Oxygen, the Invisible Orchestrator
of Metabolism and Disease:

A Focus on Mitochondrial
And Peroxisomal Dysfunction

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Submitted to the Harvard-MIT Division of Health Sciences & Technology
in partial fulfillment of the requirements for the degree in

DOCTOR OF PHILOSOPHY IN HEALTH SCIENCES AND TECHNOLOGY:
COMPUTER SCIENCE
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ABSTRACT

Variations in atmospheric oxygen levels can be traced over evolutionary time and track
closely with the development of multicellular life, speciation events, appearance of placental
mammals and the creation of a cardiorespiratory system. As the final electron acceptor for aerobic
ATP production, oxygen allows energy-intensive metabolic pathways to exist. Furthermore,
oxygen is the most utilized substrate for known biochemical reactions, surpassing even ATP and
NAD+. As a result, variations in oxygen levels have far-reaching consequences on human
physiology and health.

Mitochondrial disorders are the most common inborn errors of metabolism, affecting
approximately 1 in 5000 live births. Patients can present in infancy or adulthood with symptoms
affecting multiple organ systems including blindness, deafness, muscle weakness, developmental
delay and severe neurological impairment. Unfortunately, there are currently no proven therapies
for mitochondrial disorders. My thesis work has focused on combining systems biology, animal
physiology and cellular metabolism approaches to develop new therapies for these disorders.
More specifically, I have identified hypoxic breathing, equivalent to living at 4500m altitude, as
protective in the setting of severe mitochondrial disease.

First, I performed a genetic screen and found paradoxically, that hypoxic breathing and
hypoxia responses are protective in mitochondrial disease. I then characterized the physiology
and preclinical regimens of hypoxia therapy, laying the groundwork for translation to human
patients. Fascinated by such a vital role for oxygen in human disease, I went on to define adaptive
pathways in varying oxygen tensions. This work highlights the differential reliance on entire
organelles at extreme oxygen levels. And finally, I studied the metabolic and proteomic
consequences of defects in peroxisome metabolism and disease.

Thesis Supervisor: Vamsi Mootha
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Chapter 1: Introduction and Background
1. Introduction

1.1 Oxygen

"From the greater strength and vivacity of the flame of a candle, in this pure air, it may be conjectured that it might be particularly salutary to the lungs in certain morbid cases...but, perhaps...it might not be so proper for us in the usual healthy state of the body; for, as a candle burns out much faster in dephlogisticated than in common air, so we might, as may be said, live out too fast..." The words of Joseph Priestly echo as true now, as they did nearly two centuries ago, upon his discovery of molecular oxygen. The role of oxygen as both an essential substrate and a lethal toxin is the fundamental principle underlying this thesis.

Variations in atmospheric oxygen levels can be traced over evolutionary time and track closely with the development of multicellular life, speciation events, appearance of placental mammals and the creation of a cardiorespiratory system [1]–[5]. As the final electron acceptor for aerobic ATP production, oxygen allows energy-intensive metabolic pathways to exist. Furthermore, oxygen is the most utilized substrate for known biochemical reactions, surpassing even ATP and NAD+ [5]. As a result, variations in oxygen levels have far-reaching consequences on human physiology and health.

Countless medical crises such as myocardial infarcts, stroke, sepsis and vascular disease are associated with states of impaired oxygen delivery, utilization or sensing [6], [7]. Though the initial cause of disease varies, impaired oxygenation contributes significantly to disease pathology. This leads to some of the great unanswered questions that lie at the intersection of cellular metabolism and organism-level physiology – how is metabolism rewired as a function of oxygen tension? How are disease states affected by fluctuations in oxygen? How can this knowledge be used to understand and treat disease?

Oxygen or the "elixir of life" has fascinated chemists, physiologists and molecular biologists alike for many years. In recent times, the focus of oxygen biology has followed two parallel paths, with little crosstalk between subfields. On the one hand, the last few decades have yielded groundbreaking advances in uncovering the transcriptional response to oxygen. Beginning in the 1990s, several teams elucidated the entire signaling cascade involving the hypoxia inducible transcription factors (HIFs). In an alternate and
parallel setting, physiologists and clinical researchers have been deciphering the acute and chronic physiological response to hypoxia. Here, the focus has been on the cardiorespiratory responses to acute hypoxia, as well as chronic pulmonary and vascular adaptations to varying oxygen tensions. My PhD work lies at the very intersection of these two fields. First, I identify and develop hypoxia as a therapy for a devastating class of disorders – mitochondrial inborn errors of metabolism [8]. I then characterize the physiology and preclinical regimens of hypoxia therapy, laying the groundwork for translation to human patients [9]. Fascinated by such a vital role for oxygen in human disease, I go on to define essential genes and pathways as a function of oxygen tension. This work highlights the differential reliance on peroxisomes versus mitochondria at extreme oxygen levels. And finally, I study the metabolic and proteomic consequences of defects in peroxisome metabolism and disease.

1.2 Developing Therapies for Inborn Errors of Metabolism

Metabolic dysregulation underlies both common diseases and rare, inborn errors of metabolism. Understanding the basis of such metabolic diseases provides key insights into the biochemical roles of affected genes and pathways. Conversely, basic investigations into the metabolic roles of organelles and pathways inform the development of disease biomarkers and therapies.

The first inborn error of metabolism to be identified was phenylketonuria (PKU) in 1934, resulting from a deficiency in phenylalanine hydroxylase [10]. Simply reducing phenylalanine in the diet was found to dramatically rescue disease. In subsequent decades, many additional single enzyme deficiencies were identified as disease-causing – resulting in galactosemia, fructosemia, homocystinuria and maple syrup urine disease, to name a few. A common theme of such metabolic deficiencies is that they can be ameliorated either by supplementation of a missing reaction product or removal of a built-up substrate. We provide an unusual variation on this theme. Our work focuses on the devastating class of disorders resulting from a broken mitochondrial electron transport chain (ETC). The key substrate for the ETC is molecular oxygen. We show that simply limiting the key substrate for the ETC can dramatically improve and even reverse disease. This work provides a surprising twist to the historic theme of tweaking metabolic flux to
modulate inborn errors of metabolism. We then delve into an in-depth study of how organelle-specific functions modulate survival in extreme oxygen tensions.

1.3 Mitochondrial Metabolism

Mitochondria perform a host of essential and conserved metabolic functions, the most well-known being aerobic ATP production. In addition to energy metabolism, a functioning ETC is central to many other metabolic functions, ranging from pyrimidine biosynthesis to the TCA cycle. Furthermore, mitochondria house the biochemical pathways for lipid metabolism, calcium homeostasis, heme biogenesis and redox maintenance [11]. Before delving into disease mechanisms and therapies, we must first understand the key aspects of mitochondrial metabolism and how they interact with both the intracellular and extracellular milieu.

1.3.1 Mitochondrial Electron Transport Chain

The mitochondrial electron transport chain is composed of four protein complexes, which allow for the progressive oxidative of high energy intermediates such as NADH and FADH$_2$. Oxygen serves as the final electron acceptor, resulting in the production of H$_2$O. This oxidative process is coupled to proton pumping across the inner mitochondrial membrane, resulting in a membrane potential which is used to generate ATP via complex V. To date, about 90 different genes are known to encode subunits of the ETC [11]. These genes are encoded in both the nuclear DNA and mitochondria DNA (mtDNA), either of which can contribute to disease. While the components of the ETC and the mitochondrial proteome have been well-defined, the identification of disease genes and novel therapies lags behind [12].

1.3.2 ETC-linked Metabolism

Aside from ATP production, the ETC serves as a scaffold for many other crucial biochemical reactions. Of note, most of cellular NADH is recycled to NAD$^+$ by Complex 1. This enables the several hundred NAD$^+$-linked reactions in the cell to proceed. Succinate dehydrogenase, or Complex II, is a key component of the TCA cycle, serving as a bridge between biosynthetic and energy metabolism pathways. Furthermore, electrons can directly be donated to the mobile electron carrier, coenzyme Q, by enzymes such as dihydroorotate dehydrogenase (DHODH) and electron transfer flavoprotein dehydrogenase (ETFDH). These reactions link pyrimidine synthesis and fatty acid
metabolism to the ETC, respectively. And finally, the ETC also consumes 90% of cellular oxygen, making it the key regulator of intracellular oxygen tensions. As is evident from above, a functioning ETC is requisite for efficient energy production, among many other metabolic functions. The respective contributions of these (dys)functions to disease are unknown.

1.3.3 Reactive Oxygen Species

A significant amount of reactive oxygen species (ROS) is made as an unintentional byproduct of high flux through the ETC. Electrons can be directly donated from electron carriers to oxygen, resulting in the production of superoxide and intracellular ROS [13]. Amazingly, cytochrome oxidase, has evolved to have an extremely high affinity for oxygen, allowing for minimal exposure to free electrons. Even so, nearly 1% of oxygen consumption is believed to yield toxic reactive oxygen species [14]. To cope with such stress, mitochondria contain a range of antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidases (GPx), peroxiredoxins (Prx), thioredoxins (Trx) and glutathione reductases (GR). High flux through the ETC is what enables efficient ATP production, but also serves as the catalyst for increased ROS production. Imbalance in the ROS-producing and ROS-consuming processes leads to oxidative stress and cell death.

1.3.4 Other Metabolic Functions

While we won’t delve deep into the additional metabolic functions, we will mention that major components of heme biosynthesis, calcium homeostasis, fatty acid beta-oxidation and cholesterol biosynthesis take place within mitochondria [11]. In theory, impairment of any of these functions may contribute to disease pathology of mitochondrial disorders.

1.4 Mitochondrial Disorders

1.4.1 Respiratory Chain Disorders

Over 150 different disease genes have been linked to mitochondrial disorders, resulting in a disease incidence of 1 in 5000 live births [15]. Given the essential functions of mitochondria, it is likely that even more lesions are incompatible with life and result in in utero death. Of all mitochondrial disorders, respiratory chain diseases, comprise the largest category and are among the most devastating. Mutations have been identified in
over 110 different nuclear genes, as well as 13 genes encoded in the mitochondrial DNA (mtDNA) [16], [17]. Mitochondrial disorders can present in childhood or adulthood, often with a rapid decline, leading to death within several years. Furthermore, many neurodegenerative disorders have been associated with RC dysfunction, including Alzheimer’s, Parkinson’s and the aging process itself. Thus, the need to understand and treat RC dysfunction is paramount.

We provide a few representative examples of the most common mitochondrial disorders. Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) is a disease which primarily affects young men before the age of 40 [18]. Patients suffer from stroke-like episodes, seizures, muscle weakness and dementia. The predominant underlying cause is a mutation in the mitochondrial leucine tRNA. While the genetic causes are well-studied, there is currently no cure and patients usually die within a few years of diagnosis. As another example, Leigh syndrome is the most common pediatric form of mitochondrial disease [16]. Children are diagnosed shortly after birth and suffer from blindness, deafness, myopathies and neurodegeneration. Approximately 1 in 40,000 children are born with Leigh syndrome and usually don’t survive beyond childhood. This disorder is the initial focus of this thesis. We use unbiased, high-throughput approaches to identify and develop hypoxia as a treatment for this mitochondrial disorder [8].

**1.4.2 Disease Presentation, Current Treatment Options, and Clinical Trials**

Mitochondrial disorders can affect nearly every organ system, causing liver failure, kidney failure, blindness, deafness, diabetes, muscle myopathy and neurodegeneration [11]. The incredible heterogeneity in disease presentation for identical lesions remains unexplained. Why some tissues are protected, whereas others suffer catastrophic physiological consequences, is unknown. Such complex disease presentation, is what makes mitochondrial disorders incredibly difficult to diagnose and to treat.

Upon diagnosis, the current treatment options for mitochondrial disorders are very limited and have no proven efficacy. In a few specific cases, disease can be rescued by cofactor supplementation, such as for thiamine or biotin deficiency [15]. However, by in large, treatment options are limited or non-existent for this class of disorders. We discuss planned and existing clinical trials further in Chapters 2 and 3 of this thesis.
1.5 Oxygen sensing and adaptation

1.5.1 Hypoxia Transcriptional Response

Organisms have evolved elaborate responses to cope with changes in environmental and local oxygen tensions. The response to hypoxia involves a multifactorial adaption, including changes in transcription, metabolism and physiology [7], [19]–[21]. Over the last few decades, much progress has been made in understanding the hypoxia transcriptional program. Specifically, the hypoxia inducible transcription factors (HIF) were identified and the biochemical pathway elucidated. Briefly, this family of transcriptional factors are constitutively made and under conditions of sufficient oxygen tension, are degraded. The prolyl hydroxylase (PHD) enzymes use molecular oxygen to hydroxylate the HIF proteins, allowing for their recognition and ubiquitination by the von Hippel Lindau (VHL) factor. Under low oxygen tensions, this cascade does not take place. Instead the HIF proteins enter the nucleus and trigger the transcriptional of several hundred genes. Target genes include those that alter molecular and cellular pathways (e.g. glycolytic genes), as well as those that improve oxygen delivery on an organism-level (e.g. genes which trigger angiogenesis). While significant progress has been made in understanding the hypoxia transcriptional program, it is becoming clear that HIF proteins are just one node of the metabolic response to changes in oxygen tensions.

1.5.2 Metabolic Rewiring in Hypoxia

The most conserved and well-understood response to hypoxia is a shift from aerobic, mitochondrial ATP production to anaerobic glycolytic metabolism. This takes place both because of allosteric mechanisms and a concerted upregulation of many glycolytic enzymes by HIF1α [22]. Additionally, upregulation of pyruvate dehydrogenase kinase enzymes, causes an inhibition of pyruvate dehydrogenase activity [23], [24]. This serves as an active block of pyruvate entry into mitochondrial energy metabolism. This decreased flux through the RC is believed to decrease formation of reactive oxygen species, while also diverting valuable carbons towards glycolysis and the lactate dehydrogenase reaction. Additionally, several protein subunit switches have been noted (eg. Complex 1 subunit NDUFA4L2 protein and Complex 4 subunit COX4 splicing isoforms) [25], [26] which decrease electron transport through the RC. Altogether, these
adaptive mechanisms serve to divert fuels away from mitochondrial energy metabolism towards anaerobic pathways.

In recent years, the field of cancer metabolism has highlighted additional metabolic switches in response to tumor hypoxia. Hypoxia has been shown to trigger reductive carboxylation of the TCA cycle which results in glutamine being reductively converted to citrate, for the purpose of fatty acid synthesis without a fully-functional TCA cycle [27]. This enables lipid synthesis to proceed, even upon decreased entry of pyruvate into the TCA cycle. Recently, it was also shown that hypoxic cells rely on exogenous unsaturated lipids to compensate for inhibited synthesis of mono-unsaturated lipids [28]. Such auxotrophies highlight the plethora of metabolic consequences of low oxygen conditions, many of which remain to be systematically studied.

1.5.3 Physiological Responses to Oxygen

Acutely, hypoxia triggers a dramatic physiological response to enable an organism's survival [29]–[31]. The canonical oxygen sensor of the body is the carotid body which lies at the bifurcation of the carotid artery. This allows for the rapid sampling of arterial oxygen tensions and neuroendocrine signaling to the cardiorespiratory centers. The first response is an increase in heart rate, cardiac output and respiratory rates, all in an effort to improve oxygen delivery to hypoxic tissues. In a similar vain, hypoxia triggers cerebral and peripheral vasodilation to allow for increased oxygen delivery. In contrast, hypoxia causes acute pulmonary vasoconstriction to allow for optimal ventilation-perfusion matching. Over a longer timeframe, the increased pulmonary arterial pressure can lead to right ventricular hypertrophy. Such physiological responses to acute hypoxia have been studied by high altitude physiologists for decades. However, the metabolic and cellular changes which underlie these responses remain unknown.

