondrial genome expression is not directly sensed to trigger the ISR.

These results shed light on the complicated interplay between mitochondrial dysfunction and the ISR. They implicate diverse metabolic and bioenergetic routes to its activation whose relevance *in vivo* should be carefully evaluated in future work.

ACKNOWLEDGEMENTS

I am grateful to my thesis advisor, Prof. Vamsi Mootha, for hooking me on mitochondria and for affording me the opportunity and resources to pursue our mutual scientific interests in a cutting-edge environment and with boundless abandon. Vamsi's guiding-principles of scientific excellence, lucid thinking, effective communication and real-world relevance have inspired much of my work on this thesis.

The Mootha lab has been an incredible place both to do science and to make friends over the past few years. It was truly a privilege to be surrounded by a team of talented and motivated people with such varied expertise – biologists, chemists, clinicians, data scientists and everything in between – who taught me so much.

I owe special thanks to Denis Titov, who was a friend, mentor, partner and role model throughout much of my Ph.D. Denis taught me how to do rigorous science, focus on what matters, find and use the right tool for the job and produce results I could truly rely on. He always had patience for my fretting over everything from the big picture to the minutest of details and almost always had a reassuring and practical piece of advice to offer in response.

I am grateful for countless hours of discussions, whether serious or light-hearted, with Russ Goodman, Pat Ward, Rohit Sharma, Owen Skinner, Hoy Shen, Josh Meisel and my indispensable Broad office-mates Tslil Ast and Alexis Jourdain. I benefited from their company, perspective, advice and example on so many occasions and cannot wait to see what the future holds for these brilliant scientists. Thanks are also due to Sarah Calvo, Robert Bao and Jason Arroyo who helped initiate me into the lab as early mentors and teachers. I am indebted to Bryanna Vacanti and Olga

Goldberger for their tireless efforts to keep the trains running on time in the lab and for protecting me from the clutches of the bureaucracy in ways both small and large.

I would like to thank Prof. Joseph Avruch, Prof. Andrew Murray, Prof. Marc Kirschner and Prof. Aviv Regev, who served at various times as members of my dissertation advisory committee or thesis defense committee, for their helpful feedback and encouragement as well as their practical and flexible approach to the makings of a Ph.D. I am also thankful to the Harvard Systems Biology Ph.D. program, especially Sam Reed and Liz Pomerantz, for advice and administrative support and for fostering a community. I greatly appreciate the generous funding I received from the Harvard Herchel Smith graduate fellowship in the sciences and the Howard Hughes Medical Institute international pre-doctoral fellowship.

Perhaps the most important people who made my Ph.D. journey what it was on a daily basis were my good friends Andrew Markhard, Daniel Flicker and Isha Jain. We bonded in ways that only the interminable grind of graduate school and those many late nights in lab (and/or out to dinner) could yield. They were, each individually and all together, a bastion of support, advice, entertainment, fun and, when necessary, commiseration. It truly would not have been the same without them. This can also be said of two other special friends from my Ph.D. class, Matthieu Landon and my current roommate, Evi Van Itallie.

As an international student, I was very far away from home while pursuing my thesis. Nevertheless, I was extraordinarily fortunate to have multiple surrogate homes sprinkled throughout North America as safe havens for me to get away from it all and just relax for a while. I will forever be grateful for the fun times I had and wonderful memories I made at the homes of my best friends Assaf and Liana in San Francisco, David and Tal in New York City and Oren and

Shany in truly remote Cambridge, MA. Not to mention celebrating the Jewish holidays with my aunt Miki's family in Toronto or my cousin Michelle's family outside Boston.

But the true highlights of each year were always the visits back home or trips abroad with my amazing family: my parents, my brother Tal, his wife Maayan, my nephew Lee, my niece Lauren and my surviving grandparents. A few brief words cannot possibly do justice to the incredible support, encouragement and generosity my parents, Sarah and Natan, have shown me throughout my life and especially during the Ph.D. I was still living at home up until I hopped the short distance across the Mediterranean and the Atlantic to start graduate school, so it was quite the adjustment to be far apart. But even if we only really spoke at length once every 1-2 weeks, their unconditional love, pride in me and commitment to my success were never far from my mind and were indispensable to sustain the grit, determination and equanimity required to see through such a challenging endeavor.

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CHAPTER 1
Introduction

INTRODUCTION

The mitochondrial electron transport chain (ETC) plays key roles in the physiology of eukaryotic cells. It facilitates efficient ATP synthesis via oxidative phosphorylation (OXPHOS), maintains the balance of reducing equivalents essential for a range of metabolic reactions, supports the electrochemical gradient that drives movement of metabolites and proteins across the mitochondrial inner-membrane, and modulates cellular oxygen levels and the production of reactive oxygen species (ROS).

Genetic lesions in ETC components and in factors required for their expression and assembly, whether encoded by the nuclear genome or the small mitochondrial genome (mtDNA), underlie a class of devastating inborn errors of metabolism affecting roughly 1 in 5,000 live births [1]–[3]. These conditions display a variable age of onset and can take an acute or progressive course. They are characterized by extreme heterogeneity of disease manifestation and severity at the tissue or cell-type level depending on the underlying mutation, even when the causal gene is ubiquitously expressed. No effective treatments are currently available.

A decline in ETC function is also a hallmark of such common conditions as neurodegeneration [4]–[7], obesity and diabetes [8]–[11], some types of cancer [12], and the molecular process of ageing [13], [14]. Paradoxically, ETC dysfunction has also been shown to promote longevity or restore metabolic health in certain settings [15]–[23]. Deciphering the context-dependent manner in which ETC dysfunction affects cells, tissues and whole organisms is thus a major challenge in both basic and translational biomedical research.

The variable spectrum of repercussions stemming from ETC dysfunction may arise due to differences in how distinct cell types in their respective metabolic context rely on the multiple

intertwined functions of the ETC. However, careful manipulation of individual ETC functions is a formidable experimental challenge *in vitro*, let alone *in vivo*. Moreover, research into mitochondrial dysfunction *in vitro* is usually performed on proliferating cells whereas *in vivo* symptoms appear most prominently in post-mitotic tissues such as skeletal muscle, heart, liver and the nervous system. As a result, few broadly applicable principles have emerged that can explain the relationship between a cell's reliance on specific ETC functions and its response to ETC dysfunction.

A related source of variability, which has garnered increasing attention in recent years, is the role that cell autonomous and non-autonomous stress responses may play in driving pathological (or beneficial) outcomes of ETC dysfunction. Cells engage gene expression programs in the face of mitochondrial stress, collectively termed "retrograde signaling" [24]. Some studies have found that chronic activation of these responses, rather than the primary bioenergetic defect, can be the proximal cause of pathology in affected tissue [25]–[27]. This raises the possibility that intervening in these secondary signaling pathways could improve health outcomes.

The response most frequently observed in association with mitochondrial dysfunction across human patients, animal disease models and mammalian cells *in vitro* is the integrated stress response (ISR) [28]–[36]. The ISR is a form of "general alarm" in eukaryotic cells that can be triggered by a range of extrinsic and intrinsic insults [37]. It acutely leads to attenuation of global protein synthesis while simultaneously engaging stress-responsive transcription factors. How mitochondrial dysfunction is sensed to trigger the ISR and what the consequences of the response are in this context remain incompletely understood.

The work described in this thesis seeks to apply traditional and novel tools to delineate functional parameters tied to the ETC that can lead to ISR activation in mammalian cells, and how this may

vary among cell states. Chapter 2 provides background information on: i) the tight interdependence of functional parameters linked to the ETC, akin to a Rubik's cube, and the chemical and genetic toolbox that nevertheless allows us to disentangle it; and ii) the ISR and its connection to mitochondrial dysfunction. Chapter 3 presents the principal results of a systematic effort to isolate the functional parameters sensed to trigger the ISR during ETC dysfunction in proliferating vs. post-mitotic mouse muscle cells, including characterization of metabolite levels, gene expression and relevant signaling nodes. Chapter 4 provides concluding remarks addressing open questions and future work that could build on the original results reported herein.

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CHAPTER 2
Background

SECTION I. The bioenergetic Rubik's cube.

At the heart of mitochondrial function lies the electron transport chain (ETC). Its most prominent role is to support ATP synthesis by oxidative phosphorylation (OXPHOS), which it performs by coupling a sequence of favorable electron transfers between redox carriers with proton translocation across the inner-membrane. The resulting concentration gradient (ΔpH) and charge difference (ΔΨ; also known as membrane potential) drive ADP phosphorylation by ATP synthase (**Figure 2.1**) [1], [2].

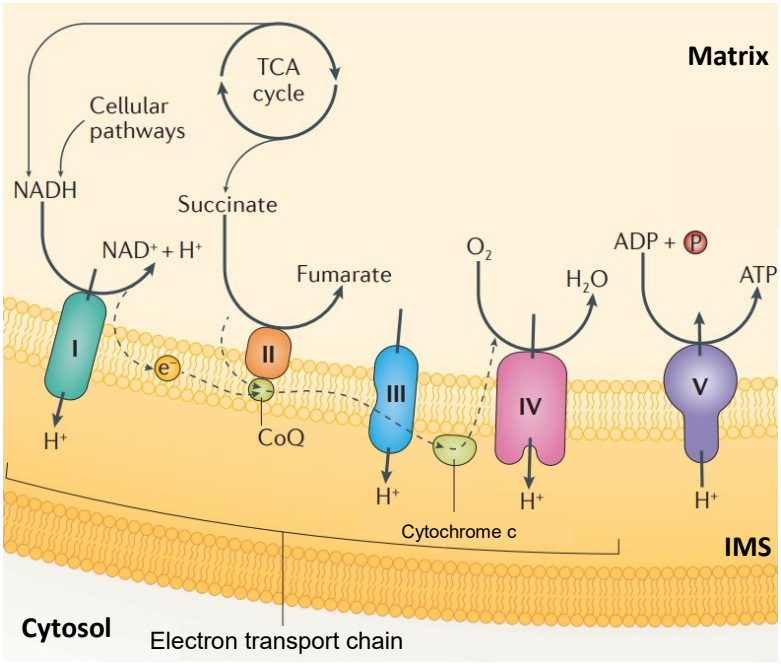

Figure 2.1: The mitochondrial electron transport chain and oxidative phosphorylation system. IMS = inter-membrane space; CoQ = coenzyme-Q.
Adapted from: Gorman et al., *Nat. Rev. Dis. Pri.*, 2016.

Electrons feed into the ETC from reducing equivalents generated by catabolic pathways and are terminally donated to molecular oxygen. The tricarboxylic acid (TCA) cycle is a major producer of the reducing equivalents NADH (reduced nicotinamide adenine dinucleotide) and $FADH_2$ (reduced flavin adenine dinucleotide) in the mitochondrial matrix. This pathway is tightly

coordinated with ETC function since one of its enzymes, succinate dehydrogenase, is also a constituent of the ETC, where it is known as complex II. Moreover, TCA cycle activity is regulated at multiple steps by the matrix [NADH]/[NAD^+] and [ATP]/[ADP] ratios [3]–[5].

Reducing equivalents produced in the cytosol – for example, during glycolysis (**Figure 2.2**) – can also flow into the matrix using a variety of redox shuttles [6]. The malate-aspartate shuttle couples exchange of aspartate and malate across the mitochondrial inner-membrane to net exchange of NADH and its oxidized form, NAD^+ (**Figure 2.3A**) [7]–[9]. Malate is a TCA cycle intermediate and aspartate derives from oxaloacetate, another cycle intermediate. The glycerol-3-phosphate shuttle feeds cytosolic reducing equivalents directly to coenzyme-Q, an electron carrier of the ETC (**Figure 2.3B**) [10].

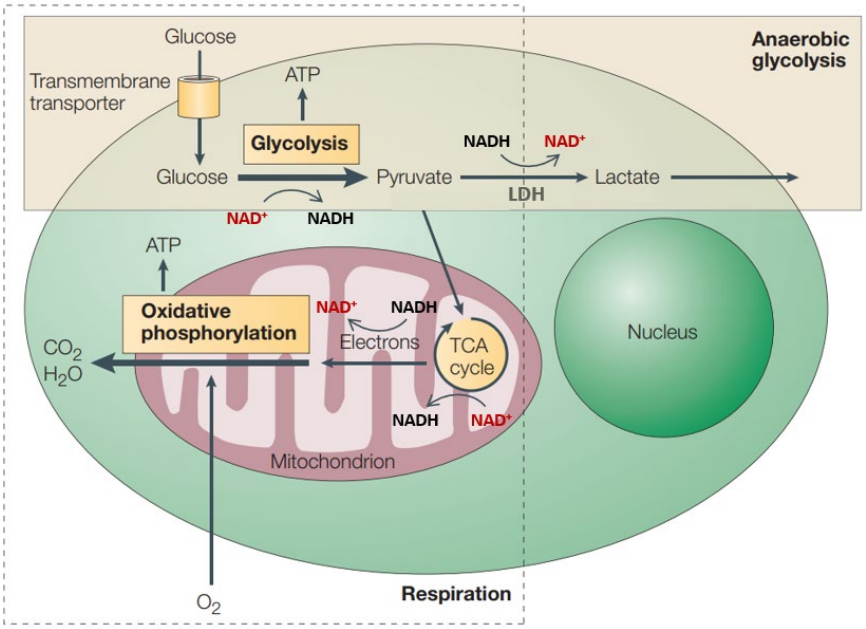

Figure 2.2: Simplified overview of central carbon metabolism, including redox co-factors.
LDH = lactate dehydrogenase.
Adapted from: Sitkovsky et al., *Nat. Rev. Immun.*, 2005.