1.6 Peroxisome Metabolism and Organelle Crosstalk

Peroxisomes are single-membrane bound organelles which are conserved across species spanning from yeast to humans. While housing only ~100 proteins, they are responsible for essential steps in lipid metabolism, amino acid metabolism and ROS homeostasis [32], [33]. For example, peroxisomes are required for degradation of very long chain fatty acids (VLCFAs), fatty acid alpha-oxidation, etherphospholipid synthesis and essential steps in cholesterol synthesis. Interestingly, peroxisomes contain their own
machinery for importing membrane and matrix proteins. Unlike other organelles, peroxisomes import fully-folded and cofactor-bound proteins into their matrix. Though peroxisomes house specific metabolic functions, there is extensive crosstalk with other organelles, including mitochondria.

As a key example, peroxisomes degrade very long chain fatty acids until they are medium or long chain fatty acids, at which point acyl-carnitines are transported to mitochondria for complete degradation. Peroxisomes, the ER and mitochondria are all involved in the synthesis of certain lipid species – e.g. steroids and cholesterol. Interestingly, a significant number of peroxisomal matrix proteins are known to co-localize between mitochondria and peroxisomes. This alludes to shared metabolic functions and evolutionary history between the two organelles. Finally, the organelle division machinery is the same for peroxisomes and mitochondria, suggesting common control of abundance and thus, function [34]. Such clues point to a close metabolic relationship between the two organelles, something which is apparent even at the level of disease and physiology.

1.7 Peroxisomal Disorders

Mutations in peroxisomal enzymes result in fatal diseases known as either peroxisomal biogenesis disorders (PBDs) or peroxisomal single enzyme deficiencies (PEDs) [35]. PBDs comprise the Zellweger syndrome spectrum, also known as the Cerebrohepatorenal syndrome. Children with these disorders have severe neurodevelopmental defects, dysmorphic features, liver failure, kidney failure and skeletal abnormalities. These disorders are uniformly fatal within a few years of life, and there are currently no available therapies.

Interestingly, patients with peroxisomal disorders also consistently show mitochondrial electron transport chain dysfunction [36]. It is likely that secondary mitochondrial dysfunction contributes to peroxisomal disease symptoms. A more detailed study of peroxisomal and mitochondrial metabolism will inform our understanding of both inborn errors of metabolism.
1.8 References


Chapter 2: Hypoxia as a Therapy for Mitochondrial Disease

Isha H. Jain, Luca Zazzeron, Rahul Goli, Kristen Alexa, Stephanie Schatzman-Bone, Harveen Dhillon, Olga Goldberger, Jun Peng, Ophir Shalem, Neville E. Sanjana, Feng Zhang, Wolfram Goessling, Warren M. Zapol, Vamsi K. Mootha

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20
2.1 Abstract

Defects in the mitochondrial respiratory chain (RC) underlie a spectrum of human conditions, ranging from devastating inborn errors of metabolism to aging. We performed a genome-wide, Cas9-mediated screen to identify factors that are protective during RC inhibition. Our results highlight the hypoxia response, an endogenous program evolved to adapt to limiting oxygen availability. Genetic or small molecule activation of the hypoxia response is protective against mitochondrial toxicity in cultured cells and zebrafish models. Chronic hypoxia leads to a marked improvement in survival, body weight, body temperature, behavior, neuropathology and disease biomarkers in a genetic mouse model of Leigh syndrome, the most common pediatric manifestation of mitochondrial disease. Further preclinical studies will allow us to assess the safety and efficacy of hypoxic exposure as a treatment for human diseases associated with mitochondrial dysfunction.

2.2 Introduction

Mitochondria are ancient organelles that are essential for normal physiology and health. The respiratory chain (RC) is crucial to mitochondrial function and generates approximately 90% of cellular ATP via oxidative phosphorylation [1]. In the oxidative step, four large protein complexes transfer electrons from NADH or FADH to oxygen while generating a proton gradient. Approximately 90% of the oxygen we breathe is utilized as a substrate for the RC [1]. In the phosphorylation step, the proton gradient is dissipated by a fifth and final complex to generate ATP. Numerous additional chemical reactions and transport processes are intimately coupled to the redox and proton pumping activities of the RC.

A spectrum of human diseases result from a faulty RC. Virtually all age-related disorders, including type 2 diabetes, neurodegeneration, and sarcopenia, are accompanied by a quantitative decline in the activity of the mitochondrial RC [2-4]. The aging process itself is associated with a gradual decrease of oxidative phosphorylation in multiple tissues. Monogenic disorders of the mitochondrial RC represent the largest class of inborn errors of metabolism. To date, lesions in over 150 genes, encoded by the nuclear or mitochondrial (mtDNA) genomes, have been identified as disease-causing. Mutations in these genes lead to a biochemical deficiency of one or more of
the RC complexes, resulting in either tissue-specific or multisystem disease with devastating effects on human health. Patients with RC chain disorders can present with blindness, deafness, gray or white matter brain disease, cardiomyopathy, skeletal muscle myopathy, GI dysmotility, anemia, ataxia, liver disease and kidney disease [2]. Management of these disorders remains incredibly challenging [5, 6]. While individual mutations are rare, the overall disease burden of mitochondrial disease is significant with an estimated prevalence of 1:4300 live births [7]. Therefore, a general and effective therapeutic is needed. The current mainstay of managing mitochondrial disease involves the use of vitamin co-factors (CoQ, α-lipoic acid, riboflavin, L-carnitine) [3, 8]. Other proposed strategies include the use of small molecule bypass of defective RC components, using electron carriers such as idebenone, and antioxidants. None of these approaches have demonstrated efficacy in randomized controlled clinical trials.

Several lines of evidence point to the existence of endogenous coping mechanisms for mitochondrial dysfunction. It is notable that mitochondrial disorders can be highly tissue-specific and episodic [4, 9]. These disorders are often triggered by drugs, alcohol, or viral illnesses, implying that a genetic lesion is not always sufficient to cause cellular dysfunction, but rather that the lesion may need to be compounded with an environmental insult. Such observations suggest the existence of cellular pathways or environments that buffer against mitochondrial lesions.

2.3 Results

2.3.1 A genome-wide screen to spotlight suppressors of mitochondrial disease

We modeled mitochondrial disease in the suspension cell line, K562, and performed a Cas9-mediated knockout screen [10, 11]. We used the natural product, antimycin, as a complex III inhibitor of the respiratory chain. In the presence of antimycin, the respiratory chain is unable to oxidize high energy reducing equivalents to power ATP production, however cytoplasmic lactate dehydrogenase maintains NAD+ redox balance. Removal of pyruvate exacerbates reductive stress, further preventing cell proliferation [12]. We modeled mitochondrial disease with the addition of antimycin alone (moderate disease) or antimycin in combination with removal of pyruvate (severe disease), using cell growth as a proxy for disease magnitude (Fig. 1A). We infected K562 cells with a ~65,000 single guideRNA (sgRNA) library, targeting ~18,000 genes [10]. After one week
of genome editing, we transferred the pool of knockout cells to experimental conditions of untreated, moderate disease and severe disease states (Fig. 1B). We collected samples for an enrichment screen by allowing the knockout pool to grow in selection conditions for three weeks. The relative growth between untreated and moderate disease conditions was 300-fold and between untreated and severe disease conditions was 7,000-fold (Fig. 1C). As expected, three weeks of genome editing in untreated cells led to a significant depletion of sgRNAs corresponding to essential genes, including those related to transcription, translation, and splicing (fig. S1). Nearly 20% of the 500 most essential genes were mitochondrial proteins, especially mitochondrial ribosomal proteins and electron transport chain subunits (table S1). As mitochondrial proteins make up approximately 5% of the proteome [13], this enrichment highlights the dramatic effects of mitochondrial dysfunction on viability.

Of the ~18,000 genes tested, the knockout screen identified inhibition of the Von Hippel-Lindau (VHL) factor as the most effective genetic suppressor of mitochondrial disease, in both the moderate and severe disease conditions (Fig. 1D). RIGER analysis ranked VHL knockout cells as the most enriched over time in both infection replicates corresponding to severe and moderate disease (table S2). The five sgRNAs spanning all three exons of VHL ranked 1, 2, 3, 12 and 14 out of ~65,000 total guides for enrichment in disease conditions relative to pre-treatment conditions (Fig. 1D-F, fig. S2-3). Furthermore, the most significant VHL sgRNAs were enriched greater than 20-fold in disease states (fig. S4). Of note, VHL knockout cells were also enriched in untreated conditions over time, reflecting an overall effect on cell growth. However, this enrichment was significantly less than in disease conditions.

VHL activity is a key regulator of the hypoxic response pathway [14-15]. Organisms have evolved elaborate mechanisms to adapt to fluctuating oxygen tensions and extreme environments. In normoxic conditions, the hypoxia inducible transcription factors (HIF) are constitutively made and hydroxylated by the prolyl-hydroxylase (PHD) enzymes (Fig. 2A) [16-18]. The hydroxylated form is recognized by the ubiquitin ligase, VHL, and targeted for degradation. In response to environmental hypoxia, the PHD reaction does not take place, allowing HIF stabilization and activation of the hypoxia transcriptional program (Fig. 2B). VHL-knockout cells show HIF stabilization, even during normoxic
conditions, thereby bypassing cellular oxygen sensing mechanisms [18-20]. Our screen suggested that harnessing innate responses to hypoxia may be protective in the setting of inherited mitochondrial disease.

2.3.2 Genetic and small molecule proof-of-concept in cellular models

We validated and characterized the hypoxic response as a therapeutic target by testing the ability of VHL-knockout cells to withstand respiratory chain dysfunction. VHL-knockout cells showed increased cell proliferation in the presence of antimycin relative to non-targeting (dummy) sgRNA-modified cells (Fig. 2C). Furthermore, there was perfect correspondence between the degree of VHL sgRNA enrichment in the CRISPR screen and the rescue effect size of individual sgRNAs (fig. S5), likely reflecting differences in genome editing efficiencies. VHL-knockout cells were also more resistant to Complex I inhibition by piericidin and complex V (ATP synthase) inhibition by oligomycin, demonstrating the potentially broad utility of our therapeutic approach (Fig. 2C).

We next explored small molecules as tools for triggering the hypoxia response. While a VHL-inhibitor has been reported [21], it is not cell permeable. PHD inhibitors have been developed as investigational drugs for anemia and ischemic disorders [22]. FG-4592 is currently in Phase III clinical trials for the treatment of anemia of chronic kidney disease and acts by upregulating the canonical marker of the hypoxia response, erythropoietin (Epo). We reasoned that FG-4592 treatment would mimic VHL-knockout, thus triggering a broader hypoxia transcriptional program. Normal growth rates were minimally increased by FG-4592. Complex I, III or V inhibition stunts cell growth, but not death (fig. S6) in most cell lines, including HT-29s, HEK 293Ts and K562s. Administering ~50µM FG-4592 in advance and during respiratory chain dysfunction nearly or completely rescued this growth defect, in a dose-dependent manner (Fig 2D-F, fig. S7). The nearly full rescue of the disease state across different cell lines and across chemical lesions highlights the general utility of our approach.

We characterized the rescue mechanism of FG-4592, by studying its effect on the hypoxia response and energy metabolism. While HIF1α is undetectable during normoxic exposure, treatment with FG-4592 stabilized the transcription factor even during normoxia. It has previously been noted that a paradox exists between severe mitochondrial dysfunction and cellular sensing of hypoxia [23]. In cell culture, full inhibition
of the RC prevents HIF stabilization, even under low oxygen conditions that would otherwise trigger the hypoxia response [23-24]. Of note, FG-4592 treatment bypassed this paradox and enabled HIF1α stabilization in the face of mitochondrial dysfunction, during states of normoxia or hypoxia (Fig. 3A). Further work is needed to determine if the paradox contributes significantly to disease pathology, or whether it is simply a feature of severe respiratory chain blockade in cultured cells.

The HIF transcriptional response is believed to be protective during states of hypoxia, at least in part by shifting the cell’s reliance away from mitochondrial oxidative energy metabolism. By shifting from aerobic respiration to anaerobic glycolysis, the HIF1α response can preserve energy supply at low oxygen tensions in a redox neutral manner. Indeed, treatment with FG-4592 for 24h, upregulated transcription of glycolytic enzymes (Fig. 3B, fig. S8) such as the glucose transporter 1 (GLUT1), hexokinase 2 (HK2), and lactate dehydrogenase (LDHA). HIF1α activation is also known to shunt the carbon supply away from the TCA cycle and towards the LDH reaction [25-28], as evidenced by the significant upregulation of pyruvate dehydrogenase kinase (PDK1) (Fig. 3B). Although lesions to the respiratory chain and hypoxia can in principle limit respiratory chain activity, cells do not mount the hypoxia response upon RC inhibition as the signal is lacking. However, FG-4592 treatment artificially triggers the hypoxia transcriptional program, even during normoxic conditions (Fig. 3B, fig. S8).

To corroborate the transcriptional changes, we also measured lactic acid production and oxygen consumption as proxies of glycolysis and oxidative phosphorylation. While glycolysis was somewhat increased by RC inhibition, likely as a result of allosteric mechanisms (Fig. 3C), treatment with FG-4592 increased glycolysis by nearly 25% in HEK293T cells under basal conditions. FG-4592 treatment also increased glycolysis beyond allosteric mechanisms in response to RC inhibition. Furthermore, basal oxygen consumption was decreased by approximately 2-fold with FG-4592 treatment (Fig. 3D). This may be protective in the setting of mitochondrial dysfunction, as it may limit the amount of ROS produced by impaired electron transport.

2.3.3 Genetic and small molecule proof-of-concept in zebrafish models

To further establish proof-of-concept, we asked whether genetic or small molecule activation of the hypoxia response would be protective against respiratory chain poisoning
in zebrafish embryos. vhl-null zebrafish continuously upregulate the hypoxia response throughout early development [29]. Just as vhl-knockout cells are protected against mitochondrial dysfunction, we asked whether vhl-null zebrafish might be more resilient to RC poisoning. Zebrafish embryos exhibit sensitivity to multiple, specific RC inhibitors including antimycin [30-32]. We demonstrated a significant improvement in the survival of vhl-null embryos exposed to 2.5nM antimycin compared to heterozygous and wild-type controls (Fig. 4A).

We then extended our small molecule approach to the zebrafish model of RC dysfunction. A previously generated zebrafish reporter strain Tg(phd3::EGFP) expresses GFP under the control of a HIF-responsive promoter, thereby enabling in vivo assessment of activating the hypoxia transcriptional response [33,34]. FG-4592 treatment of Tg(phd3::EGFP) embryos at 96hpf resulted in a time-dependent increase in fluorescence of individual reporter fish (Fig. 4B). Furthermore, in situ hybridization for the glycolytic HIF targets glut1 and Idha1, demonstrated significant upregulation upon FG-4592 treatment (Fig. 4C), confirming that FG-4592 engages the zebrafish prolyl-hydroxylases to trigger the hypoxia transcriptional program. We then demonstrated that co-treatment of embryos with FG-4592 rescued antimycin-induced death by nearly 2-fold (Fig. 4D). The genetic and small molecule experiments in zebrafish provide proof of concept that activation of the hypoxia program can protect against insults to the mitochondrial respiratory chain.

**2.3.4 Rationale for hypoxia as a therapeutic in a mouse model of Leigh syndrome**

The cellular and zebrafish models provided valuable proof-of-concept that individual components of the cellular response to hypoxia are protective against mitochondrial toxins. While small molecules are capable of activating specific branches of the hypoxia program, we reasoned that they may not have as broad and potent an effect as the naturally evolved, whole-body physiological response to hypoxia itself. Moreover, small molecule drugs for activating the hypoxia response are currently in
clinical trials for kidney disease and anemia. However, a large fraction of mitochondrial disease originates in the central nervous system, and our preliminary pharmacokinetic studies suggested limited blood-brain-barrier penetration of these drugs in mice. Higher doses resulted in whole-body toxicity. As mammals have evolved a complex homeostatic program to adapt to low oxygen tensions in their environment, we reasoned that a similarly broad hypoxic stress response could protect animal models of mitochondrial disease. Thus, we decided to test whether chronic exposure to moderate environmental hypoxia – breathing 11% O2, a level known to be tolerated by humans (equivalent to 4,500m altitude) – could alleviate the disease phenotype in a genetic mouse model of mitochondrial disease.