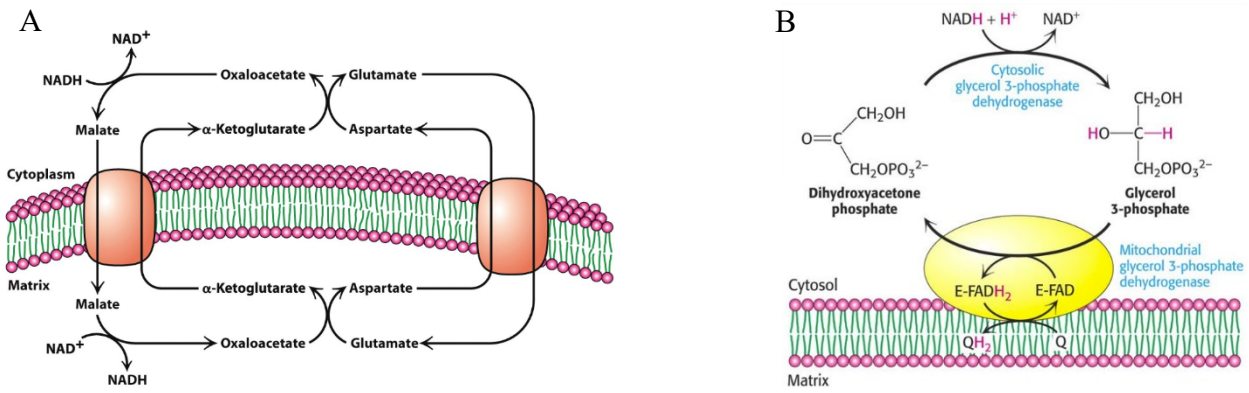

Figure 2.3: Major redox shuttles connecting the cytosol to the matrix.
(A) Schematic of the malate-aspartate shuttle. **(B)** Schematic of the glycerol-3-phosphate shuttle. From: *Biochemistry, Seventh edition.*

ATP production by OXPHOS is only one and often not the most crucial of cellular functions the ETC facilitates, as ATP can also be produced by glycolysis. Thus, NADH oxidation at complex I maintains the balance of redox cofactors on which hundreds of metabolic reactions in the cell depend [11]–[15]. Additional reactions are linked to the redox state of CoQ. In the same vein, the electrochemical gradient the ETC supports drives not only OXPHOS but also the import of ions, metabolites and some 1,000 nuclear-encoded proteins into the matrix [16]. Any attempt to dissect the contribution of individual ETC functions or their failure to cellular physiology and signaling runs headlong into the tight interdependence among these functions, akin to a Rubik's cube.

For example, while genetic and chemical tools are available to directly ablate ATP synthase activity, this will have much broader effects than just on ATP production by OXPHOS. Absent an outlet for dissipation of the proton gradient, electron transfer through the ETC becomes less favorable and oxidation of reducing equivalents like NADH stalls. NADH accumulation, in turn, inhibits the TCA cycle (**Figure 2.4**) and other redox-dependent reactions in the matrix and the cytosol, which can lead to nutrient depletion or potentially toxic accumulation. The elevated

membrane potential can itself alter metabolite and protein distributions across the inner-membrane. Conversely, specific breakdown of NADH oxidation at complex I also inhibits OXPHOS and can induce reversal of ATP synthase to defend the proton gradient, further depleting ATP [17].

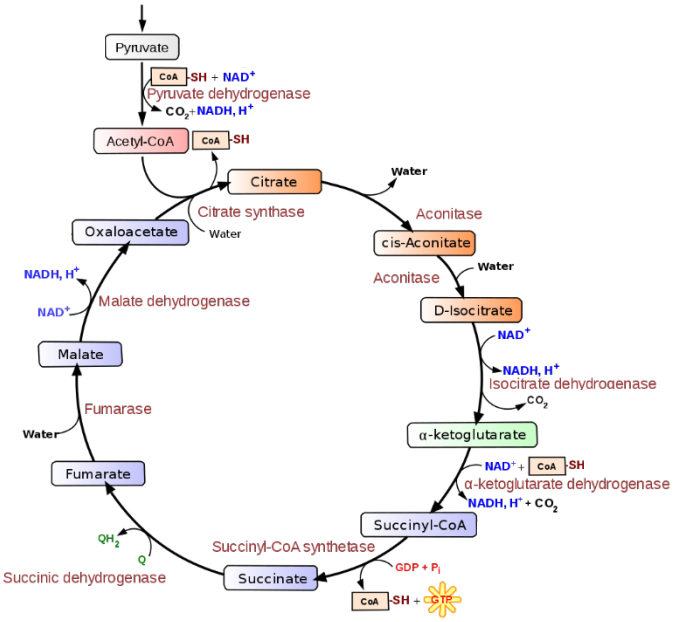

Figure 2.4: The tricarboxylic acid cycle, including essential redox co-factors.
Adapted from: https://en.wikipedia.org/wiki/Citric_acid_cycle

Several strategies have traditionally been applied to try and disentangle aspects of this bioenergetic Rubik's cube:

a. **Uncoupling.** A classic approach (inspired by physiology [18]) has been the use of uncoupling agents, which are typically weak lipophilic acids that diffuse across the mitochondrial inner-membrane and allow protons to leak back into the matrix independent of ATP synthase. Uncouplers inhibit ATP synthesis by dissipating its driving force but they prevent the depressing effect this would normally have on NADH oxidation and in fact accelerate overall

respiration [19]–[21]. However, this tool cannot separate the effects of OXPHOS inhibition from those of membrane depolarization and may depolarize other cellular membranes.

b. **Buffering.** This refers to interventions that buffer particular downstream effects of ETC dysfunction without restoring the activity of underlying ETC components. For example, researchers have long noted that mammalian cells lacking a functional ETC can only proliferate if supplemented with pyruvate and uridine [22]–[24]. Uridine was required because one of the steps in its biosynthesis is inhibited by an overly reduced CoQ, a consequence of complex III dysfunction. Pyruvate was proposed to be required to alleviate cytosolic NADH accumulation through the lactate dehydrogenase (LDH) reaction [24]. Thus, cells cultured with pyruvate and uridine are buffered against these consequences even while all ETC functions remain impaired.

c. **Protein prosthetics.** A particularly innovative approach involves expression of transgenes in ETC-compromised cells that restore functionality to certain ETC components while bypassing others. For example, yeast NDI1 is a single polypeptide substitute for the NADH oxidizing function of mammalian complex I that similarly transfers electrons to CoQ but does not pump protons. AOX is a fungal enzyme that oxidizes CoQ using oxygen as an electron acceptor, substituting for the activity of complexes III and IV other than proton pumping. Co-expression of NDI1 and AOX in mtDNA-depleted cells thus maintains the redox functions of the ETC, alleviating metabolic consequences of NADH accumulation or overly reduced CoQ, despite persistent OXPHOS deficiency and impaired membrane potential [25], [26] (**Figure 2.5A**).

Our lab has recently harnessed a class of bacterial enzymes, water-forming NADH oxidases, that perform the net redox reaction of the ETC independently of any of its components [27] (**Figure 2.5B**). We demonstrated that such an enzyme from *Lactobacillus brevis*, which we dubbed *Lb*NOX, mimics the proliferative rescue of pyruvate supplementation in ETC inhibition, directly

proving for the first time that pyruvate acts by maintaining a favorable cytosolic [NADH]/[NAD^+] ratio. Importantly, we could also target *Lb*NOX to the mitochondrial matrix (mito*Lb*NOX) to specifically oxidize this compartment. *Lb*NOX used in combination with various modes of ETC inhibition currently represents the gold standard for neatly separating the two major ETC functions: OXPHOS and maintenance of compartment-specific redox state.

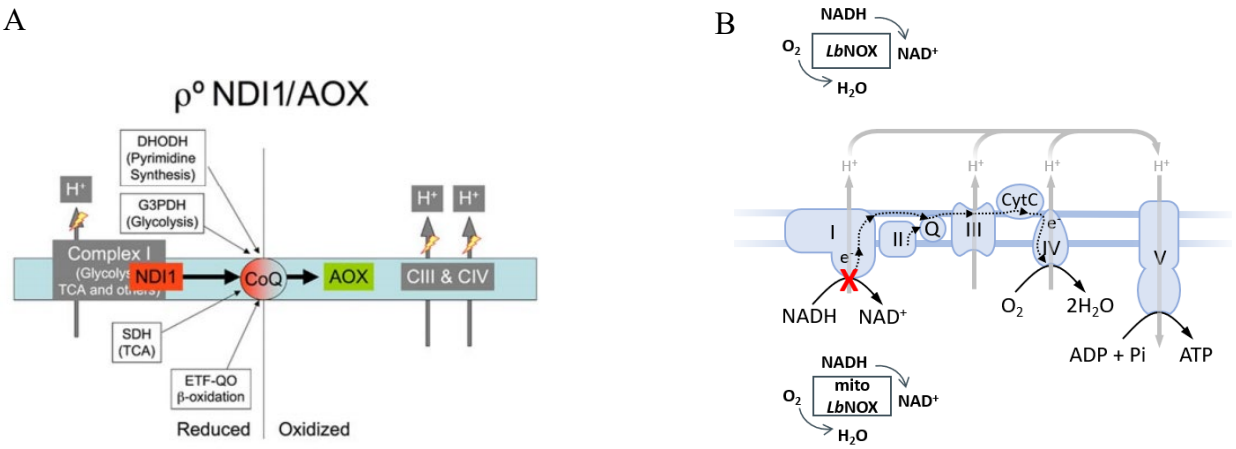

Figure 2.5: Transgene-based strategies for dissecting ETC functions.
(A) Schematic of the effects of NDI1 and AOX co-expression in mtDNA-depleted (ρ°) cells. From: Perales-Clemente et al., *PNAS*, 2008. **(B)** Net redox reaction of the ETC performed by a single soluble polypeptide, independent of the ETC, which is localized in the cytosol (*Lb*NOX) or can be targeted to the matrix (mito*Lb*NOX).

SECTION II. The integrated stress response.

The integrated stress response (ISR) is a major and widely studied pathway in eukaryotic cells triggered by a range of extrinsic or intrinsic insults, including nutrient deficiency, oxidative stress, unfolded protein stress and pathogen infection (**Figure 2.6**) [28].

Mechanism of activation

The ISR is typically activated by one or more members of a family of kinases that phosphorylate the alpha subunit of translation initiation factor 2 (eIF2α) on the residue designated serine-51 [29], [30]. While the number of eIF2α kinases varies along the eukaryotic evolutionary tree, vertebrates are known to possess four – and likely no more [31] – that are attuned to different stimuli:

a. GCN2 has been implicated principally in response to amino acid deficiency but also UV irradiation. It is activated by sensing of uncharged tRNA [32]–[36].

b. PERK is embedded across the endoplasmic reticulum (ER) membrane and is triggered by unfolded proteins in this compartment [37]–[40]. The ISR activated downstream of PERK usually comprises one of several branches of the broader ER unfolded protein response (UPR) [41].

c. PKR is activated mainly by binding of double-stranded RNA during viral infection [42]–[45]. It has also been implicated in promoting inflammatory responses to pathogen infection in immune cells, possibly independent of its eIF2α kinase activity [46].

d. HRI activity is suppressed by heme binding, which in mammals is critical for adaptation of hemoglobin producing red blood cells to fluctuations in iron availability [47]–[55].

While each kinase can be assigned stereotypical roles in sensing and transducing certain stresses, the mapping is not always one-to-one. Some conditions that lead to eIF2α phosphorylation, such as glucose limitation, severe hypoxia or oxidative stress, cannot be neatly attributed to a single kinase. This is likely because they impinge on the mechanism of activation of more than one kinase, possibly in a cell-type dependent manner. Moreover, knockout studies of individual kinases or combinations thereof have demonstrated a degree of functional redundancy among them [31], [56]. Further complicating the picture are numerous protein interactions, some of which are cell-type specific, that facilitate kinase activation or can inhibit it [57], [58].

The ISR exhibits a dynamic range of activation, not merely on/off behavior. Even in the absence of specific perturbation, a steady-state level of eIF2α phosphorylation is maintained in the cell as a balance between basal activity of the kinases and a constitutive eIF2α phosphatase (CReP) [59].

Mechanism of action

Phosphorylation of eIF2α acutely retards the initiation of cap-dependent mRNA translation, which affects most protein-coding transcripts in the cell [29]. At the same time, it activates translation of specific mRNA species encoding stress-responsive genes, including transcription factors that then induce their downstream targets [32], [60]–[62].

Both aspects of the response are elegantly coordinated by the role eIF2 plays in a ternary complex (TC) required for translation initiation, which it forms along with GTP and the charged initiator-methionine tRNA. The TC associates with the small ribosomal subunit and additional initiation factors to scan for the start codon (typically AUG) along an mRNA molecule. When the start codon is reached, GTP is hydrolyzed to GDP and the TC dissociates to allow complete assembly of the ribosome and translation elongation along the open reading frame (ORF). The protein eIF2B is

responsible for exchanging the GDP bound to eIF2 with a fresh GTP molecule so that the TC is regenerated and can participate in the next round of initiation [63]. Phosphorylation of eIF2α inhibits this nucleotide exchange, thus depleting the TC pool and impeding translation initiation [64]. This not only inhibits global protein synthesis but also facilitates sequestration of non-translated mRNA species into cytosolic stress granules [65], [66].