Leigh syndrome is the most common pediatric form of mitochondrial disease. Though relatively healthy at birth, patients develop irreversible neurodegeneration by two years of age [6]. These patients suffer bilaterally symmetric lesions in the brain stem and basal ganglia, with marked gliosis. Most patients die between the ages of 3-16 months. To date, over 75 different genes have been identified that can underlie this devastating syndrome, with Complex I deficiency being the most frequent biochemical cause of disease.

Recently, a mouse model of Leigh Syndrome has been generated by disruption of the murine Ndufs4 gene [35]. Loss of NDUFS4 in humans leads to one of the more severe recessive forms of Leigh syndrome. This murine model has been studied at atmospheric levels (21% O2) and faithfully recapitulates much of the reported human disease phenotype. Ndufs4 knockout (KO) mice breathing air display retarded growth rates, have impaired visual acuity and have a delayed startle response. Their body temperature falls progressively until reaching 32°C, shortly before death at 50-60 days of age. Diseased mice also display locomotor deficits and failure to thrive by 50d. Their neuropathology closely resembles clinical findings, with a substantial inflammatory response in the brainstem and cerebellum. Since this mouse model is well characterized and representative of mitochondrial disease progression, we tested chronic hypoxic exposure as a therapeutic strategy in this murine model.

2.3.5 Environmental hypoxia activates the hypoxic response in the Ndufs4 mouse model
We first studied whether Ndufs4 knockout (KO) mice were able to tolerate brief hypoxic exposure and mount a hypoxic response in a manner similar to WT mice. We exposed 3 wild type (WT) mice and 3 KO mice to breathing 8.5% O2 at sea level pressure for 6h. Acute exposure of wild type mice to hypoxia triggers HIF stabilization, resulting in Epo transcription and translation. After a 6h exposure, we measured Epo protein levels in plasma and showed that both WT and KO upregulated Epo production by approximately 40-fold (fig. S9). These results demonstrate that KO mice are able to mount a hypoxic transcriptional response and that the RC inhibition-HIF stabilization paradox does not extend to this disease setting.

2.3.6 Hypoxic breathing (11% O2) prevents Leigh syndrome in a mouse model

We reasoned that chronic exposure of mice to hypoxia may be protective in the setting of mitochondrial disease. Environmental hypoxia of 11% O2 was generated by adjusting the relative concentration of nitrogen and oxygen in the input gas mixture. This created environmental oxygen tensions similar to those found in the high mountain communities of Nepal and Peru (4,500m)[36]. Continuous gas flow and CO2 absorption by CaOH2 within the 11% O2 hypoxic chamber maintained CO2 levels below 0.4% with continuous monitoring. A control ambient environment for breathing 21% O2 was created with an identical chamber set-up. Ndufs4 KO and control mice were continuously exposed to breathing at normoxia or 11% hypoxia after enrollment in the experiment, excluding brief exposure to normoxia for behavior tests and maintenance 3 times per week. Untreated Ndufs4 KO mice typically begin to show disease progression after approximately 30 days of post-birth air exposure, which is about ten days after weaning. Since hypoxia-related vascular responses (constriction of pulmonary circulation, dilation of ductus arteriosus) occur in early post-natal development we initiated chronic hypoxic exposure treatments after the mice were 30 days old.

Remarkably, chronic hypoxia largely rescued this model of mitochondrial disease, preventing normal disease onset and allowing apparently healthy survival. All normoxia-exposed Ndufs4 KO mice either fulfilled criteria for humane euthanasia or died at a median age of ~60d with none surviving past 75d (Fig. 5A). However, there were no deaths in Ndufs4 KO mice that were chronically breathing 11% O2. Several mice showed
a mild clasping phenotype at ages greater than 120d. At the time of submission of this
manuscript, the oldest KO mice breathing 11% O2 were 170d old.

Hypoxia-treated mice showed an improvement in body weight gain, core
temperature maintenance, and neurologic behavior. All Ndufs4 KO mice continued to gain
weight between 30-37d of age (Fig 5B-C). At this stage, untreated KO mice lose weight,
become hypothermic and die. In contrast, Ndufs4 KO mice breathing 11% O2 gained
weight for several weeks, at which point body weight gain slowed, similar to the growth
kinetics of WT mice. The growth rate of hypoxia-treated KO mice matched that of WT
mice breathing 11% O2 upon treatment, suggesting that the primary cause of weight loss
was alleviated by hypoxic exposure. At 30d of age, untreated Ndufs4 KO mice have
similar core body temperatures to control mice. However by 50d, there is nearly a 4°C
drop in temperature (Fig. 5D). KO mice chronically breathing 11% O2 showed no
reduction of core body temperature. Thus, chronic hypoxic breathing improves the
underlying metabolic phenotype that directly or indirectly results in alterations of energy
and nutrient metabolism.

Ndufs4 KO mice, as well as patients suffering from Leigh syndrome, exhibit striking
defects in locomotor activity. Ataxia and failure to thrive are hallmarks of mitochondrial
dysfunction. Behavioral tests were performed at 10d intervals in normoxia and hypoxia-
treated, WT and KO mice. The rotarod test [37] measures the ability of mice to maintain
grip strength, balance and resist fatigue on an accelerating, rotating rod. At 30 days of
age, KO mice breathing air display a slight depression in the median time they can stay
on a rotarod (Fig. 5E). This ability declines by 40d and at 50d, untreated KO mice are no
longer able to stay on the rod for more than a few seconds, due to a combination of
muscular weakness, inability to balance and loss of visual activity. Hypoxia-treated WT
mice performed similarly to normoxia-treated control mice. KO mice breathing 11% O2
displayed a near complete rescue of this locomotor phenotype. As a further neurological
– behavioral test, spontaneous locomotor activity was measured as total distance traveled
within an hour (Fig. 5F). Untreated KO mice show drastically reduced spontaneous
locomotor activity. This defect is significantly rescued in hypoxia-treated mice, however
only to 50% of the values of normoxic WT mice (fig. S10).

2.3.7 Modest hyperoxic breathing exacerbates Leigh syndrome in a mouse model
The striking therapeutic effect of hypoxia led us to question whether oxygen itself was a key molecular parameter determining Leigh syndrome progression. Thus, we asked whether the converse environmental scenario of chronic mild hyperoxic exposure (55% O2) would affect the disease. We exposed WT and Ndufs4 KO mice to breathing 55% normobaric oxygen starting 30d of age. We observed no effect on survival of our WT mice exposed to 55% oxygen. However, all 9 Ndufs4 KO mice died after 2 to 11 days of breathing 55% O2 (Fig. 5A). On the other hand, Ndufs4 KO mice breathing air die at approximately 3-4 weeks after starting treatments. The dramatic reduction of survival in 55% O2 breathing KO mice, along with the dramatic extension of their apparently healthy survival when breathing 11% O2, points to the essential and previously unappreciated role of arterial oxygen tension in determining the progression of mitochondrial disease, and suggests that patients with mitochondrial disease may be highly sensitive to oxygen toxicity.

2.3.8 Circulating biomarkers and histopathology following treatment with hypoxia

We further characterized Ndufs4 KO mice following treatment with chronic hypoxia. As expected, the hematocrit in these mice is elevated from 40% during normoxia to ~60% during hypoxia, indicating EPO target engagement by hypoxic breathing (Fig. 6A). Given how effective hypoxia is in treating these mice, we asked whether they still retain a deficiency in complex I activity. Indeed, we find that although Ndufs4 KO mice appear quite healthy following hypoxia treatment, brain complex I activity remains dramatically reduced to the same levels as untreated Ndufs4 KO mice (Fig. 6B).

Normoxia-treated knockout mice exhibited substantial neuronal degeneration. Lesions were accompanied by Iba-1+ microglial proliferation within olfactory lobes, cerebellum and brainstem as documented elsewhere [38]. In contrast, knockout mice breathing 11% O2 exhibited minimal to no lesions (Fig. 6C), and were virtually indistinguishable histologically from WT controls.

Recently, α-hydroxybutyrate (α-HB) has been identified as a novel circulating plasma marker of Leigh syndrome [39]. Indeed, we report that α-HB is elevated in air breathing Ndufs4 KO mouse plasma. Treatment with chronic hypoxia rescued the elevation of this disease marker (Fig. 6D). Similarly, plasma lactate levels were increased
in Ndufs4 KO mice breathing air between 50-65d of age, while this was partially rescued by 11% hypoxic exposure (Fig. 6E).

Collectively, these laboratory and histopathological studies confirm that chronic hypoxic exposure to breathing 11% O2 activates the endogenous hypoxic response. Hypoxia does not fix the proximal lesion within mitochondrial complex I, but rather, prevents the onset of subsequent biochemical and histopathological defects.

2.4 Discussion

In the current study, we discover that hypoxic gas mixtures may be useful in treating or preventing mitochondrial disorders. At the surface, this approach is seemingly counterintuitive, since oxygen is a key substrate for the respiratory chain. However, hypoxia activates an evolutionarily-conserved adaptive program which allows mammals to cope with limiting oxygen levels. This program decreases an organism’s reliance on mitochondrial oxidative metabolism. Such adaptive programs are not necessarily triggered by mitochondrial disease, as the hypoxic signal is absent. Moreover, hypoxia leads to a state in which oxygen delivery and consumption are simultaneously reduced, whereas in mitochondrial disease, oxygen delivery continues in the face of impaired utilization. Such a mismatch between delivery and utilization, potentially contributes to oxygen toxicity. Hence, hypoxia may represent nature’s solution for overcoming mitochondrial disease pathology, both by triggering innate adaptive programs and by simultaneously limiting the substrate for oxygen toxicity.

Multiple cellular and systemic mechanisms are likely acting in concert to provide the remarkable therapeutic effect we observe in mice. First, hypoxia may be triggering the HIF-dependent transcriptional program that is known to activate key biochemical pathways – including glycolysis for redox neutral ATP production and decreased flux through PDH to prevent mitochondrial ROS generation. Second, breathing 11% O2 reduces the provision of oxygen to the cell, O2 that would otherwise be available as a substrate for free radical production or aberrant signaling. Many enzymes are designed to operate under reducing conditions and are highly sensitive to ambient oxygen levels. Hypoxia may establish a new oxygen set point that is better suited to the cellular environment created by impaired RC activity, whereas hyperoxia may create an environment that is less favorable. Third, it is likely that hypoxia is also operating at the
level of organ systems physiology (e.g., O2 delivery and CO2 clearance by the cardiovascular system, endocrine function, immune signaling), which are inherently non-cell autonomous and previously reported to be altered in humans living at high altitudes. Future studies will fully decipher the role of HIF, and cellular vs. cell-autonomous mechanisms underlying the pronounced in vivo therapeutic benefit.

Our study holds important therapeutic potential. However, before clinical trials in humans can begin, several key pre-clinical studies are necessary. First, studies in additional genetic mouse models will determine the generalizability of our approach to other rare mitochondrial disorders, common disorders of mitochondrial dysfunction, and disorders of oxidative stress. Second, in the current study, we have utilized a treatment regimen consisting of chronic 11% inspired oxygen. Humans can acclimate to high altitudes such as those encountered in Mount Blanc, Peru, and Nepal, where ambient oxygen tensions are comparable to those employed here. Future studies should evaluate if doses between 11%-20% may also be effective and perhaps better tolerated by patients. Third, if intermittent hypoxia proves as effective as chronic hypoxia, it may allow for a nighttime therapy for which face masks and sleeping tents have already been devised by the sports industry. Such preclinical studies that establish safety and efficacy in animals will help to optimize clinical trial design in humans.

An important principle in the management of mitochondrial disease is to avoid exposures – such as aminoglycoside or tetracycline antibiotics – that are known to be toxic to mitochondria. Patients with mitochondrial disease (like many other patients) are routinely treated with supplemental oxygen and breathe high levels of inspired oxygen during general anesthesia, recovery from surgery and during intensive care. Retrospective or prospective studies may help to extend our observations to humans, and if confirmed, would imply that caution should be exercised in O2 exposure and that administering supplemental oxygen should be limited to those instances in which it is clinically indicated. At present, how lesions in the respiratory chain lead to such diverse pathology remains a mystery. Given the striking therapeutic efficacy of hypoxic breathing and detrimental effect of moderate hyperoxia, we predict that aberrant oxygen metabolism, signaling, or toxicity lies at the heart of mitochondrial pathogenesis. The
identification of such a critical parameter suggests that a real understanding of mitochondrial pathogenesis is within reach.

The current study has focused on orphan mitochondrial disorders. It is notable that the aging process and virtually all age-related degenerative diseases are associated with secondary mitochondrial dysfunction and oxidative stress [40]. While antioxidants have been proposed for alleviating these disorders by scavenging free radicals, our current work suggests that simply limiting the substrate for oxygen toxicity may prove more effective. Moreover, hypoxia can trigger an adaptive program designed to decrease our body’s reliance on mitochondrial oxidative metabolism. It is conceivable that breathing hypoxic gas mixtures may prevent the onset of more common disorders.

2.5 Materials and Methods:

Genome-wide Cas9-mediated Knockout Screen

Virus Production

The genome-scale CRISPR knockout (GeCKO) library v1 was generously provided by the Zhang Lab. Library details have previously been published (10). For library lentivirus production, 1.2x10^7 cells were placed in each of 25, T225 flasks in 50ml of full DMEM (Life Technologies 11995) media (containing Pen/Strep, 10% FBS). 18h later, media in each flask was replaced with 13ml of DMEM (no Pen/Strep, 10% FBS) and 2h later, media was replaced with 20ml OptiMEM (Life Technologies 31985-070; no serum, no Pen/Strep). A transfection mastermix was made by combining individually prepared mastermix A (94ml of OptiMEM, 2.4ml of Lipofectamine 2000 (Life Technologies)) and mastermix B (94ml OptiMEM, 2.1ml of PLUS Reagent (Life Technologies), 240μg of pVSVg plasmid, 360μg of psPAX2 plasmid and 480μg of GeCKO plasmid library). Mastermixes A and B were combined for 20m. 8ml of the final mastermix was added to each T225 flask of HEK293T cells. After 6h, the media was changed to 30ml of DMEM media (Life Technologies 11995 w/ 1% BSA (Sigma)) and cells were incubated for 48-72h, before virus-containing supernatant was collected. Virus was concentrated by centrifugation for 2h at 24,000 rpm in a SW32Ti rotor. Virus was resuspended overnight at 4C and stored at -80C prior to use.

Knockout Screen
K562 cells were obtained from ATCC and maintained in full DMEM media (10% FBS, Pen/Strep). K562 cells were grown in 1L spinner flasks (Matrical) on magnetic stir plates (Bellco). Cells were always passaged before reaching confluency (1x106/ml) and subcultured at a concentration of 1x105/ml.

**Virus Infection**

2.5x10^8 K562 cells were resuspended to a concentration of 1.5x10^6 cells/ml. Polybrene (Sigma) was added to 120ml of the K562 cell suspension at a final concentration of 4μg/μl. 2ml of this cell suspension was placed in each well of 5, 12-well plates. 10ul of virus was added to each well for a target MOI of 0.3, ensuring that most cells incorporated 1 or 0 lentivirus particles. Plates were centrifuged for 2h at 1000g and placed in an incubator for 1h, after which media was aspirated. 2ml of full DMEM media (10% FBS, Pen/Strep) was placed in each well and cells were resuspended. 12h later, all wells were pooled into a spinner flask with 800ml of full DMEM media. A sample was taken for virus titration to ensure that the target MOI was obtained. 24h after the infection, Puromycin (Invitrogen) was added at a final concentration of 2μg/ml to begin selection for infected cells. Two independent infections (Infection 1 and Infection 2) were performed to control for variability in library infection.

**Passaging**

Infected cells were passaged before reaching 1x10^6/ml and maintained in Puromycin-containing conditions for one week after infection. At this point, 7x10^7 cells were pelleted and stored as pre-treatment (Early) samples for each infection replicate.

**Experimental Selection**

After 1 week of Puromycin selection, cells from each infection replicate were transferred to experimental conditions of (a) untreated cells, (b) moderate disease and (c) severe disease. 7x10^7 cells were pelleted and resuspended in media corresponding to each experimental condition. The untreated condition was defined as DMEM media (11965-092) with 1mM pyruvate (Invitrogen) added. The moderate disease condition was defined as DMEM media with 100nM antimycin (Sigma) and 1mM pyruvate. The severe disease condition was defined as DMEM media with 100nM antimycin, without pyruvate. All media also contained 200μM uridine (12).

**Passaging in Experimental Conditions**
Cells were subcultured at 1x105/ml and passaged before reaching 1x106/ml. At each passage, 7x107 cells were pelleted and stored for intermediate screen time points.

**Library Prep**
Sequencing libraries were prepared as previously described (10). Briefly, DNA was extracted using the Qiagen Blood and Cell Culture DNA Maxi Kit from 7x107 cells per experimental condition, for each infection replicate. DNA was then purified using Micro Bio-Spin columns (BioRad 732-6224). 25 PCR reactions were performed using Herculase II Fusion DNA Polymerase (Agilent) to amplify the single guideRNAs (sgRNAs) from genomic DNA, at a minimum coverage of 450x per guideRNA. 30μl from the first pooled PCR samples were used as input for the second PCR reaction, allowing for attachment of barcodes and sequencing adapters. Barcode replicates were included for the moderate disease condition to ensure that PCR errors did not substantially affect results. The final PCR products were run on an agarose gel and the correct size PCR products were gel extracted and sequenced on an Illumina HiSeq 2500.

**Analysis**

**Processing of sgRNA Reads**
Custom Python and Matlab scripts were written for processing of sequencing reads. Reads were trimmed to remove barcodes and sequences corresponding to the GeCKO library backbone. A custom bowtie library was created for mapping between sgRNA sequences and guide/gene names. Bowtie alignment was performed, allowing for single mismatches. Finally, guide abundance was compiled for each experimental condition and a matrix mapping guide name to abundance for all samples was created.

**Identification of Enriched and Depleted sgRNAs**

**Cell Viability Screen**
In order to identify genes which are essential to cell viability, guide abundance was first normalized to total number of reads per sample. The fold-enrichment was calculated for untreated samples (day 21 after experimental selection) relative to pre-treatment conditions (immediately before experimental selection), for both infection replicates. As infection replicates were very well correlated (r2 > 0.8), the top 500 most depleted genes (by RIGER (41) analysis) were found for each infection replicate. The 500 most essential genes were determined for both infection replicates and compared to the known list of
mitochondrial proteins, or MitoCarta to identify essential mitochondrial genes (42, 13). Genes without entrez id mappings were excluded (< 3% of list). RIGER output was generated for each infection replicate (untreated relative to pre-treated conditions) and used for Gene Set Enrichment Analysis (GSEA) (43).

**Enrichment Screen**

In order to identify gene knockouts which allow cells to cope with mitochondrial dysfunction, fold-enrichment was calculated for moderate or severe disease conditions relative to pre-treatment (early) conditions. The most enriched genes were then individually checked for their overall effect on cell viability (untreated relative to pre-treatment conditions). Alternatively, fold enrichment was also calculated for untreated vs. disease conditions. However, this form of analysis confounds genes which are enriched in disease conditions or selectively depleted in untreated conditions. The top hit was found using either approach.

**RIGER Analysis**

RIGER analysis (41) was used to generate a summary statistic by combining information corresponding to all sgRNAs for a given gene. sgRNAs were pre-ranked by fold-enrichment between two conditions. The Kolmogorov-Smirnov method was used with 1000 permutations. Gene scores were not adjusted for the number of sgRNAs corresponding to a given gene.

**GSEA Analysis**

Output from RIGER analysis was used for gene set enrichment analysis (43). All curated gene sets were used during analysis in GseaPreranked mode. All other parameters were set to default values.

**Lentiviral Cas9 Vector**

Individual sgRNAs targeting VHL from the GeCKO library were cloned into the lentiviral Cas9 vector and used for follow-up experiments. Additionally, “dummy” non-targeting sgRNAs were generated as controls. The following oligonucleotides were used for lentivirus sgRNA cloning:

**Oligo_1:** 5' – CACCG < 20bp sgRNA sequence> -3’

**Oligo_2:** 5' AAAC <complimentary 20bp sgRNA sequence> - C – 3’

**Oligo Name** Oligo Seq (5’ → 3’)
The lentiCRISPRv1 plasmid was digested with BsmBI, gel-purified and phosphorylated by T4 PNK. Above oligonucleotides were separately annealed for each sgRNA construct. The annealed product was ligated into the lentiCRISPRv1 plasmid and transformed into Stbl3 competent cells.

**Growth Curves**

All growth curves were performed in either K562, HT29 or HEK293T cells (ATCC). All conditions were counted in duplicate wells for each growth curve time point. Each growth curve was performed in independent experimental set-ups a minimum of two times in each cell type. Representative growth curves are shown in Fig 2.

**Lentivirus VHL Growth Curves**

K562 cells were infected using the lentivirus CRISPR construct containing sgRNAs targeting VHL or dummy guides, following the same protocol as used in the original GeCKO screen. Puromycin selection was started the day following the infection. Cells were allowed to grow in Puromycin-selection conditions for at least one week, before beginning growth curves. The VHL-edited cells and dummy-guide infected cells were
grown at a starting concentration of 2.5x10^4 or 5x10^4 cells/ml in 24-well plates. Cells were placed in DMEM media (Life Technologies 11995, 10% FBS, Pen/Strep) with (a) DMSO, (b) 100nM antimycin (Sigma), (c) 100nM oligomycin (Sigma) or (d) 1µM piericidin (Santa Cruz). All conditions for growth curves contained 1mM pyruvate and 200µM uridine, reflecting the moderate disease condition. Cells for each condition were counted in duplicate wells every 24h for at least 3 days.

**FG-4592 Growth Curves**

FG-4592 was purchased from MedChem Express (HY-13426) and Cayman Chemicals (CAS 808118-40-3). Drug stocks were made in DMSO at 150mM. Cells were pre-treated with 40-75µM FG-4592 for at least 24h before beginning growth curves. This allowed for the HIF transcriptional response to begin prior to treatment with respiratory chain inhibitors. All cells were resuspended at a concentration of 2.5x10^4/ml in 24-well plates and placed in (a) DMSO, (b) 100nM antimycin, (c) 100nM oligomycin or (d) 1µM piericidin. Cells were counted daily (HEK293T and K562) or every other day (HT-29). Cells in all conditions were counted in duplicate or triplicate wells. At least two independent growth curves were performed with independent cell stocks for each growth curve presented in Fig 2.

**HIF1α Immunoblotting**

Cells were exposed to DMSO, 100nM antimycin or 100nM oligomycin, ± 50µM FG-4592, in 6-well plates. Plates were then placed in normoxic (21% O2) or hypoxic (1% O2) conditions for 12-18h. Air tanks corresponding to 1% O2 (balance nitrogen) or 21% O2 were purchased from Airgas. Cell culture plates were placed in hypoxic chambers (Billups-Rothenberg), sealed and flushed with air corresponding to different conditions (normoxia or hypoxia) for 7-10m. After overnight treatment, hypoxia chambers were opened and cells were collected using standard methods and placed in RIPA buffer (Boston BioProducts). As the normoxic half-life of HIF proteins is less than 5 minutes at room temperature, all samples and buffers were handled at 4C. 30µg of protein were loaded onto an 8% SDS-PAGE gel (Invitrogen). HIF1α was detected using Bethyl A300-286A and the loading control, β-tubulin was detected using CST 2128S.

**qPCR**
24h prior to RNA collection, 2.5x10⁵ HEK293T or HT-29 cells were seeded per well in a 6-well plate in 3ml of DMEM (Life Technologies (11995), 10% FBS). Cells were incubated in 50μM FG-4592, 100nM antimycin, 1μM piericidin as indicated. Total RNA was extracted using an RNeasy Mini Kit (Invitrogen). cDNA, generated by Superscript III Reverse Transcriptase (Life Technologies), was combined with Taqman Fast Advanced Master Mix and assayed by quantitative PCR. All CT values for genes of interest were normalized against the HPRT gene (probe/control). The ratio (probe/control) was set to 1 for vehicle-treated cells. Oligonucleotide primer pairs for GLUT1, HK2, LDHA and PDK1 were purchased from Life Technologies.

**Lactic Acid Measurements**

Cells were placed in 6-well plates at a concentration of 1x10⁵/ml and pre-treated with 50μM FG-4592 or DMSO for at least 24h. After pre-treatment, media was replaced so that it contained either (a) DMSO, (b) 100nM antimycin or (c) 100nM oligomycin ± 50 μM FG-4592. Cells were incubated in given conditions for 8h. Media was then collected, spun down to remove cell debris and subsequently used for lactic acid measurements. Lactic acid concentrations were measured using the YSI 2900 Biochemistry Analyzer. Samples were run in duplicate or triplicate for each set of conditions. Each set of conditions was tested in at least two independent experiments. A representative experimental set is shown in Fig 3.

**Oxygen Consumption Measurements**

48h prior to measurement, cells were seeded at 3-4x10⁴ per well in XF24 24-well cell culture microplates in 200μl of normal growth media (DMEM (Life Technologies, 11965), 3.7g/L NaHCO3 and 10% FBS) and were incubated at 37C in a 5% CO2 incubator. 12h after seeding, 800μL of normal growth medium containing either FG-4592 or DMSO was added to each well, resulting in final drug concentration of 50μM. 15m prior to input of cell plate in XF24 Extracellular Flux Analyzer, media was replaced with 850μL of assay medium -DMEM (US Biological, D9800), 25mM glucose, 1mM pyruvate, 15.9 mg/L phenol red, 10% FBS and 25mM HEPES-KOH, pH 7.4. Assay medium contained either 50μM FG-4592 or DMSO. Measurements were performed at consecutive intervals of mixing (2m), waiting (2m) and measurement (4m). Basal measurements were collected
4 times. OCR measurements normalized to cell number were averaged from three independent trials.

**Zebrafish FG-4592 Treatment and Reporter Fish Imaging**

Fish were raised and treated according to institutional protocols and regulations on an approved IACUC protocol, HMS 04626. Transgenic reporter embryos [Tg(phd3::EGFP)] and wild-type sibling controls were exposed to 2.5D M FG-4592 in 0.1% DMSO at 96hpf. Fluorescence was assessed in vivo using a Zeiss Discovery V.8 fluorescent stereoscope equipped with an AxioCam MRc digital camera.

**Zebrafish Antimycin Survival Curves**

Wild-type zebrafish embryos (TU strain) were raised to 96hpf, and then exposed to 2.5nM Antimycin ± 2.5D M FG-4592. Embryonic death was assessed over the next 72 hours.

**VHL +/- Zebrafish Survival Curves**

vhl+/− were incrossed, and resultant embryos were raised to 48hpf (since vhl−/− show lethality at later time points) and then exposed to 2.5nM antimycin. Fish were then scored as vhl−/− or WT and heterozygotes. Embryonic death was assessed over the next 24h.

**VHL in-situ hybridizations**

In situ hybridization for glut1 and ldha1a were performed on zebrafish embryos fixed in 4% paraformaldehyde at 120hpf, after 24h of treatment with 2.5D M FG-4592. In situ hybridization was conducted using standard protocols (http://zfin.org/ZFIN/Methods/ThisseProtocol.html).

**Breedings and General Animal Care for Mouse Work**

The Palmiter lab at University of Washington generously provided us with Ndufs4 +/- mice. We then expanded our colony and bred sufficient Ndufs4 +/- and control (Ndufs4 +/- and WT) mice for experiments included in this manuscript. Pups were weaned and genotyped at ~25d after birth. All cages were provided with food and gel, as well as water bottles. Food and gel were replaced three times a week and cages were changed once a week. Mice were humanely euthanized if they lost more than 20% of peak body weight. Ndufs4 +/- and WT mice are indistinguishable in all assays we tested and were therefore combined to serve as control samples. All animals were cared for under the guidelines of Partners Healthcare. All the animal studies were approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital, Boston, MA.
Chronic Hypoxic, Normoxic and Mild-Hyperoxic Exposures

Wild type and Ndufs4 KO mice were exposed to chronic hypoxia (11% O2), normoxia (21% O2) or mild hyperoxia (55% O2) at ambient sea-level pressure. Mice were placed in 60 liter plexiglass chambers that were given a gas mixture of compressed air and 100% N2, compressed air alone or compressed air and 100% O2 (Airgas Inc.). The gas flow rates were measured and controlled with rotameters and valves. Oxygen concentrations were measured several times each day at the outlet of the chambers using an oxygen analyzer (MiniOx I Oxygen Analyzer, Ohio Medical Corporation) and the flow rates of air, nitrogen and oxygen were modified if necessary in order to obtain a stable oxygen concentration of 11% in the hypoxic chamber and 55% in the mild-hyperoxia chamber.

Soda lime (Sodasorb, Smiths Medical) (approximately 250g), was placed on the floor of each chamber to scavenge carbon dioxide (CO2) produced by the animals and replaced every 3 days. The CO2 concentration in each chamber as well as the temperature and the humidity were monitored continuously using a dedicated infrared CO2 analyzer, thermometer and humidity meter (Extech CO200 Monitor, Extech Instruments). The total flow of fresh gas flushing each chamber was adjusted between 5 and 10 L/min to maintain the chamber CO2 level below 0.4% and the relative humidity between 30% and 70%.

Mice were exposed to gas treatment continuously for 24 hours per day, 7 days a week. The chambers were briefly opened three times a week to weigh the mice, evaluate their neurological status, clean the cages and add water and food.

Due to the extremely long duration of the experiment, several accidental deaths were observed in the different conditions due to severe infection (retinal conjunctivitis), blunt trauma and accidental CO2 accumulation. As each of these were isolated incidents and unrelated to the experimental question, this data was not incorporated into the manuscript.

Blood and Tissue Collection

Mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine (120 mg kg-1) and fentanyl (0.09 mg kg-1). Following tracheostomy, rocuronium (1 mg kg-1) was injected i.p. to induce muscle relaxation. Volume-controlled ventilation was provided at a respiratory rate of 90 breaths min-1, a tidal volume of 10 ml kg-1, PEEP 1 cmH2O and inspired O2 fraction (FIO2) of 0.21 (Mini Vent 845; Harvard Apparatus). Blood was
collected by cardiac puncture after opening the chest via median sternotomy and tissues were collected and immediately frozen in liquid nitrogen.

**Rotarod Activity Measurements**

A rotarod machine (Ugo Basile) was used to measure the ability of mice to stay on an accelerating, rotating rod. Rotarod parameters were as follows: acceleration of 5rpm/m and a maximum speed of 40rpm. On each measurement day, three trials were performed, with individual trials at least 10m apart to allow mice to recuperate. The median time on rotarod is reported. If mice used their body to grasp the rod (rather than walking on it) for more than 10s, this time was recorded as time of fall. Age of measurements is +/- 5 days for practical purposes without any age bias between groups.