In parallel, reduced availability of the TC alters the way ribosomes translate a small class of transcripts that typically contain upstream ORFs (uORFs) preceding and/or overlapping the coding ORF [61], [62], [64], [67].[1] When the TC is plentiful, ribosomes preferentially initiate at the earlier uORFs instead of the coding ORF, depressing translation of the functional protein. But when the TC becomes scarce upon eIF2α phosphorylation, ribosomes switch to translating the coding ORF and the protein rapidly accumulates even in the absence of change in transcript levels (**Figure 2.7**).[2]

Among the genes whose translation is strongly upregulated by the ISR in mammalian cells are members of the activating transcription factor (ATF) family, especially ATF4[3] [68] and ATF5, and the transcription factor DDIT3/CHOP [69]. These and additional transcription factors interact, including through dimerization, to modulate levels of many target genes as well as promote their own transcription, reinforcing the translational response [70], [71]. Another prominent

[1] Though not all transcripts containing uORFs are translationally activated by the ISR. Much remains to be discovered about the mechanisms that distinguish the stress-responsive transcripts and that facilitate their stress-specific regulation, including possible involvement of mRNA modifications and RNA-binding proteins [118].

[2] eIF2α-independent ISR translation activation has been reported in the case of methionine deprivation, presumably because it directly depletes the charged initiator-methionine tRNA component of the TC [119]. Thus, some conditions may yield activation of ISR-related gene expression without involving eIF2α kinases if they otherwise impinge on TC availability [64].

[3] Mammalian homolog of GCN4 in the yeast *Saccharomyces cerevisiae*.

translationally-activated protein is GADD34, an induced eIF2α phosphatase poised to allow eventual resolution of the ISR in a negative feedback loop [72], [73].

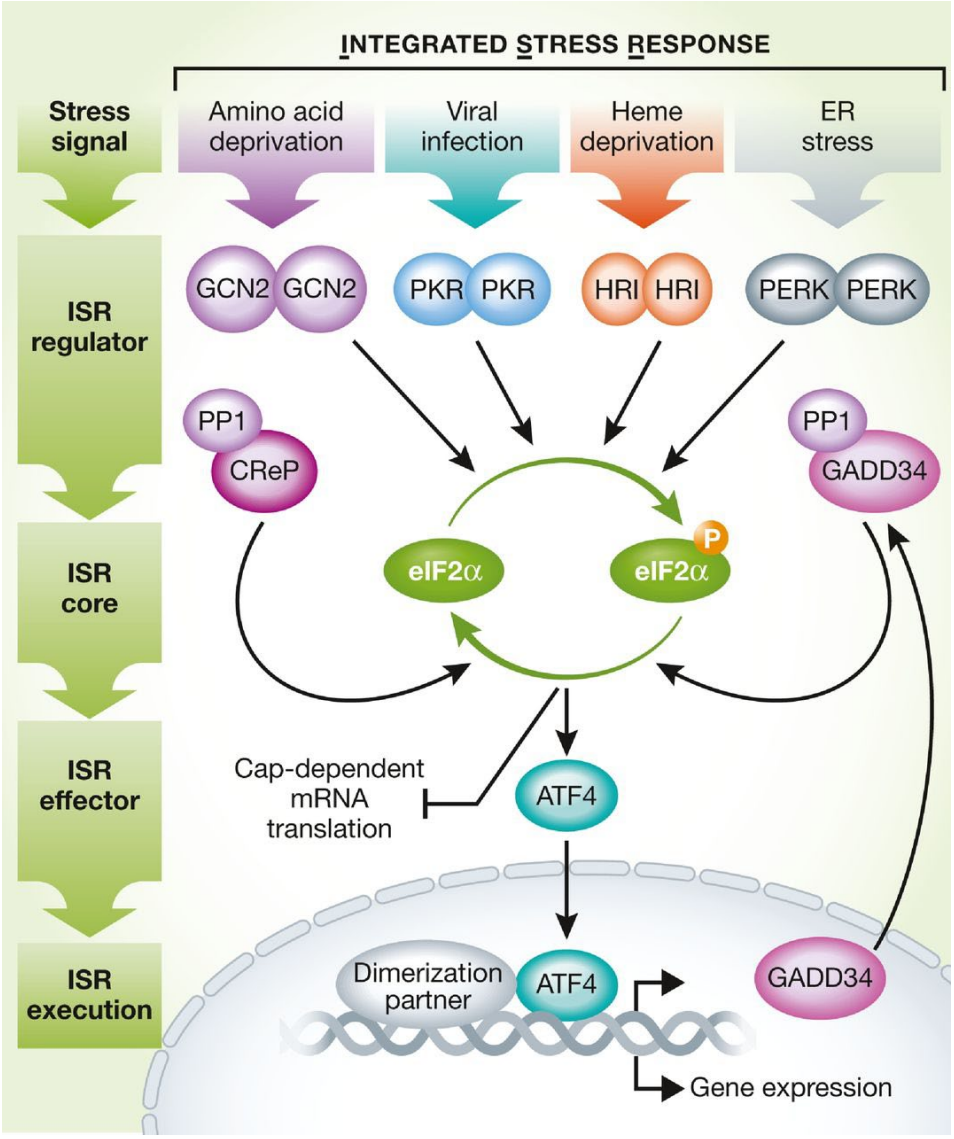

Figure 2.6: Overview of signal transduction and downstream effects during integrated stress response activation in mammalian cells.
From: Pakos-Zebrucka et al., *EMBO Rep.*, 2016.

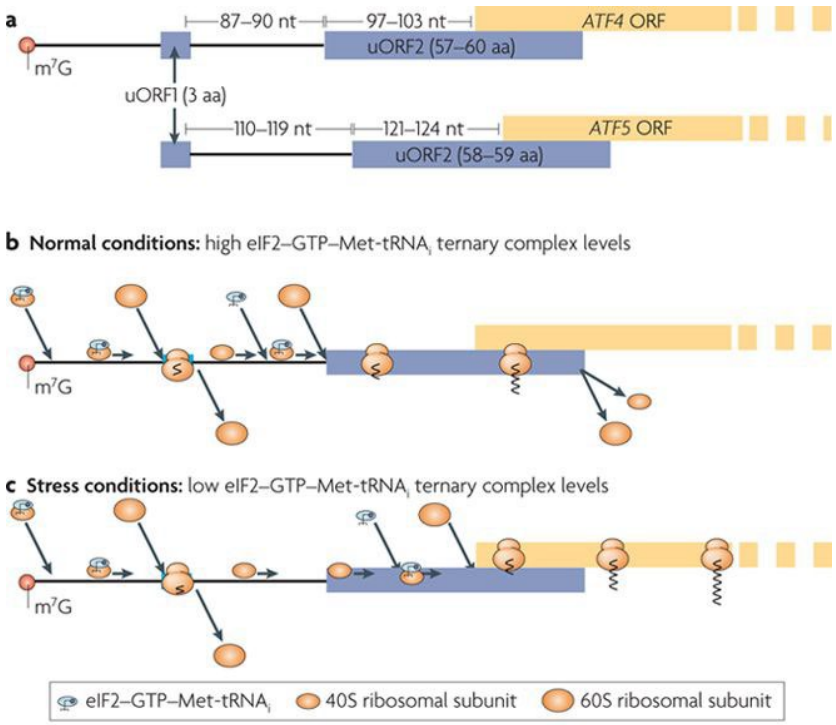

Figure 2.7: Ternary complex availability regulates translation of uORF-containing stress responsive transcripts.
From: Jackson et al., *Nat. Rev. Mol. Cell Bio.*, 2010.

It has recently emerged that mTORC1 signaling can also regulate translation of the transcription-factor ATF4 independently of eIF2α phosphorylation [74]. The mechanism underlying this regulation is not yet fully worked out but appears to also rely on the uORF structure of the transcript [75]. Upregulation of ATF4 translation by mTORC1, for example in response to growth signals, yields gene expression changes that resemble aspects of the canonical ISR [74]. However, this occurs in a very different cellular context, absent the extensive stress-related reconfiguration of the translatome and interactions with the full network of stress-induced transcription factors.

Functional logic of the ISR

Our increasingly detailed knowledge of the molecular mechanisms of ISR activation derives primarily from studies on unicellular organisms and mammalian cells *in vitro*, often utilizing

chemical inducers of the response. Naturally, these types of studies are not as well suited to elucidating the range of functional roles the ISR may play, let alone in multicellular organisms with tissue specialization and potential cell non-autonomous effects.

Nevertheless, a teleological approach can begin to paint an instructive, if simplistic, picture of the "purpose" the ISR may have originally evolved to play. The most immediate consequence of ISR activation is the attenuation of global protein synthesis. It is not difficult to imagine how this could be beneficial to cell survival during transient stress, as protein translation is one of the most demanding processes in terms of energy and resource consumption [76]. A break on protein synthesis can serve more specific protective roles in conditions that trigger the ISR, for example:

a. A pause in protein synthesis during ER unfolded protein stress will spare the organelle's chaperone and protein-folding systems further burden.

b. A pause in protein synthesis during viral infection will limit the virus's ability to hijack the cell's translation machinery for its own propagation.

c. A pause in protein synthesis during amino acid limitation will conserve cellular amino acid pools and prevent potential misincorporation of amino acids into newly-made proteins.

The transcriptional program induced by ISR-activated transcription factors, particularly ATF4 and DDIT3/CHOP, can vary somewhat between mammalian species and cell types. Nonetheless, it typically involves metabolic enzymes, amino acid transport and biosynthesis genes (particularly asparagine, serine and cysteine), cytosolic tRNA synthetases, translation factors, genes involved in antioxidant defense, chaperones, proteases, organelle quality control and autophagy-related genes, secreted factors, additional transcription factors and pro-apoptotic factors [70], [71].

This ensemble encompasses cytoprotective and compensatory elements that may help cope with stress, elements that can signal stress outside the cell, and elements that can prepare the cell for the resolution of stress and resumption of protein synthesis or for programmed cell death in case it cannot be resolved. However, the fact that a similar gene expression program is engaged in the face of a wide variety of conditions that trigger the ISR cautions against an attempt to assign it tailored adaptive purposes uniquely attuned to each one.

Animal studies have revealed that classic inducers of the ISR in unicellular organisms and cell culture are also operative in whole-body physiology. For example, dietary limitation of amino acids triggers the ISR downstream of GCN2 in several mouse tissues, including the liver and brain, and this modulates feeding behavior [77]–[79]. Activity of the ER-resident eIF2α kinase PERK is required to buffer protein folding overload in the ER of pancreatic β-cells and other secretory cells [80], [81]. And the ISR is increasingly appreciated as an integral component of the innate immune response *in vivo* [82].

However, the ISR is not only involved in coping with acute stress in animals. As a program attuned to external and internal cues and capable of orchestrating protein synthesis, metabolic remodeling and cell fate, elements of this pathway have been co-opted into the homeostatic mechanisms of various tissues. For example, ATF4 has been implicated *in vivo* in functions ranging from regulation of hepatic metabolism and glucose homeostasis to osteoblast differentiation and metabolic reprogramming during T-cell activation [83]–[86].

At the same time, ISR-related gene expression is frequently hijacked in tumor cells to support their biosynthetic requirements and cope with the stress of their microenvironment [87]–[89]. It is therefore considered a therapeutic target in this context. Moreover, chronic activation of the ISR has been associated with neurodegenerative conditions and may underlie aspects of their pathology

[90], [91]. Recent efforts have shown that inhibiting the ISR can be neuroprotective and prevent cognitive decline in some disease models, at least in part by alleviating the block on protein synthesis [92], [93]. Other neurological conditions benefit from pharmacological boosting of the ISR, underscoring the pleiotropic and context-dependent nature of this pathway [94].

The ISR and mitochondrial dysfunction

In 1996, Martinus et al. reported that depletion of mtDNA in rat hepatoma cells using ethidium bromide led to transcriptional upregulation of nuclear-encoded mitochondrially-localized protein chaperones [95]. They proposed the notion of a "mitochondrial stress response" analogous to the widely studied ER stress response. In 2002, using a model of aberrant matrix protein folding to induce mitochondrial dysfunction in cells, Zhao et al. identified the transcription factor DDIT3/CHOP as an activator of the mitochondrial chaperones [96]. These were the earliest hints that what we now refer to as the ISR is triggered in mitochondrial dysfunction.[4]

In 2007, Fujita et al. reported a microarray analysis of human cybrid cells harboring mtDNA mutations known to cause mitochondrial disease in patients [97]. They detected the upregulation of genes such as DDIT3/CHOP and ASNS that by then had been recognized as ATF4 targets in conditions that classically activate the ISR. Following additional *in vitro* studies [98], Tyynismaa et al. first observed the response *in vivo* in skeletal muscle of mice with progressive mitochondrial myopathy due to mtDNA deletions [99]. ISR activation has since been repeatedly observed in

[4] The early results involving chaperones spurred detailed studies of the transcriptional response to mitochondrial dysfunction in the worm *Caenorhabditis elegans* with an emphasis on protein folding and import as operative signals [120]–[129]. This was coined the mitochondrial unfolded protein response (mtUPR), analogously to the ER-related one. Some aspects of the worm mtUPR are reminiscent of observations in other studied models, principally mammalian cells, which has led to inconsistent terminology in the literature at times conflating and at times distinguishing the concepts of "mtUPR", "mitochondrial stress response" and "ISR". The focus of this thesis is on mammalian systems and I refer throughout to ISR activation by mitochondrial dysfunction, rather than to mtUPR, as this conceptualization more precisely reflects, in my view, the currently available evidence on the pathway and eschews the implicit assumption of a proteostasis-related signaling mechanism.

different modes of mitochondrial dysfunction across human patients, animal models and mammalian cells in culture [100]–[108], firmly establishing it within the scope of "retrograde signaling": communication from mitochondria to the nucleus [109].

The ISR is now understood to underlie the secretion, especially from skeletal muscle, of circulating metabolic cytokines FGF21 and GDF15 in some mitochondrial disorders [104], [106]. These proteins affect systemic metabolism and are increasingly used as sensitive (though not specific) biomarkers of disease. In addition, the ISR strongly induces expression of the mitochondrial 1-carbon pathway, which is paradoxically inhibited in mitochondrial dysfunction due to excess matrix NADH, the *de novo* serine biosynthesis pathway and the transsulfuration pathway, which can support biosynthesis of glutathione, a potent cellular antioxidant [99], [103], [110]. It can also promote expression of mitochondrially-targeted chaperones and proteases.

Despite a decade of studies into the ISR in mitochondrial dysfunction, it remains unclear whether its activation serves a protective role or represents aberrant signaling for which evolution has not had occasion to account. The seemingly more tractable question of how mitochondrial dysfunction signals to activate the response similarly remains unresolved.

Mitochondria are intimately integrated into many cellular processes whose failure could theoretically impinge on the mechanism of activation of eIF2α kinases. Thus, many reactions of amino acid metabolism occur in mitochondria (GCN2), mitochondria physically interact with the ER network (PERK), mitochondria possess a repertoire of "foreign" mtDNA-encoded RNA species (PKR), and critical steps in heme biosynthesis also take place in mitochondria (HRI). Signaling routes beyond canonical ISR triggers, including ROS and perturbed mitochondrial proteostasis, have also been proposed, as well as involvement of non-canonical regulation of ATF4 by mTORC1 [111], [112].

Several factors complicate the study of this question. First, mitochondrial functions are tightly interconnected and ISR signaling is frequently redundant. Second, signaling in chronic models of mitochondrial dysfunction is difficult to interpret because of the potential for secondary and feedback effects. Acute models, on the other hand, typically involve chemical inhibitors that do not necessarily mimic physiological conditions and whose off-target effects can act as confounders. More fundamentally, both mitochondrial dysfunction and cellular signaling display extensive variability among cell types and disease models that defies simple generalization.

While multiple routes from mitochondrial dysfunction to ISR activation likely exist, newly available tools for manipulating ISR signaling ([67], [113], [114]) and for manipulating specific mitochondrial functions – from genetically-encoded metabolic modifiers ([27], [115]) to inhibitors of specific ROS production sites ([116], [117]) – hold great promise for achieving a more refined mapping of the range of levers that mitochondria can pull to communicate their dysfunction to the rest of the cell.

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CHAPTER 3

The integrated stress response is triggered by distinct metabolic consequences of electron transport chain dysfunction in proliferating and post-mitotic cells

The integrated stress response is triggered by distinct metabolic consequences of electron transport chain dysfunction in proliferating and post-mitotic cells

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(Original research article, in preparation for submission. Author contributions at end of chapter.)

ABSTRACT

Mitochondrial dysfunction is frequently associated with activation of the integrated stress response (ISR) in human patients, animal models and mammalian cells *in vitro*. What functional consequences of mitochondrial dysfunction can trigger the response is incompletely understood. We compared the sensitivity of the ISR to electron transport chain (ETC) dysfunction in proliferating and terminally-differentiated muscle cells using a combination of acute ETC inhibitors and genetically-encoded tools for buffering their effects. In proliferating cells, complex I inhibition potently triggered the ISR specifically due to failure of NADH oxidation which sharply depressed cellular aspartate levels and activated the eIF2α kinase GCN2. This could be prevented by expressing a protein prosthetic that restored cytosolic NAD^+ recycling or by aspartate supplementation. Strikingly, this route to ISR activation became inoperative in differentiated myotubes where only ATP synthase inhibition triggered the ISR through a distinct route dependent on residual ETC activity. The ISR was largely agnostic to defects in ATP synthesis by oxidative phosphorylation. Finally, we did not observe direct surveillance of mtDNA gene expression by the ISR. These results provide clarity as to bioenergetic and metabolic ramifications of mitochondrial dysfunction that trigger the ISR and how this may vary with metabolic context. Our findings can inform ongoing work to unravel the basis for ISR activation during mitochondrial dysfunction *in vivo*, which likely involves multiple signaling routes.

INTRODUCTION

The mitochondrial electron transport chain (ETC) plays key roles in the physiology of eukaryotic cells. It facilitates efficient ATP synthesis via oxidative phosphorylation (OXPHOS), maintains the balance of reducing equivalents essential for a range of metabolic reactions, supports the electrochemical gradient that drives movement of metabolites and proteins across the mitochondrial inner-membrane, and modulates cellular oxygen levels and the production of reactive oxygen species (ROS).

Genetic lesions in ETC components or in factors required for their expression and assembly, whether encoded by the nuclear genome or the mitochondrial genome (mtDNA), underlie a class of devastating inborn errors of metabolism affecting roughly 1 in 5,000 live births [1]–[3]. These conditions exhibit extreme heterogeneity of disease severity and manifestation among tissues and cell types, even when the causal gene is ubiquitously expressed. Deciphering how cells sense and respond to an impaired ETC is thus a major challenge in mitochondrial medicine and can also inform common conditions that exhibit a decline in ETC function, such as neurodegeneration [4]–[7], obesity and diabetes [8]–[11], certain types of cancer [12] and the ageing process [13], [14].

A fundamental distinction among cells is whether they are proliferating or post-mitotic. The metabolism of the two cell states is geared toward different purposes – nutrient uptake and biosynthesis versus self-maintenance and specialized functions [15], [16]. Mitochondrial disease typically manifests in post-mitotic tissues, such as skeletal muscle, heart, liver and brain, yet most studies *in vitro* utilize proliferating cells. Possible differences in the response to mitochondrial dysfunction between proliferating and post-mitotic cells have not been systematically addressed.

One way that cells react to mitochondrial dysfunction is by engaging gene expression programs. The program most frequently observed in this context, both *in vivo* and *in vitro*, is the integrated stress response (ISR) [17]–[28]. The ISR can be triggered by diverse insults such as nutrient deficiency, unfolded protein or pathogen infection [29]. In mammalian cells, it is typically activated by a family of four kinases that can phosphorylate the alpha subunit of translation initiation factor 2 (eIF2α). This attenuates global protein synthesis while simultaneously activating translation of transcription factors like ATF4 and DDIT3/CHOP [30]–[33].

ISR activation in tissues affected by mitochondrial disorders is thought to underlie the secretion of circulating metabolic cytokines, such as FGF21 and GDF15, that serve as potential biomarkers of disease and may mediate systemic effects of mitochondrial dysfunction [34], [35]. It also activates the *de novo* serine biosynthesis, transsulfuration and mitochondrial 1-carbon pathways [17], [25], [36]. It remains unclear whether chronic ISR activation in this setting is protective or ultimately detrimental [18], [37], [38].

Moreover, how mitochondrial dysfunction is sensed to trigger the ISR is incompletely understood. Evidence *in vivo* suggests defects in mtDNA maintenance and gene expression are most frequently associated with ISR activation [28], [34]. However, such defects are expected to have multiple intertwined effects on bioenergetics, metabolism and proteostasis that could potentially trigger the ISR but are very difficult to disentangle in chronic genetic models.

To shed light on potential routes of ISR activation by mitochondrial dysfunction, we tested whether specifically perturbing bioenergetic parameters tied to the ETC can trigger the response in muscle cells that were proliferating (myoblasts) or terminally-differentiated (myotubes). We used a panel of small-molecule inhibitors to acutely manipulate ETC functions and measured the effects on metabolism and global gene expression. We used *Lb*NOX, a bacterial enzyme that performs the

net redox reaction of the ETC independently of it [39], to neatly separate the contribution of two principal ETC functions: compartment-specific NADH oxidation and ATP synthesis by OXPHOS.

Our experiments revealed two paths to ISR activation through bioenergetic perturbation. In myoblasts, impaired NADH oxidation was responsible for triggering the ISR during complex I inhibition, mostly due to the resulting sharp drop in aspartate levels which activated the eIF2α kinase GCN2. This path became inoperative in myotubes where only ATP synthase inhibition elicited a significant response. This was not due to impaired ATP production by OXPHOS but was rather dependent on residual ETC activity in this setting. Finally, inhibiting translation of mtDNA-encoded transcripts failed to trigger the ISR in myotubes, and did so only upon emergence of a redox defect in myoblasts, arguing against direct surveillance of mtDNA gene expression.

RESULTS

ETC inhibition in C2C12 myoblasts potently triggers the ISR and depresses proliferative gene expression

We used RNA sequencing to profile changes in global gene expression in rapidly proliferating sub-confluent C2C12 mouse muscle cells (myoblasts) following a 10-hour treatment with small-molecule inhibitors of ETC complex I (piericidin, 0.5uM), complex III (antimycin, 0.5uM) or ATP synthase (oligomycin, 1uM) (**Figure 3.1A**). We confirmed these inhibitor concentrations yielded the expected effects on cellular respiration (**Figure 3.1B**). The cells were cultured in DMEM containing 10% dialyzed fetal bovine serum, 10mM glucose and no pyruvate or uridine.

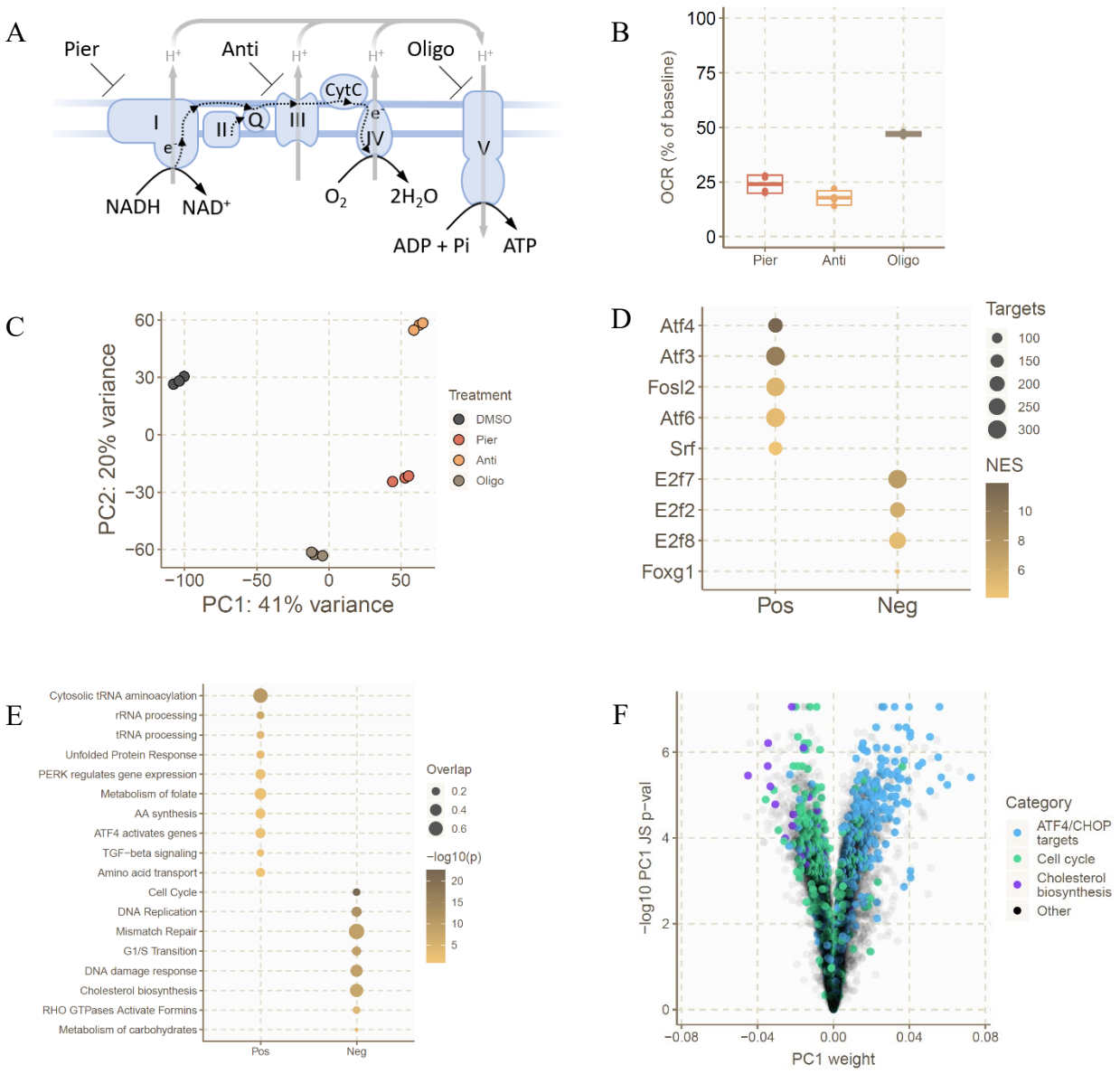

Figure 3.1: ETC inhibition in C2C12 myoblasts potently triggers the ISR and depresses proliferative gene expression. **(A)** Schematic of experimental design. **(B)** Fraction of cellular oxygen consumption rate remaining following treatment with ETC inhibitors (Seahorse). **(C)** Principal components analysis of global gene expression levels in ETC treated cells inferred from RNA sequencing. **(D)** iRegulon analysis of putative transcription factor binding sites in genes positively or negatively associated with PC1. NES = normalized enrichment score. **(E)** Functional enrichment of REACTOME pathways in genes positively or negatively associated with PC1. **(F)** Volcano plot highlighting enriched functional categories among genes significantly associated with PC1. JS = jackstraw.