**Spontaneous Locomotor Activity Measurements**

The open field instrument (Med Associates Inc.) was used to measure spontaneous locomotor activity. Mice were blindly chosen for a given day and placed in open field chambers for 1h. Spontaneous locomotor activity was measured based on beam breaks and recorded by the instrument. The traces shown in Fig. 5E are representative of a sick, Ndufs4 -/- mouse exposed to 21% O2 (age of such sickness varies slightly between mice) and age-matched KO and WT mice exposed to 11% O2, and WT mice exposed to 21% O2. Age of measurements is +/- 5 days for practical purposes without any age bias between groups.

**Histology**

Mice were anesthetized as during tissue collection (see above). The chest cavity was opened and a catheter was placed in the left ventricle. The whole body was perfused with ice cold PBS and then with 4% PFA. The brain was dissected out, stored overnight in 4% PFA and then placed in 30% sucrose (in PBS) for two days.

Formalin-perfused brains were sectioned parasagittally at the olfactory lobes. Two transverse sections of cerebellum and brainstem were also collected: a rostral section with subjacent pons, and a more caudal section with medulla oblongata. Immunohistochemistry was performed on adjacent tissue sections using an antibody recognizing the microglial marker Iba-1 (Wako; 2µg/ml) according to methods described elsewhere (40).

**Complex I Activity Assay**
Complex 1 Activity was measured in cerebellum tissue from mice. Tissue was homogenized in 1ml of ice-cold PBS using a Qiagen TissueLyser II. Approximately 50-100mg of tissue was used as input material for the Complex I Enzyme Activity Microplate Assay Kit (ab109721, Abcam). Absorbance was read at 450nm wavelength and recorded every 30s for 135 total measurements. Background signal was not subtracted in data shown.

**Erythropoietin (Epo) Measurements**

Mice were exposed to 21% O2 or 8.5% O2 for 6h. Blood was collected as described above in EDTA-containing tubes. Plasma was then used for detection of Epo using the Mouse Erythropoietin Quantikine ELISA Kit (EP00B, R&D Systems).

**Plasma Metabolomics**

Lactate and α-hydroxybutyrate were quantified in mouse plasma by spiking in each corresponding isotope labeled standard (CDN isotope). A series of standard solutions of metabolites at 7 different concentrations were prepared in a surrogate matrix buffer (PBS buffer with 30g/L human serum albumin). 30uL of the mouse plasma sample were combined with 20uL of isotope labeled internal standard, vortexed for 10s and spun down for 10s. Metabolite extractions were performed using 70% acetonitrile. A Q Exactive Plus Orbitrap Mass Spectrometer coupled to a Dionex UltiMate 3000 UHPLC system (Thermo Fisher Scientific) was used for LC-MS. The Xbridge amide HILIC column (2.1 X100 mm, 2.5 μM particle size, from Waters 186006091) was used to separate metabolites and MS was acquired under the negative ionization mode. The column was maintained at 27°C during runs. The mobile phase A was 20mM ammonium acetate, 0.25 % ammonium hydroxide pH adjusted to 9. The mobile phase B was 100% acetonitrile. The MS data acquisition was full scan mode in a range of 70–1000 m/z, with the resolution set at 140,000, the AGC target at 3E6, and the maximum injection time at 400 msec.
2.6 References

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Figure 1. Genome-scale Cas9-mediated knockout screen identifies VHL inhibition as protective during states of mitochondrial dysfunction. (A) Mitochondrial disease was modeled with the addition of the complex III inhibitor, antimycin (moderate disease) or antimycin and removal of pyruvate (severe disease). (B) K562 cells were infected in duplicate with the genome-scale Cas9-mediated knockout library, and separated into conditions of untreated, moderate disease or severe disease. Samples were taken at a pre-treatment time point, as well as after three weeks of selection. (C) Growth curves for cumulative differences in growth rates in different experimental conditions for both infection replicates. (D) RIGER output based on enrichment of sgRNAs in severe disease condition relative to pre-treatment conditions. Each row denotes a single gene, with ranks of corresponding sgRNAs in middle column. Ranks for individual sgRNAs are out of ~65,000 total sgRNAs in library. (E) sgRNA enrichment magnitude vs. rank, with most enriched sgRNA shown to the far right. sgRNAs corresponding to VHL in red. (F) Guide abundance in pre-treatment conditions (Infection 1 vs. Infection 2) shown in grey for each sgRNA, representative of experimental noise. Guide abundance in severe disease condition vs. pre-treatment condition in black, with VHL sgRNAs in red.
Figure 2. Genetic or small molecule activation of the HIF response is protective against multiple forms of RC inhibition, in multiple cell types. (A) Schematic for HIF degradation during normoxia. (B) Schematic for induction of hypoxia transcriptional program during hypoxia. (C) Growth curves for K562 VHL-knockout cells (cyan, blue) or non-targeting sgRNA cells (black, red) for untreated or disease conditions (mean shown). Disease conditions correspond to inhibition of Complex I (piericidin), Complex III (antimycin) or Complex V (oligomycin). Growth curves for (D) K562 cells, (E) HEK293T cells and (F) HT-29 cells FG-4592, in combination with untreated or disease conditions (inhibition of complex I, III and V). All time points were measured in duplicate and all growth curves are representative of 2-3 independent experiments (mean shown). All final cell counts of FG-treated rescue (or VHL-KO rescue in 2A) in presence of RC inhibitor was statistically significant (one-sided t-test p-value < 0.05).
Figure 3. FG-4592 causes normoxic stabilization of HIF1α and rewire energy metabolism. (A) Immunoblot against HIF1α ± RC inhibition with antimycin or oligomycin, ± FG-4592 under normoxia (21% O2) or hypoxia (1% O2). RC inhibition prevents HIF1α stabilization during hypoxia. FG-4592 administration overcomes this paradox and stabilizes HIF1α even during normoxia. Immunoblot is representative of independent experiments done in duplicate in multiple cell types. (B) Normalized expression for known HIF targets glucose transporter 1 (GLUT1), hexokinase 2 (HK2), lactate dehydrogenase A (LDHA), and pyruvate dehydrogenase kinase 1 (PDK1) +/- RC inhibition, +/- FG-4592 in HT-29 cells. Data shown as mean of two independent experiments and normalized so vehicle-treated expression (probe/control) is 1. (C) Mean concentration of lactic acid secreted by cells treated with FG-4592 or DMSO ± RC inhibitors as proxy for anaerobic glycolytic flux. Data shown for HEK293T cells (without pyruvate to eliminate contribution from LDH reaction) and is representative of at least two independent experiments (D) Basal oxygen consumption rates for HEK293T cells treated with FG-4592 or DMSO for > 24h, averaged across three independent experiments (Mean ± S.E.). (one-sided t-test p-value < 0.05 for all pairwise comparisons ± FG-4592 in figures 3B-3D).
Figure 4. vhl knockout or FG-4592 treatment activates the HIF response in zebrafish embryos and alleviates death caused by respiratory chain inhibition. (A) 48hpf vhl-null zebrafish are less sensitive to RC inhibition than control (WT and Het) fish, n > 75 per treatment, p < 0.001 by Mantel-Cox test. (B) FG-4592 treatment activates expression of HIF-responsive promoter in Tg(phd3::EGFP) embryos. Images are shown for embryos treated with either DMSO or 2.5pM FG-4592 from 96 to 102hpf. Embryos were assayed for GFP expression at 0 hours post treatment (hptx) and 6hptx. DMSO treatment fails to activate GFP expression beyond autofluorescence in Tg(phd3::EGFP) transgenic embryos, while FG-4592 robustly initiates GFP expression by 6hptx. (C) Known Hif targets, glut1 and idha1 are overexpressed in 96hpf zebrafish embryos treated with FG-4592 for 6h. (D) Exposure to FG-4592 rescues antimycin-induced zebrafish embryonic death. Respiratory chain inhibition by 2.5nM antimycin in 4dpf (days post fertilization) embryos results in significant death within the first 24 hours of treatment. Co-exposure of antimycin with FG-4592 (2.5μM) doubles embryo survival, while FG-4592 alone has no impact. n=75 per treatment, p < 0.0001 by Mantel-Cox test.
Figure 5. Chronic hypoxia extends lifespan and alleviates disease in a mouse model of Leigh syndrome, whereas chronic hyperoxia exacerbates disease. (A) Ndufs4 KO mice of both genders were chronically exposed to hypoxia (11% O₂), normoxia (21% O₂) or hyperoxia (55% O₂), at 30d of age and survival was recorded (n = 12, n = 12, n = 9 mice respectively). Cyan bars represent current age of hypoxic KO mice. (B) Body weights were measured in WT and KO mice exposed to normoxia or hypoxia, three times a week upon enrollment in the study. Weights are shown as mean ± S.E. (C) Representative images of 50d-old KO mice exposed to normoxia or hypoxia. (D) Body temperature was measured in KO mice exposed to normoxia or hypoxia at age ~30d, 40d and 50d. Temperatures are shown as mean ± S.E. (n ≥ 7 for all groups) (E) Latency to fall on an accelerating rod was measured as median values of triplicate trials per mouse for WT and KO mice, exposed to normoxia or hypoxia at different ages (n ≥ 7 for all groups). (F) Representative 1h locomotor activity traces of sick, normoxia-treated KO mice and age-matched hypoxia-treated KO mice, as well as controls. All data shown as normoxia KO (maroon), hypoxia KO (blue), normoxia WT (black) and hypoxia WT (grey). *denotes t-test p-value < 0.05.
Figure 6. Hypoxia exposure of Ndufs4 KO mice alleviates metabolic disease markers, as well as neuropathology, without rescuing Complex I activity. (A) Hematocrit values for WT and KO mice treated with normoxia or hypoxia for ~3 weeks (n = 3-4 per group, test p-value < 0.05 for normoxia vs. hypoxia for both WT and KO). (B) Complex I Activity is significantly reduced in KO mice relative to WT mice, in both normoxic and hypoxic conditions (n = 3-4 per group, t-test p-value < 0.01). (C) Representative images for immunostaining against the inflammatory marker, Iba-1, in the olfactory bulb and cerebellum of Ndufs4 KO mice treated with hypoxia or normoxia and WT mice exposed to normoxia breathing. The number of Iba-1 positive cells per 10 random fields of view shown for each treatment group (Mean ± S.E., t-test p-value < 0.01 for normoxic vs. hypoxic KO, n = 3-4 per group) (D) Plasma α-HB levels in WT and KO mice, exposed to hypoxia or normoxia (n = 4-8 per group). Median shown as horizontal bar. (E) Plasma lactate in WT and KO mice, exposed to hypoxia or normoxia (n = 4-8 per group). Median shown as horizontal bar.
Chapter 3: Hypoxia treatment reverses neurodegenerative disease in a mouse model of Leigh syndrome


This chapter is presented in the context of its contemporary science and will appear as Ferrari*, Jain* et al., PNAS. 2017 [Epub ahead of print]
3.1 Abstract

Inherited defects in mitochondrial energy metabolism give rise to devastating human disorders for which there are no proven general therapies. The most common pediatric mitochondrial disease is Leigh syndrome, an episodic, subacute neurodegeneration that can lead to death within the first few years of life. Mice lacking the complex I subunit, Ndufs4, develop a fatal progressive encephalopathy resembling Leigh syndrome and die at approximately 60d of age. We previously showed that continuously breathing normobaric 11% O₂ from an early age prevents neurological disease in Leigh syndrome mice and dramatically improves survival. Here, we report three key advances. First, we report updated survival curves and organ pathology in Ndufs4 KO mice exposed to hypoxia or hyperoxia. It is known that normoxia-treated KO mice die from neurodegeneration at about 60d. We find that hypoxia-treated mice die at about 270d, likely from cardiac disease, while hyperoxia treated mice die within days of exposure from respiratory distress. Second, we demonstrate that more conservative hypoxia regimens, such as continuous normobaric 17% O₂ or intermittent hypoxia, are ineffective in preventing Leigh syndrome neuropathology. Finally, we show that breathing normobaric 11% O₂ in mice with late-stage encephalopathy reverses established neurological disease, evidenced by improved behavior, circulating disease biomarkers, and survival. Importantly, the pathognomonic T2-hyperintense MRI brain lesions and neurohistopathologic findings are reversed after four weeks of continuously breathing 11% O₂. Upon return to normoxic breathing, Ndufs4 KO mice die within a few days. To our knowledge, hypoxia is the first experimental therapy capable of reversing neurodegeneration in an animal model of Leigh syndrome. Future work is required to establish the generalizability of hypoxia therapy in additional animal models of mitochondrial disease, and to determine if it can be provided in a safe and practical manner to allow in-hospital human therapeutic trials.

3.2 Introduction

Defects in mitochondrial function, due to mutations either in the nuclear genome or in the mitochondrial DNA (mtDNA), result in severe diseases that can present at any point from infancy through adulthood [1]. The most common biochemical class of mitochondrial disorders arises from genetic mutations affecting the mitochondrial
respiratory chain (RC) with an incidence of approximately 1 in 4300 live births [2]. The management of these disorders is challenging in part because of their clinical and genetic heterogeneity. Virtually any organ system can be impacted, and to date, more than 250 genes encoding mitochondrial proteins are known to be disease-causing [3]. Of these, at least 150 can underlie disorders of oxidative phosphorylation. The mainstay of therapy involves the use of vitamin cocktails that have little or no proven efficacy, as well as avoiding drugs that are known to be mitochondrial toxins.

Leigh syndrome is the most common pediatric manifestation of mitochondrial disease and is characterized by bilaterally symmetric lesions in the gray matter of the brainstem, basal ganglia or cerebellum. It affects approximately 1 in 40,000 live births and can be due to mutations in any of 75 different genes [4]. Children with this disorder often become hypotonic, and can develop vision and hearing loss. While the disease course can vary widely between individuals, in severe cases, affected children can present with developmental delay, and ultimately succumb to respiratory failure within the first few years of life. These patients are typically healthy at birth, but then present with neurometabolic crises, often in the context of a febrile illness, resulting in psychomotor regression and death within the first few years of life. Diagnosis is based on clinical presentation and classic findings on T2-weighted brain MRI. A small subset of these disorders are due to defects in vitamin transport and alleviated with dietary supplementation such as in the case of riboflavin, thiamine and biotin deficiencies [5, 6]. However, there are no proven therapies for the vast majority of these disorders. Several new experimental strategies are currently under investigation in preclinical and clinical settings, including agents that target oxidative stress, the mTOR pathway, NAD+ pool sizes, and Complex 1 bypass (idebenone) [7-10].

We recently demonstrated the therapeutic potential of hypoxia in preventing mitochondrial disease. We reported that chronically exposing the Ndufs4 mouse model of Leigh syndrome [11, 12] to breathing 11% O₂ at normobaria prior to disease onset markedly extended lifespan, improved behavior and prevented neurodegeneration [13]. Furthermore, we demonstrated that breathing 55% O₂, a level that is well tolerated by WT mice, leads to rapid death of the diseased mice.
The goal of our current study is to build on this initial work [13] in order to better understand the disease course and pathophysiology of these mice when breathing different O₂ levels and to explore alternative hypoxia regimens. We provide an update of the survival curves under hypoxia and carefully assess how variations of O₂ concentration and exposure duration affect organ pathology. We show that intermittent or more moderate hypoxia regimens are ineffective, and we report that the effects of breathing 11% O₂ chronically are not durable if mice return to breathing 21% O₂. Importantly, we demonstrate that breathing 11% O₂ not only prevents, but can also reverse established neurodegenerative disease in this murine model.

3.3 Results
3.3.1 Breathing 11% O₂ prevents the development of brain lesions throughout the life of Ndufs4 KO mice

To investigate the therapeutic potential of hypoxia treatment on mitochondrial dysfunction, we studied whether hypoxia was capable of preventing the neurodegenerative disease rather than simply delaying its onset. KO and WT controls were randomized to breathe either 21% or 11% O₂ starting at 30d of age. As previously reported [13], our initial cohort of control KO mice breathing 21% O₂ all died between the ages of 42d and 75d, with a median of 58d. In contrast, KO mice breathing 11% O₂ were still alive at 170d [13]. Here, we report an updated survival curve, showing that KO mice breathing 11% O₂ had an overall median survival duration of 270d (logrank, p<0.0001 vs 21% O₂ controls, Fig. 1A). To investigate the chronic physiological adaptation to hypoxia we measured the venous hematocrit of mice after nine months of breathing 11% O₂. Venous hematocrit was elevated, albeit to a lesser extent in KO mice compared to WT controls (47±2 vs 55±5 %, respectively, p<0.05, n=8, Fig.1 B). The circulating plasma disease biomarkers, lactate and alpha-hydroxybutyrate, are elevated in both humans with Leigh syndrome and in the Ndufs4 mouse model [14]. In our original report [13], both markers were partially normalized in KO mice breathing 11% oxygen. At 250d, alpha-hydroxybutyrate trends towards a partial rescue in KO mice breathing 11% oxygen (39±8 μM, n=7) relative to normoxia breathing KO mice at day 50 [13], though not reaching significance (Fig. 1D). At 250d, plasma lactate levels in knockout mice breathing 11% O₂ are similarly ameliorated relative to 50d [13] normoxia breathing KO mice.
To study whether hypoxic breathing delays death by delaying the onset of CNS lesions, we performed T2-weighted brain MRI studies of lightly anesthetized mice chronically breathing either 21 or 11% O₂ (n=4). KO mice breathing 21% O₂ developed lesions in the postero-lateral portion of the brainstem, localized to the vestibular nuclei. Lesions became evident at 55d of age (Fig. 2B). In contrast, brain MRI scans performed in KO mice breathing 11% O₂ showed no sign of neurological lesions even at 250d. We assessed neuropathology in the same cohort of mice. Brains were stained for the inflammatory activation marker lba-1 (Fig. 2A). KO mice breathing 21% O₂ displayed significant microglial activation in both the cerebellum and olfactory bulbs (OB) by 50d. In contrast, KO mice breathing 11% O₂ displayed no accumulation of lba-1 in the cerebellum, olfactory bulb or brainstem at 250d. In the latter group, brain sections were comparable to hypoxic, age-matched, WT mice (n=4). These findings suggested that the cause of death in older, hypoxia-treated KO mice might differ from the progressive neurodegenerative disease of normoxia-treated KO mice. This led us to explore the functional alterations of other organs known to be involved in mitochondrial disease pathology.

3.3.2 Ndufs4 KO mice breathing 11% O₂ for over 150 days exhibit mild left ventricular dysfunction and AV blockade

A common presentation of pediatric mitochondrial disease is cardiomyopathy, with left ventricular (LV) dysfunction negatively affecting prognosis in up to 20% of cases [15]. The pathogenesis of cardiac pathology is incompletely understood. The sudden death of some of our greater than 170d old KO mice breathing 11% O₂ led us to investigate their cardiac function (Fig. 3A-F). We obtained echocardiograms in mildly-sedated mice at 200d (Fig. 3A, C). Using ultrasound, we detected decreased LV fractional shortening of KO mice compared to WT mice breathing 11% O₂ at 200d (37±4 vs 57±9 %, respectively, p<0.05, n=6 in each group, Fig. 3A, C). We also examined ventricular function by MRI at 9.4T with a gradient echo cine sequence (Fig. 3F) [16]. The aged-matched WT mice were anesthetised with 1-2% isoflurane, while the KO mice were anesthetised using 0.3% isoflurane, due to their increased sensitivity to anesthetics [17]. Scans were performed while breathing 21% O₂. Body temperature was maintained in the physiological range with a warm air blower. Heart rates averaged approximately 400bpm in WT mice and
300bpm in KO mice. Left ventricular function assessed by cardiac MRI demonstrated reduced LV ejection fraction (EF) in KO mice treated by breathing 11% O₂ at greater than 200 days of age, compared to WT controls breathing 11% O₂ at the same age (56±7 vs 69±2 %, n=3 in each group, p<0.05, Fig. 3D) confirming the echocardiographic findings (Fig. 3). Right ventricular EF was also assessed and we detected no significant differences between KO and WT mice (58±1 vs 58±1 %, n=3 in each group, p=0.97, Fig. 3E). Of note, ECGs sporadically revealed intermittent atrioventricular blockade in hypoxia-treated KO mice under isoflurane anesthesia. Furthermore, no differences of LV fractional shortening were detected between hypoxia treated KO mice at 50d and age matched KO controls breathing 21% O₂ (Fig. 3B).

3.3.3 Breathing 55% O₂ in Ndufs4 KO mice causes acute lung edema and isolated olfactory bulb lesions

To further understand the critical role of inhaled oxygen concentration on mitochondrial disease progression, we investigated the physiological effects of breathing 55% O₂. We previously reported the remarkable sensitivity of KO mice to mild hyperoxic breathing (13). Upon exposure to breathing 55% O₂, KO mice died between 48h and 10 days, resulting in a median survival of five days. To assess whether supplemental O₂ exacerbates the original neurological disease, we obtained brain MRI scans on KO mice that had been breathing 55% O₂ for 24h. Imaging showed hyperintense lesions in the olfactory bulbs (OB), but no lesions in the brainstem, suggesting augmented O₂ sensitivity of the olfactory cells of mice exposed to hyperoxia via the nasal epithelium, but not in the brainstem via the arterial circulation. We then focused our attention on the lung, an organ known to be sensitive to supplemental O₂ toxicity. It is known that breathing 100% O₂ results in pulmonary edema within several days in WT mice [18]. Thus, we wondered if similar pathology ensued at a more moderate hyperoxia exposure in KO mice. To estimate pulmonary edema we assessed the wet-to-dry lung weight ratio (WD)[19]. Mice were exposed to 55% O₂ for 24h starting at 30d. WD was elevated at 5.63±0.6 vs 4.38±0.02, in KO and WT controls, respectively (p<0.001, n=7, Fig. 4A) revealing significant acute pulmonary edema. Furthermore, lung myeloperoxidase activity at 24h was 4.6-fold that of WT controls (23±8 vs 5±4 U/g, p<0.001, n=7, Fig. 4B), suggesting significant neutrophil infiltration. To further investigate lung pathology, we performed H&E
staining at 48h of mice breathing 55% O₂. We observed the presence of alveolo-capillary membrane swelling, inflammatory cell infiltration, extensive capillary disruption and hemorrhagic extravasation of red cells into alveoli (n=4). This was specific to KO mice breathing 55% O₂ (Fig. 4C).

3.3.4 Intermittent 11% O₂ breathing is not effective

Intermittent hypoxic exposure is currently used in sports training, aviation and in clinical studies of sleep apnea [20-22]. Because nighttime hypoxia therapy would allow unhindered patient mobility during the day, we tested whether such a regimen would have a therapeutic effect on KO mice. Starting at 30d, Ndufs4 KO mice breathed 11% O₂ for ten hours per day from 9am to 7pm (while they slept), and 21% O₂ for the remaining 14 hours (while awake nocturnally). Daily intermittent exposure to breathing 11% O₂ increased the venous hematocrit to 56±4 % within three weeks (n = 4), indicating a systemic physiological response to hypoxia. However, the survival time of these mice was not increased relative to mice continuously breathing 21% O₂ (58.5 vs 58.5 days, respectively, log-rank p=0.77, HR 1.15 0.45-2.96, n=8, Fig. 5A). During exposure to intermittent hypoxia, body weight decreased until humane euthanasia criteria were eventually met. Of note, KO mice undergoing the intermittent hypoxic protocol had a significantly lower body weight at 60d compared to control mice breathing 21% O₂ (10.6±0.8 vs 12.1±1.1 g, p<0.05, n=6, Fig. 5B). Core body temperature at 50d was lower, 32.4±0.9 vs 35.0±1.5 °C, in the two groups respectively (p<0.06, n=8, Fig. 5C). We tested the ability of KO mice to remain on an accelerating, rotating rod. We recorded no significant difference when comparing KO mice after three weeks of intermittent hypoxia treatment with control KO mice breathing 21% O₂ (Fig. 5D). We further studied whether brain lesions in the Leigh syndrome model were being ameliorated by intermittent 11% O₂ hypoxic breathing. All four MRI scans of KO mice receiving intermittent treatment revealed hyperintense lesions in the brainstem and olfactory bulbs by 60d, resembling the neurological lesions of mice breathing 21% O₂ at the same age (Fig. 5F). Thus, although intermittent 11% O₂ hypoxia is sufficient to trigger certain aspects of hypoxia adaptation, such as hematocrit elevation, intermittent hypoxia did not prevent the progression of KO neuropathology.

3.3.5 Milder hypoxic breathing regimens are not effective
We previously reported the results of KO mice breathing normobaric 11% O₂, equivalent to the partial pressure of oxygen found at 4500m altitude. Our current study investigated whether chronic exposure to a milder level of hypoxia would be sufficient to prevent encephalopathic disease. Breathing 17% O₂ for three weeks, equivalent to living at 1800m, commencing at 30d did not prevent Leigh syndrome in KO mice (Fig. 6A). After three weeks of treatment, the core temperature of 17% O₂ breathing mice and normoxic controls was reduced to 35.9±0.7 and 35.0±1.5 °C, respectively (p=ns, n=6, Fig. 6C). In the same mice, body weight was not significantly changed at 60d (12.5±1.3 vs 12.1±1.1 g, respectively, p=0.54, n=6, Fig. 6B). Moreover, all KO mice reached humane euthanasia criteria by 90d (HR 0.47 0.20-1.13, 17% vs 21% O₂, log-rank p=0.07, n=6).

To assess the levels of hypoxemia resulting from changes in inhaled oxygen levels, we measured both murine PaO₂ (arterial partial pressure of oxygen) and SpO₂ (peripheral saturation of oxygen) in WT animals. Mice that were acutely breathing 17% O₂ had a PaO₂ of 77.5±8 mmHg and transcutaneous SpO₂ of 88.5±3 %. Breathing 11% O₂ resulted in a PaO₂ of 45±4 mmHg and SpO₂ of 61.3±2.5 % (n=5, Table 1), resulting in a similar PaO₂ as in humans breathing equivalent O₂ pressures [23, 24]. After three weeks of chronic 17% O₂ breathing, the value of venous hematocrit in KO mice was 51±3 %, as compared to 60±6 % at 11% O₂ (normoxic control level was 44±6 %), (p<0.05, between groups, n=6, Fig. 6D). These findings suggest that breathing 17% O₂ triggered an intermediate physiological hematocrit response but did not prevent neurologic disease manifestation.

We tested the durability of the effects of hypoxia on neurodegenerative disease. We transitioned three KO mice that were breathing 11% O₂ for greater than 100d back to breathing 21% O₂ air. These KO mice then lost weight and died within 4, 5 and 9 days after transitioning to normoxia. This finding highlights the importance of maintaining hypoxic exposure throughout the lifetime of the murine disease model.

3.3.6 Breathing 11% O₂ reverses clinical manifestations of Leigh syndrome

Leigh syndrome patients are typically diagnosed after an acute metabolic crisis with the concomitant presence of bilateral lesions detectable by brain MRI [25]. Thus, the degenerative brain disease usually manifests prior to diagnosis. We previously demonstrated that commencing hypoxic breathing at an early age (30 days) can prevent
neuropathologic disease onset in the KO mouse model [13]. An even more clinically-relevant question is whether neuropathologic disease can be reversed after its onset. In order to assess this, we initiated therapeutic hypoxia exposure just prior to death in untreated KO mice breathing 21% O2 (55 days). We randomly assigned KO mice to one of three treatment groups: group A, starting 11% O2 breathing (hypoxia) at 30d, group B, commencing 11% O2 breathing at 55d, and group C as 21% O2 normoxic breathing controls, (Tab. 2). Mice in all three cohorts appeared healthy at 30d. By age 55d, group A mice weighed an average of 16.1±3.7 g, while groups B and C weighed 11.6±1.1 and 11.6±0.9 g, respectively (p<0.05, group A vs B and A vs C, n=8). Mice in group A demonstrated an upward growth curve trajectory, while mice in groups B and C had an identical deterioration of body weight (p<0.05, Group A vs B and Group A vs C, Fig. 7A).

At 60d of age, mice in Group B were on the fifth day of breathing 11% O2, and showed an upward weight gain trajectory, while mice in group C continued to rapidly lose weight. By 100d, the body weight of early hypoxia-treated mice, Group A, was 17.7±2.5 g, while the body weight of delayed hypoxia treatment mice from Group B had increased to 16.5±1.9 g (p=0.33, n=8). We measured the rectal temperature five days before and five days after initiation of 11% O2 breathing in group B mice (33.5±1.2 and 35.3±0.9 °C, respectively, p<0.01, n=8). Temperature remained stable at subsequent times (Fig. 7B). Mice in Group B could run on an accelerating, rotating rod for 38±23s at 50d of age. At the age of 100d, endurance on the rotating rod had partially recovered to 82±73 s (p<0.05, n=9, Fig. 7C). Ultimately, greater than 70% of the mice in this group were alive at 210d, as opposed to a median survival of 55d in group C (HR 9.7 (3.1-30.1), logrank p<0.001, n=13, Fig. 7D). Furthermore, we obtained plasma samples at 40, 50, 60 and 70d of age from mice in Group B. Two previously reported Leigh disease biomarkers [13, 14], alpha-hydroxybutyrate and lactate were both progressively elevated in normoxia with neurodegenerative disease, and decreased after 5 or 15d of 11% O2 breathing (t test p<0.05, 50d vs. 70d, n = 5-8 per group, Fig. 7E-F). Thus, treating diseased mice with hypoxic breathing after they manifest neurological disease at 55d leads to body weight gain, increasing core temperature, and improved endurance and stability running on a rotating rod with a significantly increased duration of survival.
3.3.7 Breathing 11% O₂ reverses brain lesions in Ndufs4 KO mice with late-stage disease

Leigh syndrome patients develop symmetric, bilateral neurological lesions, leading to respiratory failure during childhood. A similar mode of pathogenesis has previously been demonstrated in KO mice breathing 21% O₂, with mice developing disturbances of their normal respiratory pattern causing death due to respiratory failure between 55d and 70d [11, 12]. To determine whether rescue of lifespan and behavior are accompanied by a reversal of the brain lesions, we performed sequential brain MRI scans in four KO mice that developed the encephalopathic disease breathing 21% O₂ and then breathed 11% O₂ after developing late-stage neurological disease (treatment group B, Tab. 2). After their first MRI brain scan, at 55d, mice commenced breathing 11% O₂. After two and four weeks breathing 11% O₂, brain MRI scans were repeated (Fig. 8). Neuroimaging demonstrated a progressive reduction of the intensity and the size of lesions in the brainstem and olfactory bulbs, which we observed after the first two weeks of hypoxic breathing. In late-stage brain disease mice, the IV ventricle appeared more diffuse, likely as a consequence of parenchymal atrophy. This abnormal morphology was reversed upon breathing 11% O₂. The aforementioned disease pattern was reversed in all four mice that were studied. Finally, in order to verify our neuroradiological results, we assessed Iba-1 staining in the same mice treated for over 150d. The inflammatory response was no longer present and hypoxia-rescued brains appeared histologically similar to WT controls (Fig. 9). Future studies will investigate the biochemical nature of pathology reversal. The ability of hypoxia to reverse neurological lesions may have significant implications for clinical applications, as well as our understanding of the rescue mechanism.

3.4 Discussion

We previously demonstrated that early onset chronic exposure of the Ndufs4 KO mouse model of Leigh syndrome to breathing 11% O₂ prevented the onset of neurodegenerative disease and extended the duration of KO survival. The goal of our current study is to better understand the disease course and pathophysiology of these mice when exposed to breathing different oxygen tensions, and to determine whether alternative hypoxia exposure regimens could be useful. To our knowledge, no other
therapeutic approach has demonstrated such high levels of efficacy in mouse models of mitochondrial encephalopathy.