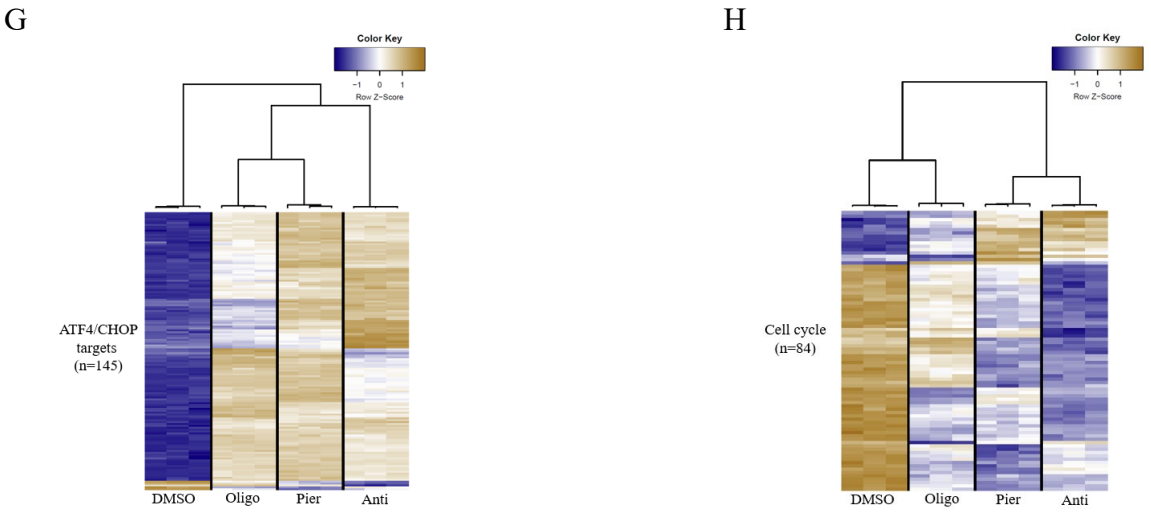

Figure 3.1 (continued): (G) Hierarchically-clustered, row-normalized expression heatmap of ChIP-seq derived ATF4 and DDIT3/CHOP targets among the genes significantly associated with PC1. **(H)** Hierarchically-clustered, row-normalized expression heatmap of REACTOME cell cycle pathway members among genes significantly associated with PC1.

Principal components analysis of the myoblast gene expression data revealed a dominant first principal component (PC1; 41% of the variance) along which all three inhibitors progressed, with piericidin and antimycin displaying a stronger effect and oligomycin lagging (**Figure 3.1C**). To gain insight into genes driving variation along PC1, we first applied the jackstraw method to select genes significantly associated ($p < 0.001$) with PC1 [40]. We then subjected the 500 genes with the most positive PC1 weights (upregulated along PC1) and the 500 genes with the most negative weights (downregulated along PC1) to analysis of *cis*-regulatory motifs using iRegulon [41] (**Figure 3.1D**). We also subjected the top 1000 genes in each direction to pathway enrichment analysis based on the REACTOME database [42], as implemented in Enrichr [43] (**Figure 3.1E**).

Both approaches converged on activation of the ISR as a major trend driving gene expression following ETC inhibition. Thus, *cis*-regulatory analysis identified binding motifs for the ISR master regulator ATF4 and related family members as the most enriched in promoters of genes

upregulated along PC1 while pathway analysis identified multiple gene sets controlled by ATF4, such as cytosolic tRNA synthetases, 1-carbon (folate) metabolism and amino acid synthesis and transport, as well as gene sets representing effectors of the ISR.

At the same time, the analyses highlighted downregulation of proliferation-related gene expression. Downregulated gene promoters were enriched for binding motifs of E2F family transcription factors – master regulators of the cell cycle – while genes in pathways related to cell cycle progression, DNA replication and cholesterol biosynthesis were the most sharply depressed. This is consistent with the proliferative arrest ETC inhibition is expected to impose in our media conditions. Of note, the ISR itself promotes cell cycle arrest, mainly by repressing translation of short-lived cyclin D1 [44], though it is likely not the only contributor to this outcome.

We annotated a volcano plot, showing the significance and magnitude of association with PC1 for each gene in our dataset, with *bona fide* ATF4 and DDIT3/CHOP target genes obtained from mouse ChIP-seq data [45] as well as the REACTOME cell cycle and cholesterol biosynthesis pathways (**Figure 3.1F**). This further illustrated the striking enrichment of these gene categories among the most differentially expressed genes upon ETC inhibition. We then performed hierarchical clustering on expression levels of PC1-associated genes ($P < 0.0001$) belonging to these categories. The resulting heatmaps confirmed that the gene expression signatures of ISR activation and proliferative arrest mirrored one another (**Figure 3.1-G, H**).

Oxidizing the cytosol is sufficient to ablate ISR activation by complex I inhibition

We wondered whether the variation in the magnitude of ISR activation among the inhibitors could inform the search for signals that trigger the response. Inhibition of complex I and complex III triggered the ISR more potently than ATP synthase inhibition. We thus reasoned that lack of ATP

synthesis by OXPHOS was less likely to play the operative role in ISR activation in our experimental setup. NADH oxidation, on the other hand, should be affected more drastically by inhibition of complex I or complex III and so presented the more compelling candidate.

To facilitate specific dissection of the contribution of redox perturbation to ISR activation by ETC inhibition, we generated myoblasts expressing untargeted *Lb*NOX or a matrix-targeted variant (mito*Lb*NOX) under a doxycycline (DOX)-inducible promoter. After 24 hours of induction, these cells maintain the ability to oxidize NADH in the cytosol or in the matrix, respectively, independent of the ETC (**Figure 3.2A**). We then measured the effect of acute ETC inhibition on: i) whole-cell [NADH]/[NAD^+], which primarily reflects the matrix ratio [39], [46], [47]; and ii) secreted [lactate]/[pyruvate], which is a proxy for cytosolic [NADH]/[NAD^+] through the equilibrium of the lactate dehydrogenase (LDH) reaction [48].

We confirmed that antimycin and piericidin caused a significantly larger increase in whole-cell [NADH]/[NAD^+] as compared to oligomycin (**Figure 3.2B**), likely due to the ability of leak respiration to maintain residual matrix oxidation in the latter case. The increase in the ratio was effectively prevented by mito*Lb*NOX, as expected, whereas *Lb*NOX could only partially buffer it, reflecting the smaller cytosolic contribution to the whole-cell signal.

Interestingly, the inhibitors increased secreted [lactate]/[pyruvate] in a similar pattern (**Figure 3.2C**). This is likely due to differential effects on the shuttles that facilitate oxidation of cytosolic reducing equivalents in the mitochondria. Matrix NADH buildup is expected to stall the tricarboxylic acid (TCA) cycle, and consequently the malate-aspartate shuttle, in both piericidin and antimycin. Only antimycin, however, is also expected to cause over-reduction of CoQ, which both inhibits the TCA cycle and blocks the glycerol-3-phosphate shuttle. The lesser effect of oligomycin could again be attributed to proton leak.

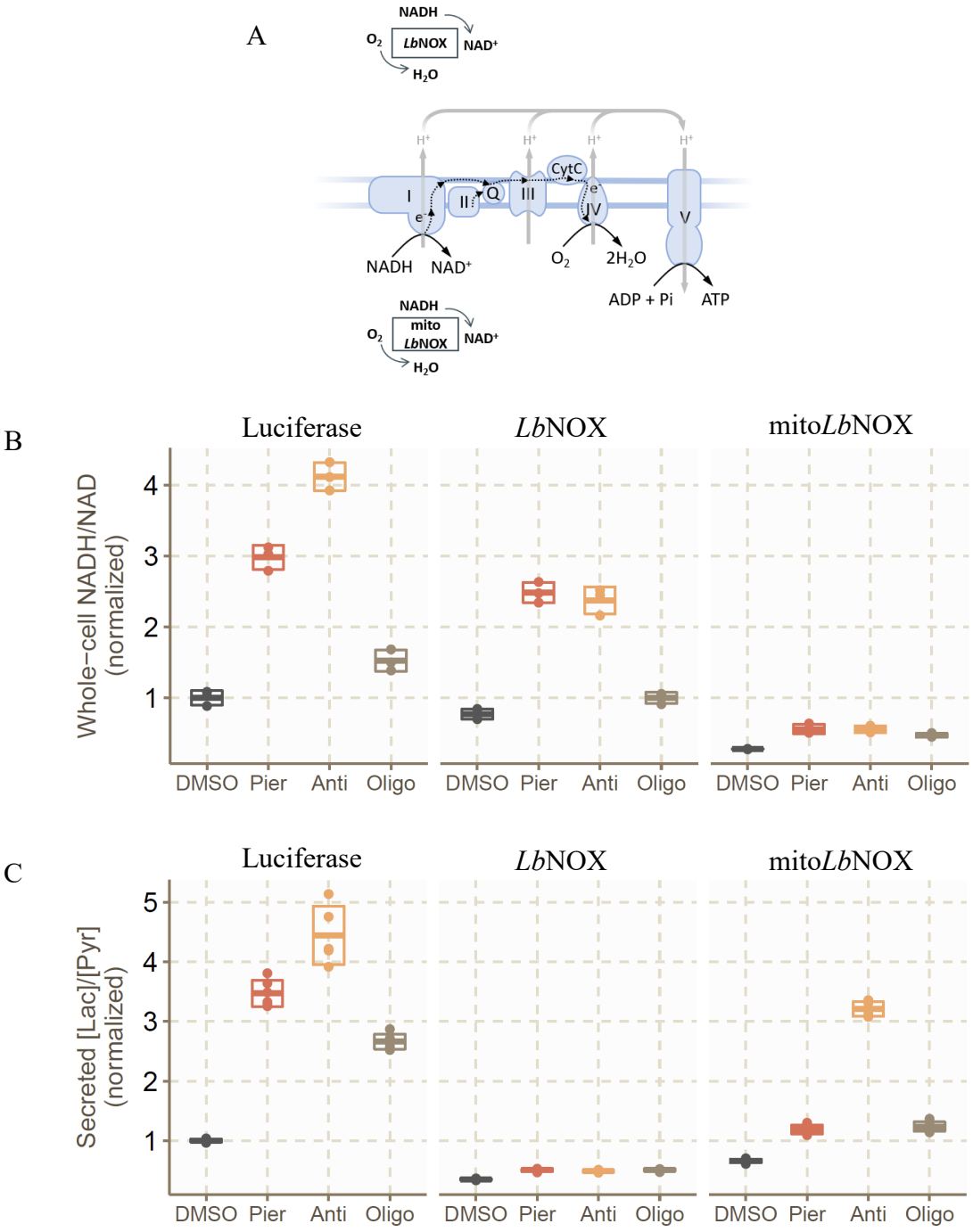

Figure 3.2: (A) Schematic of *Lb*NOX and mito*Lb*NOX activity and localization. **(B)** LC/MS-based [NADH]/[NAD$^+$] measurements from cell extracts of myoblasts expressing DOX-inducible Luciferase, *Lb*NOX or mito*Lb*NOX and treated with ETC inhibitors for 1 hour. **(C)** LC/MS measurements of [lactate]/[pyruvate] in spent media of myoblasts expressing Luciferase, *Lb*NOX or mito*Lb*NOX and treated with ETC inhibitors for 2 hours.

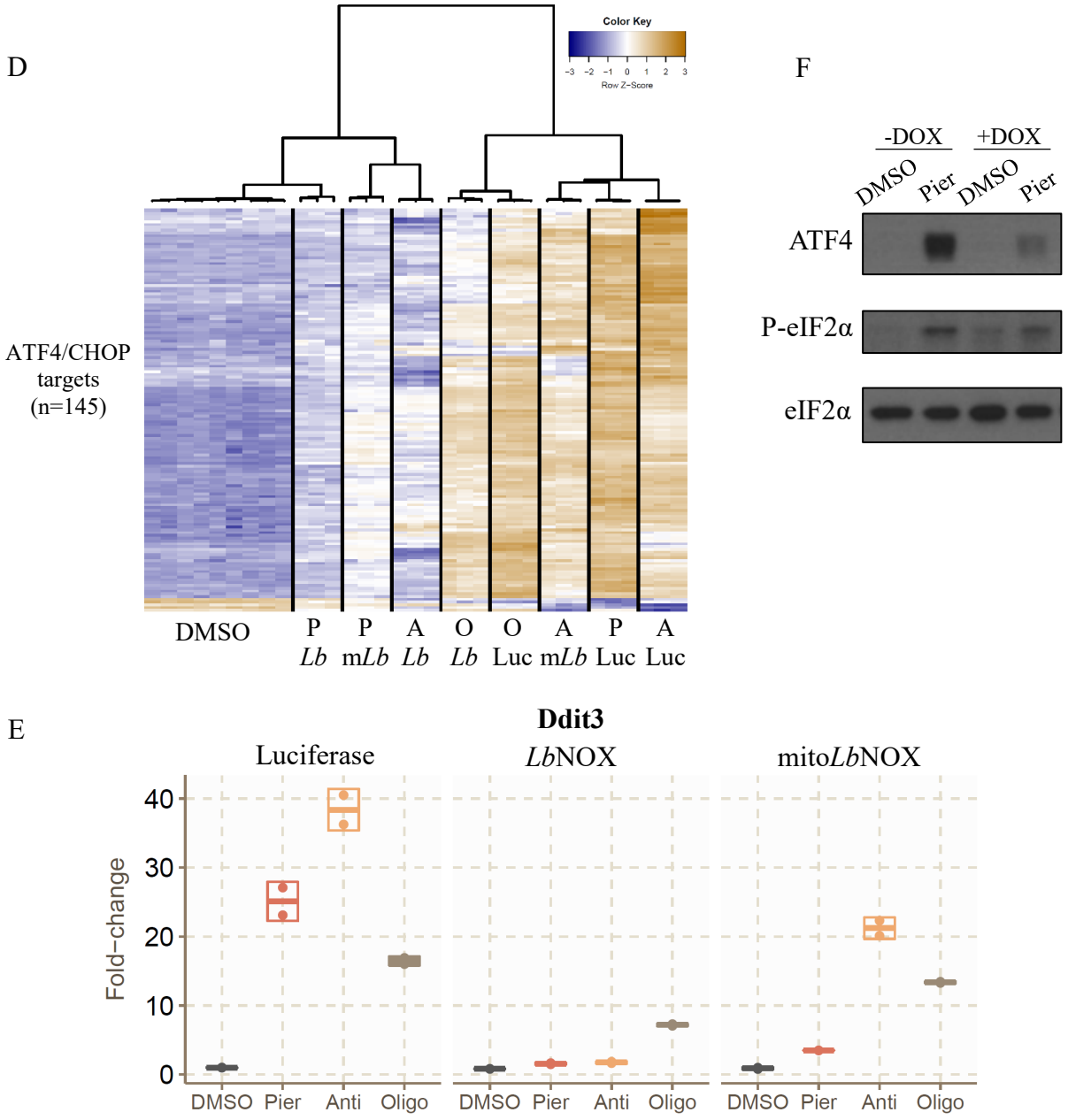

Figure 3.2 (continued): (D) Hierarchically-clustered, row-normalized expression heatmap of the same ATF4 and DDIT3/CHOP target genes from Figure 3.1G in myoblasts expressing DOX-inducible Luciferase, *Lb*NOX or mito*Lb*NOX and treated with ETC inhibitors for 10 hours. P = piericidin, A = antimycin, O = oligomycin. **(E)** Validation by qPCR of Ddit3 gene expression levels in the same samples as in D. **(F)** Representative Western blot of ATF4, P-eIF2α and total eIF2α in myoblasts treated for 6 hours with piericidin after 24 hours of DOX-inducible *Lb*NOX expression or in the absence of DOX induction.

As expected, *Lb*NOX expression maintained a low [lactate]/[pyruvate] ratio in all conditions since it directly oxidizes the cytosol. In contrast, mito*Lb*NOX only significantly buffered the effect of piericidin and oligomycin. Its effect on the cytosol remains dependent on redox shuttles and while it can alleviate matrix NADH buildup, it cannot correct over-reduction of CoQ by antimycin.

Having confirmed the redox buffering effects of *Lb*NOX and mito*Lb*NOX, we compared ISR gene expression in these cells and in control cells treated with ETC inhibitors (**Figure 3.2D**). Strikingly, *Lb*NOX expression was sufficient to almost completely ablate ISR activation upon complex I or III inhibition but only partially reduced activation by ATP synthase inhibition. mito*Lb*NOX also significantly attenuated ISR activation with complex I inhibition but proved far less effective with complex III, possibly due to its inability to oxidize the cytosol in the latter case. As validation, we performed qPCR on the Ddit3 transcript, itself a sensitive marker of ISR activation (**Figure 3.2E**).

We wondered at which regulatory step *Lb*NOX expression ablated ISR gene expression. We blotted for eIF2α phosphorylation and for ATF4 protein, which are upstream events in ISR activation. Cells where *Lb*NOX expression was induced showed markedly less ATF4 protein accumulation and less eIF2α phosphorylation after piericidin treatment than cells where *Lb*NOX expression was not induced (**Figure 3.2F**). This suggested oxidizing the cytosol prevented the signaling whereby cells otherwise sensed complex I inhibition to trigger the ISR.

Aspartate deficiency is a direct activator of ISR gene expression in complex I inhibition

We sought to identify the direct functional consequence of complex I inhibition that is sensed to trigger the ISR and that can be buffered by oxidizing the cytosol. One possibility was that glycolysis was inhibited by an elevated cytosolic [NADH]/[NAD^+] ratio, limiting the ability of myoblasts to support their energy charge in the absence of ATP synthesis by OXPHOS.

Using lactate secretion as a proxy for glycolytic flux, we found that only complex III inhibition led to a drop in flux in control cells (**Figure 3.3A**). Moreover, complex I and ATP synthase inhibition were indistinguishable in their effects. While *Lb*NOX expression did stimulate glycolysis, this did not translate into increased energy charge, and in fact the opposite was true in the case of complex I inhibition (**Figure 3.3B**). Thus, energy charge did not correlate with ISR activation despite its well-established roles in acute regulation of metabolism and signaling.

Another possibility was that the combined effects of increased matrix and cytosolic [NADH]/[NAD^+] depleted a critical nutrient, which then triggered the ISR. It has recently emerged that aspartate becomes a limiting factor for cell proliferation during ETC inhibition. This results from depletion of its precursor oxaloacetate, which itself derives from redox coupled reactions in the matrix (TCA cycle) or the cytosol. Aspartate is required in proliferating cells for both protein and nucleotide biosynthesis [49]–[51].

We therefore measured steady-state aspartate levels in control and *Lb*NOX-expressing cells (**Figure 3.3C**). Inhibition of complex I or complex III led to a steep drop in aspartate in control cells (17-fold and 32-fold, respectively), whereas ATP synthase inhibition caused less than a 2-fold drop. Oxidizing the cytosol using *Lb*NOX supported aspartate levels, likely by facilitating flux through the NADH-dependent cytosolic MDH1 (malate dehydrogenase 1) reaction to produce oxaloacetate, which GOT1 (aspartate aminotransferase 1) converts to aspartate [49].

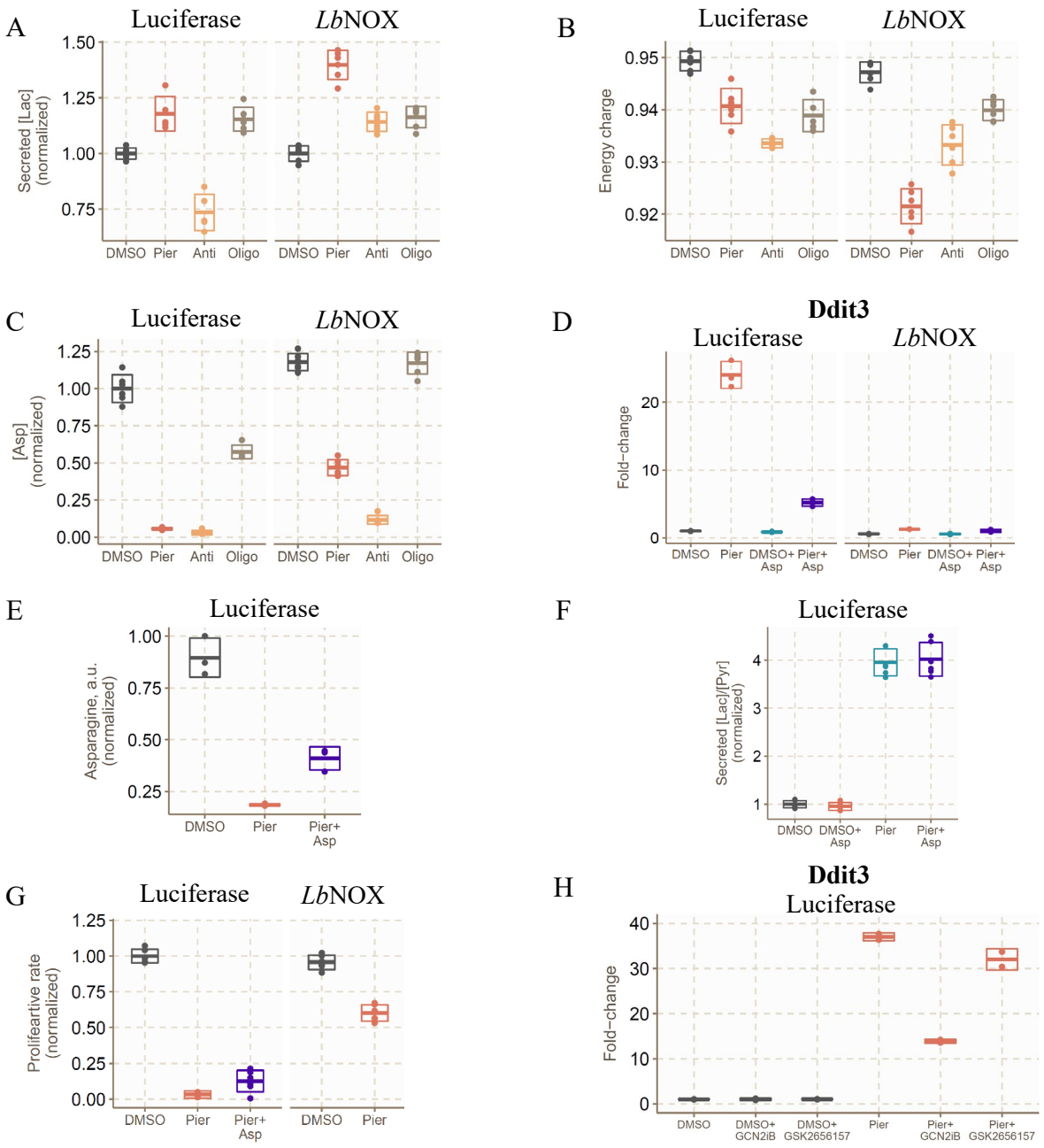

Figure 3.3: (A) Lactate secretion following 2-hour treatment with ETC inhibitors. **(B)** Adenylate energy charge following 1-hour treatment with ETC inhibitors, calculated as ([ATP]+0.5*[ADP])/([ATP]+[ADP]+[AMP]) from LC/MS-derived absolute concentrations. **(C)** Intracellular [aspartate] (absolute) following 1-hour treatment with ETC inhibitors. **(D)** Ddit3 transcript levels upon 10-hour complex I inhibition with 10mM aspartate (qPCR). **(E)** Intracellular asparagine (relative) upon complex I inhibition with 10mM aspartate. **(F)** Secreted [lactate]/[pyruvate] upon complex I inhibition with aspartate. **(G)** Proliferative rate (doublings per 24 hours) of complex I inhibited cells with aspartate. **(H)** Ddit3 transcript levels upon 10-hour complex I inhibition with GCN2 or PERK inhibition (qPCR).

If aspartate indeed signals ETC inhibition to activate the ISR in myoblasts, then exogenous supplementation of this amino acid should abrogate the response. Most mammalian cells do not transport aspartate efficiently across the plasma membrane, which is why media concentrations as high as 10-20mM are required to rescue proliferation in ETC inhibited cells [49], [50]. Using qPCR on the Ddit3 transcript, we found that 10mM aspartate significantly attenuated ISR activation upon complex I inhibition, though not to the same extent as *Lb*NOX expression (**Figure 3.3D**).

10mM aspartate was sufficient to support cellular levels of a downstream metabolite like asparagine during complex I inhibition (**Figure 3.3E**) but it did not oxidize the cytosol (**Figure 3.3F**) nor restore cell proliferation to an appreciable extent (**Figure 3.3G**). This suggests aspartate supplementation attenuated the response because the amino acid deficiency was directly sensed to trigger the ISR. Aspartate (and asparagine) deficiency could plausibly be sensed by the amino acid sensitive eIF2α kinase GCN2. To test this, we used a recently described specific inhibitor of the kinase (GCN2iB) [52]. Co-treatment of cells with GCN2iB significantly depressed ISR gene expression in response to complex I inhibition whereas interfering with signaling by the ER-resident eIF2α kinase PERK using GSK2656157 [53] was ineffective (**Figure 3.3H**).

Lack of ISR activation by complex I or complex III inhibition in myotubes reflects reduced reliance on ETC NADH oxidation

We wondered whether similar patterns of ISR activation with ETC inhibition would be observed in both dividing and non-dividing cells since they have distinct metabolic programs that could be differentially impacted by mitochondrial dysfunction [15], [16]. Our model system provided a natural juxtaposition of these two cell states as C2C12 myoblasts can be induced to exit the cell cycle and then terminally-differentiate into myotubes. We therefore treated myotubes that had been differentiated in low serum for 4 days with the ETC inhibitors. Global gene expression profiling

following 10 hours of treatment revealed that differentiated myotubes only minimally activated the ISR in response to complex I or complex III inhibition, in striking contrast to proliferating myoblasts. In this cell state, only ATP synthase inhibition significantly triggered the response (**Figure 3.4A**).

We reasoned that the muted response to complex I or complex III inhibition in differentiated myotubes stemmed from their reduced reliance on the NADH oxidizing function of the ETC, as compared to myoblasts. In support of this, the pattern of ISR activation with the different inhibitors in myotubes was similar to that in myoblasts expressing *Lb*NOX, such that the cytosolic redox perturbation was corrected (see Figure 3.2E).