We previously reported that hypoxia extends, and hyperoxia reduces, the lifespan of mice with Ndufs4 deficiency, but eventual lifespan and organ pathologies were not yet known. Here, we have shown that while Ndufs4 KO mice breathing 21% O₂ develop subacute neurodegeneration, 11% O₂ breathing Ndufs4 mice do not show signs of neurodegeneration at greater than 200d of age. Rather, they develop mild left ventricular dysfunction and eventually die at a median age of 270d. Previous studies have shown that cardiac-specific Ndufs4 KO mice breathing 21% O₂, developed mild cardiac dysfunction [26, 27]. While we cannot conclusively define the cause-of-death in our hypoxia-treated KO mice, we did observe intermittent AV block at greater than 200d of age. In contrast, untreated KO mice breathing 21% O₂ did not display cardiac dysfunction at 50d, just prior to their death. One interpretation of these findings is that hypoxic breathing at 11% O₂ is able to prevent neurodegeneration, but not eventual cardiac dysfunction.

We find that breathing 55% O₂ results in a rapid death of Ndufs4 KO mice due to acute pulmonary edema resulting from acute pulmonary O₂ toxicity, without MRI evidence of brainstem lesions that are responsible for the death of the untreated mice. Lungs of WT mice exposed to breathing similar O₂ levels for similar periods were normal. This observation is consistent with hyperoxic pulmonary pathology in WT mice that are exposed to breathing much higher O₂ levels (eg. 100% FiO₂) or even at hyperbaric oxygen pressures [18]. It is notable that the respiratory epithelium, and the primary olfactory neurons, are two tissues in direct contact with extremely high inhaled oxygen tensions (not requiring blood oxygen delivery). Our pathophysiological findings highlight the variable tissue-specific oxygen sensitivities of the nose, lung, brain and heart of Ndufs4 KO mice with diffusely impaired oxidative metabolism across all of their tissues. We speculate that these differences arise from variations in metabolic flexibility such as the ability to rely on glycolytic ATP production [28-30], or alternatively, differences in the ability of these tissues to compensate for oxidative stress [31].

Perhaps the most exciting finding of our current study is that hypoxia can reverse brain radiographic lesions of established Leigh syndrome. Most mitochondrial disorders
are diagnosed after the first metabolic crisis, and often, it is T2-intense lesions on brain MRI that are indicative of mitochondrial disease and help to define the diagnosis. It is notable that inherited deficiencies of vitamins such as thiamine, riboflavin and biotin can also lead to Leigh syndrome. Such deficiencies in mitochondrial metabolite transporters are one of the few truly treatable forms of Leigh disease [5, 6]. Our finding that body weight, body temperature, behavior and even neuroradiographic and histopathologic lesions can be reversed within a few weeks by 11% O₂ breathing, offers hope more generally for diverse genetic etiologies of established Leigh disease. We hypothesize two means by which this could take place: (i) either hypoxia is removing the proximal cause of the damage and thus endogenous repair mechanisms can occur, or (ii) hypoxia actively triggers repair mechanisms which restore damaged tissue. It is known that cardiomyocytes are capable of regeneration and proliferation when exposed to very low oxygen levels even after completion of fetal development [32]. Furthermore, hypoxia has recently been shown to improve adult neurogenesis [33-35].

An important question is whether the doses of hypoxia used in mice in the current study may extrapolate to humans. We are optimistic about these prospects. Our healthy hypoxic mice breathing 11% oxygen had an average PaO₂ of 45 mmHg (Table 1). Human volunteers at the Mont Blanc research station (4559m), equivalent to breathing 11% oxygen at sea level, have been reported to have an average PaO₂ of 46 mmHg [36]. Despite having similar PaO₂, the ability of hemoglobin to bind oxygen is lower in mice than humans, which means that murine arterial oxygen saturation is lower than humans when breathing 11% O₂. However, the partial pressures of oxygen in the tissues of all mammals can only be at or lower than the partial pressures of oxygen found in arterial blood. Therefore breathing 11% oxygen poses an overall upper limit to the oxygen tension in any body compartment and is equal in humans and mice. Furthermore, in murine models of chronic hypoxia [37], breathing 11% oxygen at sea level produces pulmonary hypertension similar to humans, suggesting that the pulmonary vasoconstrictor response to breathing 11% oxygen is conserved across both species. Thus, several major aspects of oxygen transport and physiology show comparable dose responses between humans and mice.
While chronic, continuous 11% O₂ breathing has a dramatic effect in Ndufs4 KO mice, more practical regimens are desirable. In the current study, however, we report that neither (i) more moderate, chronic hypoxic breathing of 17% O₂ nor (ii) intermittent 11% O₂ breathing for a duration of 10h each day is effective. We also note that the therapeutic effects of chronic hypoxia in mice do not appear to be durable, i.e., when hypoxia treated mice are returned to breathing at normoxia, they die after 1 week of air-breathing. Hence, the only regimen at present that prevents and reverses Leigh syndrome in the Ndusf4 KO mouse is continuously breathing 11% O₂. Although the Ndufs4 KO mouse is perhaps the most accurate mouse model of Leigh syndrome, its disease trajectory is distinct from that of humans. In particular, this mouse has a very uniform, downward trajectory of body temperature, body weight, and activity, typically dying at day 55. The disease course in humans tends to be more variable and episodic, and in fact, it may be possible that more practical hypoxia regimens will be intermittently effective in helping to repair neurodegenerative lesions in humans. Future investigations will be required to determine whether hypoxia can be delivered safely and effectively as a therapy for mitochondrial diseases.

3.5 Methods

Animal Care

Ndufs4+- mice were generously provided by the Palmiter lab at the University of Washington. We continuously bred heterozygous mice to provide enough Ndufs4-/- and control (Ndufs4+- and WT) mice for experiments detailed in this manuscript. As Ndufs4+- and WT mice are identical in all reported assays performed by us and others, we treated both groups as controls. Pups were weaned and genotyped at ~25d after birth. All cages were provided with daily food, water and hydrated gel (Hydrogel). Food and gel were replaced 3x/wk and cages were changed 1x/wk (additional food was placed on the bedding). Body weights were recorded regularly and mice were humanely euthanized upon losing 20% of peak body weight, in accordance with the most recent AVMA guidelines. Animals were randomized on a 1:1 basis, balanced by age and gender. All the animal studies were approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital, Boston, MA and the Institutional Animal Care and Use Committee at MGH.
Hypoxic chambers

Mice were housed in 80L transparent acrylic boxes. The desired FiO2 was obtained by mixing a constant flow of medical air with pure nitrogen from a liquid nitrogen tank (Airgas) or by direct separation of nitrogen and oxygen present in room air by a nitrogen generator (MAG-20, Higher Peak, Winchester, MA). The total gas flow through the chamber was measured using flowmeters (Cole Parmer) and adjusted to between 5-10L/m to maintain chamber CO2 concentrations below 0.4% (CO200, Extech, NH, USA). Soda lime was also added to the chambers as a CO2 scavenger. The oxygen concentration was measured at the outlet port of the chamber using an O2 sensor (MiniOx I, Ohio, USA) which was calibrated weekly using an 8.55% O2 reference tank from Airgas. Oxygen levels inside the chambers were tolerated within a 0.4% offset from target concentration, by adjusting nitrogen flow as needed. Temperature was maintained between 24-26°C and humidity between 30-70%. A standard light-dark cycle of ~12h light exposure was used. Mice were housed in cages with standard bedding and given unlimited access to food and water.

Magnetic Resonance Imaging

MRI scans of the brain were performed during general anesthesia with isoflurane 0.5-1.5% in 21% O2. T2 weighted RARE (rapid acquisition of refocused echoes) images were acquired on a 4.7 Tesla small-animal scanner (Pharmascan, Bruker, Billerica MA) with a transmit-receive volume coil and the following parameters: TR 6000 ms, TE 60ms, Rare factor 10, slices 24, matrix192 x 192, voxels 0.130 x 0.130 x 0.7mm, 8 averages. The data were converted from the Raw Bruker format into Dicom images and visualized with a freeware Dicom reader (Osirix, University of Geneva).

Cardiac MRI was performed on a 9.4T horizontal-bore small animal MRI scanner (Biospec, Bruker, Billerica MA) equipped with a 1500mT/m gradient system. Images were acquired with an MR-compatible cardio-respiratory gating system (SA Instruments, Stonybrook, NY) and a transmit-receive surface coil. Cine imaging was performed in the short-axis of the heart from base to apex with a gradient echo cine sequence and the following parameters: Field-of-view 25x25mm2, slice 1mm with no gaps, matrix 200 x 200, 20 frames per cycle, TR = RR interval/20, TE 1ms, flip angle 30°, 4 averages. The
data were converted in Matlab (Mathworks, Natick MA) into the Dicom format and analyzed with a freeware Dicom reader (Osirix, University of Geneva).

**Brain histology**

Mice were placed under deep anesthesia with ketamine and fentanyl and a needle was inserted into the left ventricle. The whole body was perfused with ice cold PBS, followed immediately by 4% PFA. The brain was removed, stored in 4% PFA for 24-48h and then placed in 30% sucrose (in PBS) for 48h. Fixed brains were sectioned parasagittally. Additionally, two transverse sections of cerebellum were prepared – (i) a rostral section with subjacent pons, and (ii) a more caudal section with medulla oblongata. Immunohistochemistry was performed using an antibody against the microglial marker Iba-1 (Wako) at 2μg/ml.

**Echocardiography**

Mice were scanned using a 13 MHz probe (Vivid 7, GE, Milwaukee, MN) for LV studies. Scans were performed by an experienced echocardiographer who was blinded to the study group assignments. During the procedure, all mice were breathing 21% O2 and were anesthetized with isoflurane (0.5-1.5%) titrated to maintain a heart rate between 500 and 600bpm.

**Pulmonary studies**

The wet to dry ratio was calculated by dividing the wet lung tissue weight by dry lung tissue weight. Organs were left to dry for 24h at 60°C to obtain dry lung tissue. Frozen lung samples were stored at -80°C and MPO activity was assessed as previously described (38). Lungs of mice were perfused for H&E staining using a wash solution of ringer’s lactate followed by 4% PFA. Perfusions were performed during deep anesthesia followed by open chest puncture of the cardiac apex using a 20 gauge needle. A 1 mm incision was made in the right atrium to allow outflow of blood from venous return. After one minute, lungs were inflated with 4% PFA through the trachea allowing one minute for proper filling of alveoli. A pressure of approximately 30cm H2O was obtained by gravity flow. Organs were stored overnight in PFA and processed on slides by the histopathology core at MGH.

**Behavior**
Behavioral experiments were performed using a Rotarod (Ugo Basile) machine with the following parameters: (i) acceleration of 5rpm/m and (ii) maximum speed of 40rpm. Mice were placed on the accelerating, rotating rod while breathing room air. Latency to fall was measured until a maximum time of 300 seconds elapsed. Tests were performed three times in a row, allowing a minimum of 10min between sessions to allow for recovery. The median latency to fall was reported to avoid the incorporation of aberrant behavior trials. If mice latched onto a rotarod, rather than walking on the rod for more than 10s, this is recorded as the latency to fall.

**Biomarker Measurements**

Isotope labeled standards (CDN isotope) of lactate and α-hydroxybutyrate were used to generate a standard curve. This allowed for absolute quantification of metabolites in mouse plasma. 30μL of the mouse plasma sample were combined with 20μL of isotope labeled internal standard. 70% acetonitrile was used for metabolite extraction. LC-MS was performed on a Q Exactive Plus Orbitrap Mass Spectrometer coupled to a Dionex UltiMate 3000 UHPLC system (Thermo Fisher Scientific). The Xbridge amide HILIC column (2.1 X100 mm, 2.5 μM particle size, from Waters 186006091) was used for separate in negative ionization mode. Mobile phase A was: 20mM ammonium acetate and 0.25 % ammonium hydroxide (pH 9). Mobile phase B was 100% acetonitrile. Data acquisition was performed in full scan mode, selected for a range of 70–1000 m/z, with resolution of 140,000, the AGC target at 3e6, and the maximum injection time at 400 msec.

**Blood and Tissue Collection**

For collection of plasma and tissues, mice were anesthetized with an intraperitoneal injection of ketamine (120 mg/kg) and fentanyl (0.09 mg/kg). After tracheostomy, volume-controlled ventilation was maintained at a RR of 90 breaths/m, a tidal volume of 10 m/kg, PEEP 1 cmH2O and inspired O2 of 21% (Mini Vent 845; Harvard Apparatus). Whole blood was collected by cardiac puncture and placed in an EDTA-containing tube. Plasma was obtained by centrifuging whole blood at 2000g for ~20m. Tissues were harvested and immediately flash frozen in liquid nitrogen. When repeated samples were required, a small tail knick was performed and blood was collected using heparinized glass capillaries. Blood samples were then centrifuged as described above.
Transcutaneous awake SpO2 and arterial blood gas tensions at various FiO2.

Healthy, unanesthetised, adult C57Bl6 mice were placed inside a 5L chamber, gas flow was set to maintain chamber CO2 concentration below 0.1% (CO200, Extech, NH, USA). Oxygen concentrations were measured at the chamber inlet and outlet using O2 analyzers (MiniOx I, Ohio, USA). A murine transcutaneous oximeter collar probe was placed on mice to allow for unrestrained measurements (MouseOx plus, Starr Life Sciences, Oakmont, Pennsylvania, USA). The chamber FiO2 was adjusted to obtain 11%, 17% and 21% O2 sequentially. Mice were allowed to adapt for 5 minutes at each oxygen level. The SpO2 values were recorded continuously for the following minute and averaged.

For arterial measurements, different mice were anesthetised using Sevoflurane 3.5%. A PE 10 catheter was used to cannulate the carotid artery after ligation. Mice were allowed to recover for 1h after surgery. At the end of the procedure, mice were placed in a 5L acrylic chamber which was constantly ventilated with the desired FiO2. Mice were allowed a 5 minute adaptation to each FiO2 (randomized sequence), then heparinized arterial blood was sampled and analysed using the ABL 800 Radiometer (Brea, CA, USA).

Statistics

Data in text are shown as mean ±SD. Analyses were performed using GraphPad Prism v6.0 software. A two-sample student t-test was used for two group comparisons. One way Anova was conducted for multiple comparisons using Bonferroni’s correction. Log-rank test was performed and hazard ratios were reported to compare survival rates with a 95% confidence interval. Statistical significance was considered with p<0.05.