To examine this further, we measured secreted [lactate]/[pyruvate] in spent media of myotubes treated with ETC inhibitors. These cells did experience a significant increase in cytosolic [NADH]/[NAD^+] upon ETC inhibition, which we could reverse with *Lb*NOX (**Figure 3.4B**). However, it did not stem from failure of complex I to oxidize cytosolic reducing equivalents, as in myoblasts, since maintaining matrix NADH oxidation by mito*Lb*NOX did not impact the cytosolic ratio. Thus, cytosolic and matrix redox pools became weakly connected in myotubes, and only the glycerol-3-phosphate shuttle, which is uniquely sensitive to antimycin, appeared operative.

Instead, the increase in cytosolic [NADH]/[NAD^+] appeared driven mostly by a ~2-fold increase in glycolytic flux, regardless of the specific inhibitor used (**Figure 3.4C**). While glycolysis is net redox neutral at steady-state, increased flux elevates that steady-state according to LDH equilibrium. In the absence of ATP synthesis by OXPHOS, increased glycolytic flux was likely required to support the energy charge, which was stabilized at the same level across all the inhibitors (**Fig. 3.4D**).

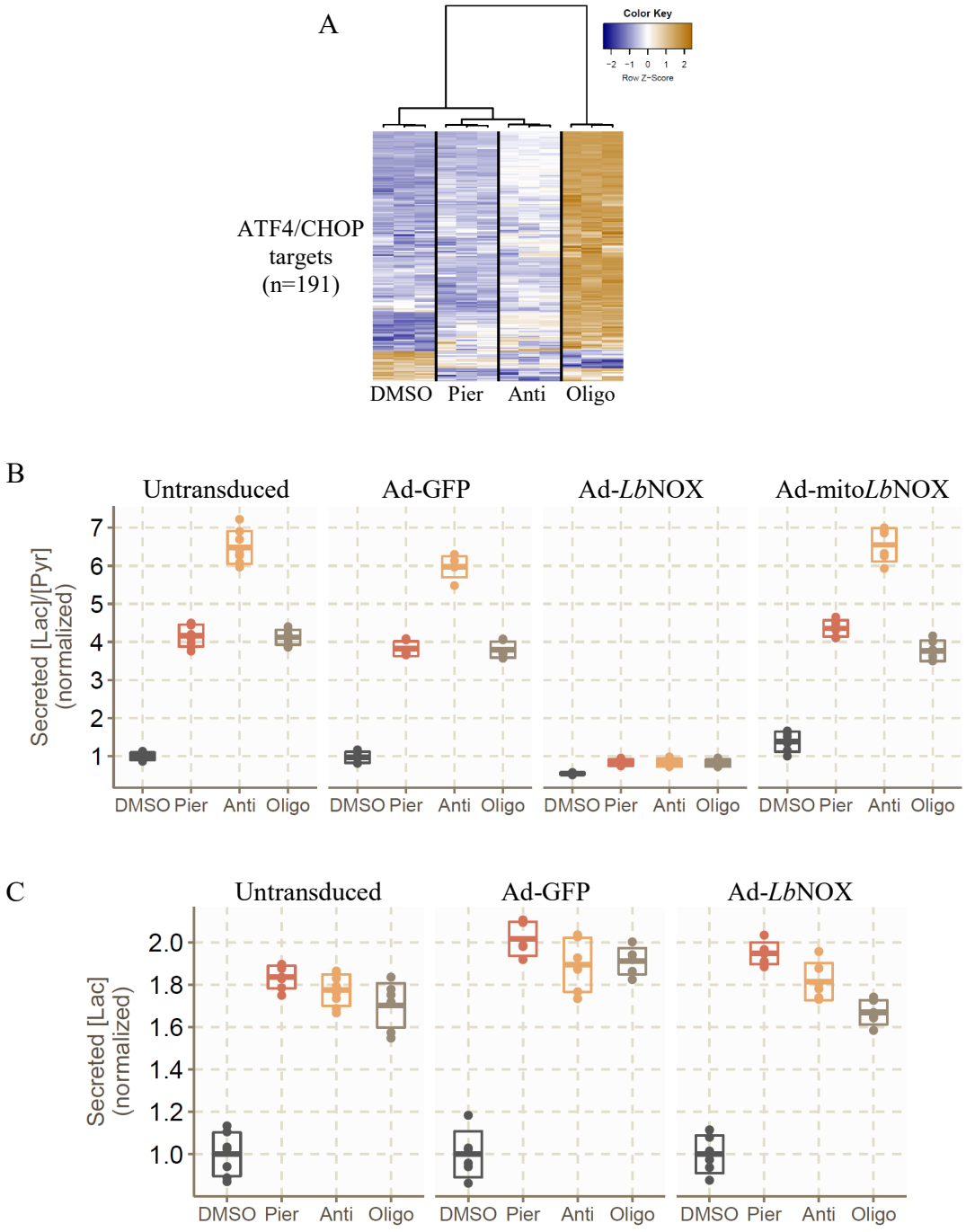

Figure 3.4: (A) Hierarchically-clustered, row-normalized expression heatmap of ATF4 and DDIT3/CHOP targets among all differentially expressed genes (FDR < 0.001) following 10-hour treatment with any of the ETC inhibitors in myotubes. **(B)** LC/MS measurements of [lactate]/[pyruvate] in spent media of myotubes expressing GFP, *Lb*NOX or mito*Lb*NOX using adenovirus and treated with ETC inhibitors for 2 hours. **(C)** Lactate secretion following 2-hour treatment with ETC inhibitors.

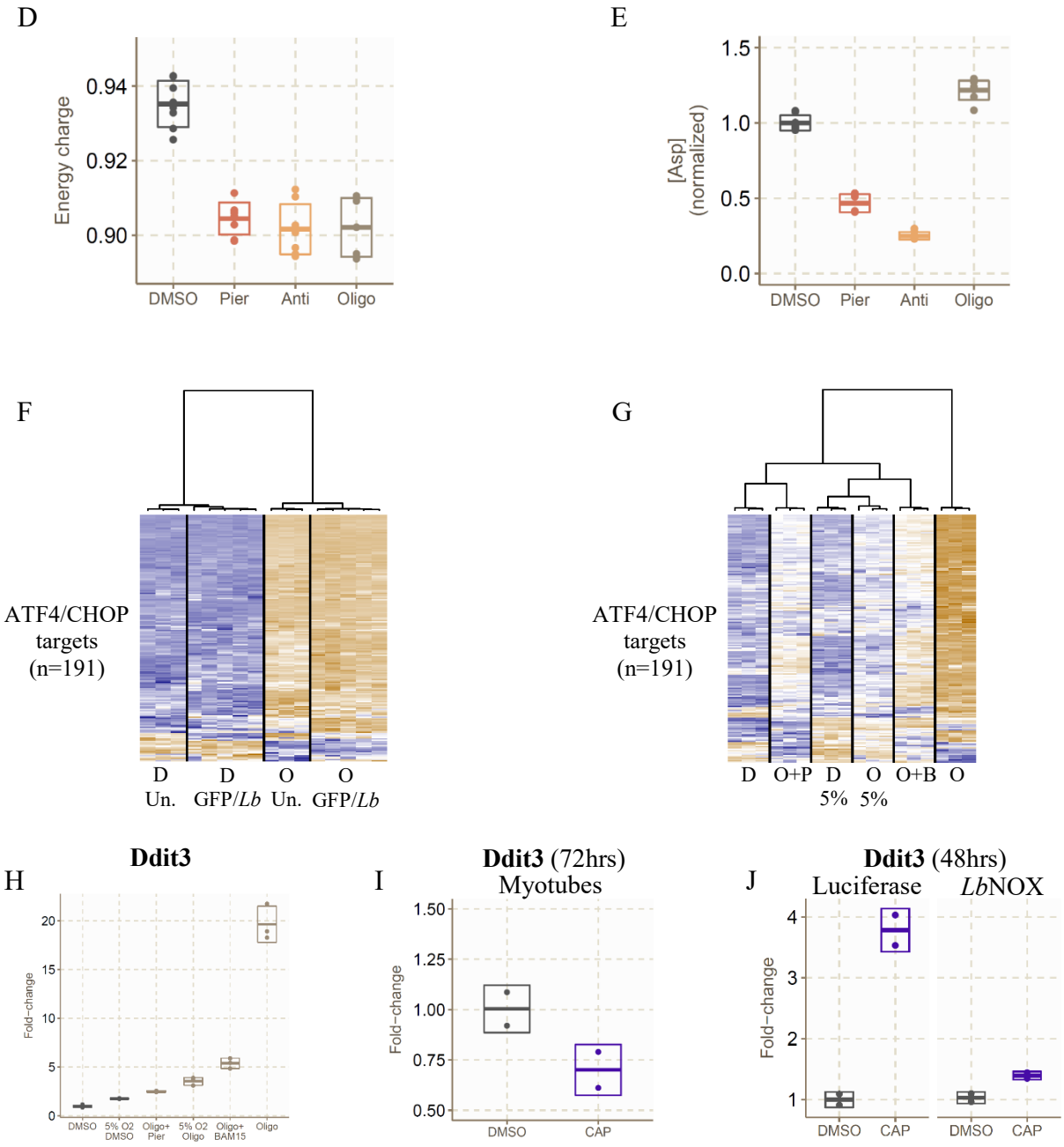

Figure 3.4 (continued): (D) Adenylate energy charge in myotubes following 1-hour treatment with ETC inhibitors. **(E)** Intracellular [aspartate] in myotubes. **(F)** Hierarchically-clustered, row-normalized expression heatmap of the differentially expressed ATF4 and DDIT3/CHOP targets in myotubes untransduced, expressing GFP or *Lb*NOX in the indicated conditions. **(G)** Hierarchically-clustered, row-normalized expression heatmap of differentially expressed ATF4 and DDIT3/CHOP targets in the indicated conditions. **(H)** Ddit3 transcript levels in indicated conditions (qPCR). **(I)** Ddit3 transcript levels in myotubes following 72hrs treatment with chloramphenicol (qPCR). **(J)** Ddit3 transcript levels in myoblasts following 48hrs treatment with chloramphenicol (qPCR).

We also measured aspartate levels in myotubes treated with ETC inhibitors and found that complex I or complex III inhibition caused only a modest decrease (2-fold and 4-fold, respectively), as compared to their drastic effect in myoblasts, whereas oligomycin even led to a slight accumulation (**Figure 3.4E**). The limited drop in aspartate may explain why these cells do not significantly activate the ISR following complex I or complex III inhibition.

ISR activation by ATP synthase inhibition in myotubes requires residual ETC activity

We wished to explore what drove the seemingly unique contribution of ATP synthase inhibition to ISR activation, as oligomycin was indistinguishable from piericidin and antimycin in its effects on energy metabolism and did not negatively impact aspartate in myotubes. We also confirmed that ISR activation by oligomycin in myotubes was entirely independent of cytosolic redox state, as *Lb*NOX expression was without effect on ISR gene expression (**Figure 3.4F**).

A distinctive feature of ATP synthase inhibition is residual respiration due to proton leak, albeit against greater resistance from the membrane potential (hyperpolarization). To test if this underlies the separate path to ISR activation, we treated myotubes with oligomycin in combination with piericidin, to eliminate residual ETC activity, or with the mitochondrial uncoupler BAM15 [54], to negate hyperpolarization. We also treated with oligomycin after pre-conditioning in mild hypoxia (5% O_2), which shifts cells away from oxidative metabolism and reduces electron flux into the ETC through physiological mechanisms [55], [56].

Strikingly, all these interventions significantly attenuated ISR activation due to ATP synthase inhibition (**Figure 4.4G**), which we also confirmed by qPCR on the Ddit3 transcript (**Fig. 4.4H**). This demonstrates that residual leak respiration is required for ISR activation during ATP synthase inhibition.

Inhibition of mtDNA gene expression is not directly sensed by the ISR

Finally, we wondered whether breakdown in mtDNA gene expression is directly sensed to activate the ISR, or indirectly sensed through the resulting ETC dysfunction. Dividing cells treated with chloramphenicol, which acutely inhibits translation by mitochondrial ribosomes, gradually develop a combined respiratory defect as existing ETC complexes are diluted out and newly synthesized ones lack key mtDNA-encoded subunits. Non-dividing cells may be less susceptible to a dilution effect, depending on their rate of ETC complex turnover.

Chloramphenicol treatment for up to 3 days failed to activate the ISR in myotubes (**Figure 4.4I**), consistent with the reported stability of ETC complexes in skeletal muscle in the face of mitochondrial translation defects [19], [57]. Myoblasts, on the other hand, showed initial signs of ISR activation within 2 days. This was suppressed by *Lb*NOX, indicating that initial ISR activation in this setting stemmed largely from a defect in NADH oxidation, similarly to complex I inhibition (**Figure 4.4J**). These results are inconsistent with direct surveillance of mtDNA expression.

DISCUSSION

We systematically interrogated the sensitivity of the ISR to bioenergetic perturbation by harnessing the gamut of congruent and incongruent effects of acute ETC inhibitors alongside classic and novel tools for selectively buffering their impact. We applied this toolkit in a comparative fashion across two cell states, proliferation and differentiation, whose metabolism reflects altered reliance on ETC functions.

Our results imply that in both cell states, abrogation of ATP synthesis by OXPHOS and the resulting reconfiguration of energy metabolism do not trigger the ISR, at least so long as glucose

is available. Future work would be required to explore whether this is also true in cells of different tissue origin, which may not be as metabolically flexible as muscle cells.

We demonstrate that the unique vulnerability of proliferating cells to redox perturbation in ETC dysfunction is also reflected in activation of the ISR, which at least in part follows from the resulting drop in aspartate levels. Aspartate has been shown to become generally limiting for cell proliferation in ETC dysfunction but its effect on the ISR is direct. Aspartate deficiency is sensed by GCN2, the eIF2α kinase attuned to amino acid levels. The connection between redox perturbation and the ISR may have previously been obscured by standard culture of ETC-compromised cells at supraphysiologic concentrations of pyruvate [58], which can oxidize the cytosol through LDH.

The ISR lost its sensitivity to redox perturbation upon cell cycle exit, which correlated with a significantly smaller drop in aspartate levels. A likely explanation for this could be a diminished biosynthetic rate in differentiated myotubes, and in particular a reduced requirement for nucleotides. This would act to conserve aspartate since it is a critical precursor for both purines and pyrimidines. It is also possible that differentiated myotubes are better able to buffer their aspartate levels through increased proteolysis and/or autophagy.

A second path to ISR activation, observed in myotubes, involved inhibition of ATP synthase and was dependent on residual ETC activity. It is not presently clear what about residual ETC activity in this setting triggers the ISR, though one possibility is ROS production which may be promoted by hyperpolarization [59]. Newly developed tools to dissect the contribution of specific ROS-producing sites in the ETC could prove useful for testing this hypothesis [60], [61]. Alternatively, redistribution of a protein or metabolite between the matrix and the cytosol could also be involved.

Interestingly, our results also identify parameters that do not seem to play a role in ISR activation. For example, we did not detect contributions to ISR activation by metabolic consequences tied to elevated matrix [NADH]/[NAD^+], other than aspartate biosynthesis. Thus, oxidizing the cytosol was sufficient to ablate the ISR during complex I inhibition in myoblasts even while the matrix remained overly reduced. Similarly, membrane depolarization on its own exerted a minor effect on the ISR in myotubes while hyperpolarization exerted a large effect [62].

What triggers the ISR during more complex scenarios of mitochondrial dysfunction, such as chronic defects in mtDNA expression, is more difficult to isolate. ISR activation has been reported in this setting *in vitro* even when cells were cultured with pyruvate in the media, such that the cytosolic redox perturbation was at least partially buffered. Nevertheless, our results argue against the possibility that impaired mtDNA gene expression activates the ISR directly. It is likely that a combination of signaling routes is involved, depending on the specific defect. In addition to metabolic effects, these may act through perturbed inner-membrane integrity, ROS or perturbed proteostasis.

In a similar vein, it is difficult to directly compare our results on ISR activation *in vitro* with what occurs during mitochondrial dysfunction *in vivo.* Acute ETC inhibition in cultured cells is an imperfect model for subtler genetic defects in a whole-organism setting whereas careful control (or even measurement) of specific mitochondrial functions is difficult to achieve in an animal. However, recent work has raised some doubt as to whether ISR activation *in vivo* is the result of bioenergetic/metabolic defects at all.

For example, Kühl et al. showed that heart-specific knockout of the mitochondrial RNA processing factor LRPPRC induces the mitochondrial 1-carbon pathway, a hallmark of the ISR, earlier than biochemical ETC defects can be detected [28]. A similar sequence of events was inferred in a heart

knockout of a mitochondrial tRNA synthetase [19]. Lehtonen et al. measured circulating FGF21 and GDF15, markers of ISR activation mainly in muscle, across mitochondrial disorders, and found them elevated when the mutation was in mtDNA maintenance and expression genes but not in structural ETC subunits [34]. On the other hand, Khan et al. reported that the ISR in mitochondrial myopathy caused by progressive mtDNA deletions was related to mTORC1, a metabolic master regulator, and did correlate with severity of ETC deficiency [18].

Thus, it is clear that much remains to be learned regarding the intricate relationship between mitochondrial dysfunction and the ISR, which likely encompasses multiple potential signaling routes. Our approach of careful monitoring of, and intervention in, specific functional outputs in order to conclusively relate them to gene expression changes offers a promising avenue for elucidating the full range of these connections. It can be adapted in future work to tackle more physiological modes of mitochondrial dysfunction in cells of affected post-mitotic tissues.

MATERIALS AND METHODS

Cell culture

C2C12 cells were obtained from ATCC. DOX-inducible cell lines were generated as previously described [39]. Cell culture media consisted of DMEM with 10mM glucose and no sodium pyruvate or uridine. This base media was supplemented with different types and concentrations of serum, as follows: i) DOX-inducible myoblasts were cultured in media supplemented to 10% dialyzed fetal bovine serum (dFBS). Up until cells were seeded in plates for experiments, the media was also supplemented with 1ug/ml puromycin and 0.5mg/ml geneticin to maintain selection of the DOX-inducible system; ii) Myoblasts intended for differentiation were cultured in media supplemented to 20% dFBS. After cells became confluent, they were switched to media

supplemented to 2% dialyzed horse serum. Cells were considered differentiated myotubes after 4 days in low-serum media. Spontaneous contractions were usually observed by this time.

Inhibitor treatments for RNA

DOX-inducible myoblasts were seeded at 20,000 cells per well in 24-well plates with 0.5ml/well of media without selection antibiotics. Approximately 3 hours later, an additional 0.5ml/well of media supplemented with DOX was dispensed in each well, such that the final DOX concentration was 300ng/ml. ETC inhibitor treatments were started with complete replacement of the media (including 300ng/ml DOX) 24hrs after initial DOX addition. Piericidin was used at 0.5uM, antimycin at 0.5uM and oligomycin at 1uM.

Cells were seeded for differentiation at 50,000 cells per well in 24-well plates with 1ml/well of media. 2 days later, cells became fully confluent and were switched to low-serum media. Myotubes (4 days after media switch) were treated with complete replacement of the media. Piericidin was used at 0.5uM, antimycin at 0.5uM and oligomycin at 1uM.

Cells were lysed for RNA after 10hrs of treatment with ETC inhibitors in 150ul/well buffer RLT (Qiagen). The lysate was frozen at -80C until RNA isolation. RNA isolation was performed using Qiagen RNeasy or RNeasy96 kits.

RNA sequencing and data analysis

RNA was quantified using Nanodrop and normalized to 5ng/ul in 10ul for submission to library preparation and sequencing. This was performed at the Broad Institute Technology Labs using the Smart-seq2 protocol which includes polyA selection. Libraries were sequenced on 2 flow-cells of an Illumina NextSeq500 instrument, generating 2 x 37bp paired-end reads. Sequencing reads were pseudo-aligned to the mouse transcriptome (ENSEMBL, v. 93) with kallisto. Read counts were

summarized to the gene level with tximport in R. Differential expression analysis was performed using DESeq2 in R.

qPCR

Isolated RNA was annealed to random primers for 5 minutes at 70C, then reverse transcribed for 1hr at 37C. The resulting cDNA was assayed with TaqMan gene expression probes on a BioRad CFX96 instrument. Raw amplification cycle data was produced by the accompanying analysis software using default parameters. Cycle differences among tested conditions and the baseline condition were normalized against changes in the gene Ubr3, which was selected as it showed no response to ETC inhibition in our system based on the RNA sequencing experiment. Fold-changes were calculated by exponentiation of the normalized cycle differences from baseline.

[Lactate]/[Pyruvate] in spent media

Cells were cultured and treated in the same way as for RNA extraction. 100ul of the media in the well was collected 2hrs after the start of treatment and immediately frozen at -80C. 30ul of each media sample was combined on ice with 70ul extraction buffer (22.5% methanol:67.5% acetonitrile:10% water) including heavy labels for pyruvate, lactate and glucose. Samples were run through an Amide HILIC column on a Q Exactive Plus Orbitrap Mass Spectrometer coupled to a Dionex UltiMate 3000 UHPLC system (Thermo Fisher Scientific). Absolute concentrations for pyruvate and lactate were obtained based on a standard curve.

Cell extract metabolite profiling

DOX-inducible myoblasts were seeded at 200,000 cells per dish in 6cm dishes with 4ml/dish of media without selection antibiotics. Approximately 3 hours later, 2ml/dish were removed and replaced with 2ml/dish of media supplemented with DOX, such that the final DOX concentration

was 300ng/ml. 24hrs after DOX addition, the media was fully replaced with 3ml/dish fresh media (including DOX). 2hrs after the media replenishment, 1.5ml/dish was removed and replaced with 1.5ml/dish of media supplemented with ETC inhibitors at 2x the final concentration. Beginning at 1hr after the treatments were started, dishes were serially removed from the incubator, placed on ice, the media was aspirated, cells were washed in 1ml/dish PBS and this was aspirated, and finally 800ul/dish metabolite extraction buffer (40% methanol:40% acetonitrile:20% water + 0.1M formic acid) was introduced. The extraction buffer included heavy labels for AMP, ADP, ATP and aspartate. The cells were thoroughly scraped, the lysate was transferred to a fresh tube, vortexed briefly and placed on ice. ~2-3 minutes later, 70ul ammonium bicarbonate was pipetted into the tube to neutralize the pH, the tube was vortexed and placed at -20C until all extractions were completed. Then, all the tubes were spun at 4C at maximum speed for 10mins to pellet the cellular debris. 100ul of the supernatant was transferred into LC-MS tubes, which were placed into the Q Exactive instrument and run through a ZIC-HILIC column in alternating negative/positive mode. Absolute concentrations for AMP, ADP, ATP and aspartate were calculated based on a standard curve.

Myotube cell extracts were collected in the same way, except they were seeded at 200,000 cells per dish in 35mm dishes with 3ml/dish of media and differentiated for 4 days in low-serum media upon becoming confluent. 500ul/dish extraction buffer was used.

Cell proliferation

DOX-inducible myoblasts were seeded at 5,000 cells per well in 24-well plates with 0.5ml/well of media. Approximately 3 hours later, an additional 0.5ml/well of media supplemented with DOX was dispensed in each well, such that the final DOX concentration was 300ng/ml. ETC inhibitor treatments were started with complete replacement of the media (including 300ng/ml DOX) 24hrs

after initial DOX addition. Piericidin was used at 0.5uM. Baseline counts for each condition were collected immediately after the start of treatments, on separate wells. Final counts were collected 24hrs after start of treatments. The proliferative rate was calculated as the number of cell doublings between final and baseline counts. Counts were obtained using a Z2 Coulter Particle Count and Size Analyzer.

Author contributions

Eran Mick and Vamsi Mootha designed and oversaw the overall project. Eran Mick designed and performed all cell culture experiments and data analysis. Denis Titov generated DOX-inducible cell lines and consulted on design of experiments and data interpretation. Owen Skinner and Rohit Sharma assisted in technical preparations for metabolite profiling experiments, operated the LC/MS instrument and performed raw data acquisition and initial analysis. Eran Mick wrote the manuscript with input from all authors.

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CHAPTER 4
Conclusion

CONCLUSION

The work described in this thesis presents a template for isolating the impact that failure of specific mitochondrial functions has on mammalian cell physiology, signaling and gene expression. It argues for renewed focus on primary bioenergetic defects as triggers of retrograde signals, such as the integrated stress response, and highlights the cell-state dependence of these triggers.

The study of retrograde signaling in mitochondrial dysfunction is another lens through which to interpret the differential reliance of cell types and tissues on mitochondrial functions. Even among post-mitotic cells, variability in the degree of metabolic plasticity, the connectivity between cytosolic and matrix redox pools and the turnover rate of mitochondria and of specific ETC complexes likely modulates the response to mitochondrial dysfunction. It would thus be of interest to replicate our experiments in cells of different tissue origin (neurons, hepatocytes, cardiomyocytes), which frequently exhibit distinct stress response patterns *in vivo* [1], [2].

Our approach relied on chemical inhibitors of specific ETC functions, acute treatments, genetically-encoded tools and sensitive measurement techniques. Such careful control is much more difficult to achieve in chronic models, let alone *in vivo*. Nevertheless, ISR activation is largely cell autonomous and so even complex scenarios of mitochondrial dysfunction are amenable to dissection *in vitro* if performed along an appropriate time-course. Our approach can thus be adapted to gain considerable insight in these models as well.

Recent work in animals suggests there are still missing links in our understanding of why mitochondrial dysfunction triggers the ISR and through what signaling routes. In particular, the fact that defects in “mtDNA central dogma” activate the response prior to biochemically detectable

ETC dysfunction begs explanation [3], especially since our results do not support direct surveillance of mtDNA gene expression.

One compelling avenue for connecting mtDNA gene expression defects with subtler early effects on signaling and metabolism is through inner-membrane integrity. The mtDNA-encoded subunits of ETC complexes are co-translationally inserted into the membrane due to their hydrophobicity. Defects in mitochondrial RNA processing or translation lead to accumulation of aberrant products that can compromise membrane integrity and result in depolarization, leakage of matrix contents into the cytosol or production of ROS [1], [4]–[6]. If more firmly established, this route may prove the missing link that reconciles contradictory observations in the field.

The second major open question, which was not touched upon in this thesis, is the extent to which the ISR and ISR-related gene expression play adaptive versus maladaptive roles *in vivo*. While recent work has identified aspects of pathology that may be exacerbated by the response [7], the answer in general is likely to be highly context-specific. Nevertheless, identifying those contexts in which ISR activation or inhibition is beneficial may prove of therapeutic value in mitochondrial disorders given the growing arsenal of tool compounds for manipulating ISR signaling.

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