Author Contributions

MF, IHJ, VKM, and WMZ conceived and designed the experiments. MF, IHJ and ER performed the experiments. OG assisted with mouse breeding and genotyping. RT, KHC and MSC performed rodent echocardiography. DES acquired and analyzed the cardiac MRI data. MF, IHJ, VKM, and WMZ wrote the manuscript. All authors participated in interpreting the results and revising the manuscript.
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3.6 References

Figure 1. Survival, hematocrit and disease markers in older Ndufs4 KO mice breathing 11% O₂. (A) Survival rates of mice breathing various oxygen levels. Hypoxia-treated Ndufs4 KO mice were all alive at 170d, n=12. The experiment was interrupted upon reaching 50% survival. All WT mice breathing 11% and 21% O₂ survived 300 days, n=14 per group. Kaplan-Meier log-rank between KO, 11% O₂ vs. KO, 21% O₂, p<0.0001, HR 7.58 (2.75-20.9), n=12 per group. (B) After 30 weeks of treatment, hematocrit was elevated in KO (n=7) and WT mice (n=8) breathing 11% O₂. (C) Body weight of KO (n=7) and WT (n=9) controls at 250 days of age. Plasma levels of (D) lactate and (E) alpha-hydroxybutyrate in hypoxic KO mice at 250 days of age, as compared to age-matched WT mice and KO mice breathing 21% O₂ at 50d, data at 50d taken from (4). Data mean±SE; *p<0.05 vs WT, 21% O₂; #p<0.05 vs WT, 11% O₂, One-Way Anova, Bonferroni's correction.
Figure 2. Absence of neurodegenerative pathology in 250d old hypoxia-treated Ndufs4 KO mice. (A) Representative images with staining for the microglial activation marker, Iba-1 (n=3). Normoxic KO mice at 50 days have a significant inflammatory response in the cerebellum and olfactory bulb. Analogous images in 250 day old hypoxic KO mice and WT mice do not show brain inflammation. (B) Axial MRI head scans showing bilateral, symmetric hyperintense lesions in the olfactory bulbs (white arrow, left panel) and vestibular nucleus (white arrow, right panel) of normoxic breathing KO mice at 55 days. These lesions were not present in hypoxic KO mice at 250 days of age. See methods section for MRI technique.
Figure 3. Cardiac ultrasound (US) and cardiac MRI reveal depressed left ventricular myocardial function in *Ndufs4* KO mice breathing 11% O₂ at greater than 200 days of age. (A) US left ventricular fractional shortening (LVFS) of mice breathing various oxygen concentrations at 200 days of age, n=6. (B) US LVFS of mice breathing various oxygen concentrations at 50 days of age, n=6. (C) Representative M-mode scans of the left ventricle in WT and KO mice breathing 11% O₂ at 200 days. (D) MRI left ventricular ejection fraction (LVEF) of WT and KO mice breathing 11% O₂ at 300 days of age, n=3. (E) MRI right ventricular ejection fraction (RVEF) of WT and KO mice breathing 11% O₂ at 300 days of age, n=3. (F) Representative MRI sections of WT and KO mice breathing 11% O₂ at 300 days of age. Images display end diastolic (ED) and end systolic (ES) reconstructions. Scans were obtained during sedation with isoflurane while breathing 21% O₂. Data mean±SE; *p<0.05 vs WT; One Way Anova with Bonferroni's correction for multiple comparisons.
Figure 4. Detrimental effects of breathing 55% O_2 on lungs and olfactory bulbs of *Ndufs4* KO mice. (A) Pulmonary wet to dry weight ratio of KO and WT mice breathing various levels of oxygen. Lungs of mice were weighed after 24 hours breathing 55% O_2 at an age of 30 days, n=7. (B) Myeloperoxidase activity in the lungs of mice after 24h breathing 55% O_2 at age 30 days, n=7. (C) H&E stains of lungs exposed to 55% O_2 for 48h at age 30 days (n=3, representative images, see text for description). (D) Axial MRI scans of murine brains showing olfactory bulbs (top row) and brainstem (bottom row) of KO mice breathing 21% at 60d vs Ndufs4 KO mice breathing 55% oxygen for 24h at 30 days (representative images, n=3). See methods section for MRI technique. Data mean±SE; *p<0.05 vs other groups, One Way Anova, Bonferroni’s correction.
Figure 5. Intermittent hypoxic (11%) breathing (10h/day) does not alleviate mitochondrial disease in Ndufs4 KO mice. (A) Survival rates for Ndufs4 KO mice breathing intermittent hypoxia (Int) versus 21% O₂, (log-rank p=0.77, HR 1.13 (0.47-2.73), n=8). (B) Body weights after breathing at various oxygen levels and during intermittent hypoxic breathing starting at the age of 30d, n=8. (C) Core temperature and (D) falling latency from an accelerating, rotating rod for KO mice breathing various oxygen levels or receiving intermittent hypoxia starting at 30 days of age, n=8. (E) Hematocrit levels for WT and KO mice following three weeks of exposure to normoxia, hypoxia or intermittent hypoxic breathing, n=4. (F) Representative MRI of a 60d KO mouse exposed to intermittent hypoxic breathing. Arrows denote lesions in vestibular nuclei. Data mean±SE; *p<0.05 vs KO, 11% O₂; #p<0.05 vs KO, 21% O₂.
Figure 6. Breathing at moderate hypoxia (17% O₂) does not alleviate mitochondrial disease. (A) Survival rates for Ndufs4 mice breathing various oxygen levels starting at age 30 days. (B) Time course of body weight (n=6) and (C) body temperature for 17% oxygen breathing, compared with breathing 21 or 11% oxygen for 30, 40 and 50 days (n=6). (F) Venous hematocrit after three weeks of exposure to various oxygen levels (n=6). Data mean±SE; *p<0.05 vs 11% oxygen, # p<0.05 vs 17% oxygen.
Figure 7. Hypoxic breathing (11% O₂) rescues survival, body weight and behavior of Ndufs4 mice with late-stage neurologic impairment. (A) Growth curves of n=8 Ndufs4 KO mice exposed to early hypoxic breathing, Group A, starting at 30 days of age (grey triangles), late hypoxic breathing, Group B, starting at 55 days of age (black circles) and control Ndufs4 KO mice breathing normoxia (orange squares), Group C. (B) Body temperature and latency of falling from an accelerating rotating rod (C) in Ndufs4 KO mice with late-stage disease, Group B, and WT controls exposed to breathing 11% O₂ starting at 55 days of age, n=8. (D) Survival rates of hypoxic breathing mice with late-stage disease, treated with hypoxia at the age of 55 days, Group B (black, n=17) and controls breathing air, Group C (orange, n=13), Log-rank p<0.0001. Data mean±SE. *p<0.05 vs 50 days. (E) Lactate and (F) alpha-hydroxybutyrate timecourse in Group B mice and WT controls. *p<0.05 and **p>0.05 for comparison to WT 40d. Median shown as bar.
Figure 8. Breathing 11% O₂ in late-stage neurological disease reverses the neurological lesions of *Ndufs4* mice at MRI. Four *Ndufs4* mice were breathing 21% oxygen until they developed late-stage neurological disease (55 days). They were MRI scanned to document bilateral lesions in the vestibular nuclei (top row, white arrow). Subsequently they commenced breathing 11% oxygen. The mice were scanned again at two and four weeks of hypoxic breathing (middle and bottom rows respectively). Neurological lesions disappeared by four weeks of hypoxic breathing. The section of the fourth ventricle, displaying at the center of the brainstem, appears enlarged in the early scans, suggesting parenchymal atrophy. After four weeks of treatment the area of the IV ventricle appears reduced and morphologic relationships are restored. MRI technique is described in method section.
Figure 9. Breathing 11% O₂ in late-stage neurological disease reverses pathological inflammation in the brain of Ndufs4 KO mice. Representative images with Iba-1 staining of olfactory bulbs (OB) and cerebellum (CB) of KO mice and WT controls (n=3 per group). Iba-1 is a marker of inflammation in the brain, indicative of microglial activation. Images demonstrate the reversibility of the neuropathological pattern by breathing 11% O₂ at the late-stage of disease (55d). (A) KO mice breathing 21% O₂. (B) KO mice breathing 21% O₂ until 55d and subsequently 11% O₂ breathing (160d). MRI reversal of the lesions reported in Fig. 8 was observed in these same mice. (C) normoxic and hypoxic (D) WT controls.
Chapter 4: Mitochondria and Peroxisomes are Differentially Essential at Varying Oxygen Tensions

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4.1 Abstract

Oxygen is the most utilized substrate in known biochemical reactions. Its presence in the atmosphere allowed for the evolution of aerobic ATP production, which enabled the formation of complex life. Given its crucial role in metabolism and physiology, it follows that variations in oxygen levels underlie many disease pathologies. Thus, understanding the pathways which sense and adapt to variations in oxygen levels is paramount. Here we identify the list of essential genes as a function of physiologically-relevant oxygen tensions. We find that many mitochondrial electron chain subunits are essential in 21\% oxygen, but not in 1\% oxygen. This signal is dominated by subunits of complex 1. We go on to show that the rescue mechanism involves shunting of pyruvate away from a broken respiratory chain, likely alleviating mitochondrial oxidative stress. On the other hand, we identify peroxisome biogenesis genes, and specifically the plasmalogen synthesis pathway, as being more essential in 1\% or 5\% oxygen than 21\% oxygen. These findings point to previously unappreciated pathways in oxygen metabolism and provide candidate lists for hypoxia and hyperoxia therapy.

4.2 Introduction

Oxygen is a key substrate for efficient ATP production. It is also a lethal toxin which leads to the production of reactive oxygen species. This Jekyll-and-Hyde role of oxygen is reflected in its importance for essential redox reactions, as well as its contribution to disease. Atmospheric oxygen levels have varied dramatically over the last 300 million years, ranging from 10\% to an estimated peak of 35\% during the Carboniferous era (300 Ma) [1]. Such variations have placed constraints on animal size and reproduction, led to the development of a cardiorespiratory system and evolved elaborate physiological responses to varying oxygen tensions [2]. In mammals, careful titration of oxygen tensions is crucial to life – the precise tissue oxygen tensions are regulated through a balance of oxygen delivery and oxygen consumption. Within the human body there are great variations in local oxygen tensions, with the brain being exposed to 1-3\% oxygen and alveoli, nearly 21\% oxygen [3]. Furthermore, disease states can lead to profound hypoxia and ischemia, culminating in necrosis, organ failure and even death [4]. Thus, understanding the molecular adaptations and responses to varying oxygen levels is of
utmost importance to our understanding of redox biochemistry, as well as disease pathologies.

Countless medical crises such as myocardial infarcts, stroke, sepsis and vascular disease are associated with states of impaired oxygen delivery, utilization or sensing ([2], [4]). Though the initial cause of disease varies, hypoxia contributes significantly to disease pathology. On the other hand, we have recently shown that relative hyperoxia can exacerbate disease in settings of impaired mitochondrial function, whereas hypoxia can alleviate disease. Indeed, decreasing oxygen levels has shown to extend healthy lifespan in lower organisms such as \textit{C. elegans} [5]. Finally, tumor hypoxia is one of the worst prognosis factors for patients with solid tumors and is a key target for cancer therapeutics [6]. Such biomedical implications of high and low oxygen lead to some of the great unanswered questions at the intersection of cellular metabolism and organism-level physiology – how do tissues sense and adapt to variations in oxygen levels? What are the metabolic and genetic pathways underlying such adaptations?

Genetic screens in yeast, flies and worms have all pointed to genetic programs which confer resistance to varying oxygen tensions. For example, genome-wide screens performed under hypoxia in \textit{Drosophila} showed significant enrichment for the Notch signaling pathway [7]. Exposing the yeast deletion library to hyperoxia demonstrated the role of antioxidant defenses in protecting against oxygen toxicity [8]. Forward genetics approaches have also highlighted the active selection of genetic programs to protect against hypoxic environments. As an example, flies maintained in hypoxia for 200 generations show significant positive selection for loci regulating the Notch pathway, consistent with genetic screening results [9]. Human populations who have been living at high altitudes for generations show selection for variants of HIF2α and its key regulator PHD2 [10]. And most strikingly, the hypoxia inducible factor (HIF) proteins have evolved to regulate the transcription of several hundred genes in response to low oxygen levels [11]. These examples suggest that precise genetic programs and even individual genes modulate a biological system’s tolerance to variations in oxygen levels.

In the current work, we use genome-wide approaches to carefully delineate essential genes and pathways as a function of oxygen tension in a mammalian cell culture system. Surprisingly, we identify a differential reliance on entire organelles as a function
of oxygen tension – mitochondrial respiratory chain subunits are selectively essential at high oxygen tensions (21% oxygen), whereas peroxisomal etherphospholipids are essential at low oxygen tensions (1-5% oxygen). These findings enhance our basic understanding of oxygen adaptation and metabolism, while also highlighting potential disease targets for hypoxia and hyperoxia treatment.

4.3 Results

4.3.1. Genome-wide screen for essential genes as a function of oxygen tension

The identification of essential genes is crucial for the (i) elucidation of novel biological pathways, (ii) prioritizing potential disease genes and (iii) understanding the safety of drug targets. In recent years, two groups published high-quality lists of essential genes using genome-wide CRISPR screens and haploid insufficiency screens ([10],[11]). Both groups annotated approximately 10% of the genome as being essential in mammalian cell culture. However, traditional cell culture is notoriously misrepresentative of in-vivo metabolism. Growth and media conditions were historically optimized for the maintenance and proliferation of immortalized cell lines. An Achilles heel of mammalian cultures is the presence of unphysiologically high amounts of oxygen. Cells are usually cultured at ambient oxygen tensions (21% at sea level). However, most cells in the body “see” oxygen tensions ranging from 0.1-10% oxygen (Fig. 1A). The importance of oxygen in determining cellular metabolism is evident in the fact that many cell types (e.g. stem cells) grow faster when cultured at 1-5% oxygen [14]. Furthermore, increased oxygenation can change the differentiation status of many cell types, as stem cells often reside in hypoxic niches within the body. Here, we perform genome-wide screens of essential genes at a range of physiologically-relevant oxygen tensions. We highlight differences between essential pathways under states of relative hypoxia and hyperoxia.

We used the recently developed Brunello library, composed of >70,000 sgRNAs, with four different guideRNAs targeting each annotated human gene [15]. We infected a pool of the leukemic cell line K562, which we have previously shown to have a canonical hypoxia response – HIF stabilization, increased expression of target genes, increase in lactate production and a decrease in basal oxygen consumption [16]. We initially grew the infected pool of knockout cells at ambient oxygen tensions to allow for sufficient genome editing. We then divided the edited pools into three different oxygen tensions –
1%, 5% and 21% oxygen (Fig. 1B). These values were chosen to represent a range from physiological hypoxia to normoxia to hyperoxia. Pools were maintained for 15d after infection. This intermediate duration of the screen enabled us to study both positive selection and negative selection. All experiments were performed in triplicate infection replicates.

4.3.2. Mitochondria and peroxisomes are differentially essential at different oxygen tensions

We sequenced libraries from day 0, day 9 and day 15 after infection in all three oxygen tensions. This approach enabled us to use temporal kinetics as a secondary metric for true hits. Genes that were progressively eliminated over increasing cell passages were more likely to represent real signal. We used the MAGeCK software to generate gene-level summaries of KO abundance [17]. As many genes are essential across conditions, we performed the final analysis by comparing guide abundance in 1% oxygen vs. 21% oxygen at day 15. We then looked at individual sgRNA trajectories for gene sets we studied in further detail.

To identify metabolic pathways that are differentially essential across different oxygen tensions, we performed gene set enrichment analysis (Fig. 1C-F). Using GO terms, there were two very clear signals across infection replicates. All 8 of the top gene sets corresponding to genes essential in 21%, but not 1%, were related to the mitochondrial electron transport chain and specifically complex 1 subunits. 5 of the 8 top gene sets corresponding to genes that are essential in 1%, but not 21% were related to peroxisome biogenesis genes. This striking reliance on the metabolism of distinct organelles across oxygen tensions, has never been previously reported.

We compared our list of essential genes across oxygen tensions. There was 87% overlap among the top ~2500 most essential genes between 21% and 5% oxygen. Similarly, there was 83% overlap between the analogous lists for 21% and 1% oxygen. In considering cell culture conditions, disease genes and therapeutic targets, such differences should be kept in mind.

4.3.3. Selectively essential genes are not necessarily part of the hypoxia transcriptional program
In the last two decades, the hypoxia transcriptional response has been carefully elucidated. During states of high oxygen tension, the family of hypoxia-inducible transcription factors (HIFs) are continuously made, hydroxylated by the prolyl hydroxylase enzymes (PHDs) and then marked for degradation by the E3 ubiquitin ligase, VHL [11]. Under states of hypoxia, this cascade does not take place. Instead the HIF proteins enter the nucleus and trigger the concerted transcription of nearly 5% of the genome. The transcriptional response causes a shift towards anaerobic metabolism, while also increasing oxygen delivery at the tissue and organism-level. We were interested in understanding whether essential genes in hypoxia and hyperoxia intersect with key targets of the hypoxia transcriptional program.

A lesser appreciated fact about the hypoxia transcriptional response is the presence of strong negative feedback loops. Acutely, hypoxia triggers a dramatic increase in HIF stabilization, which then dampens to a new steady-state over time [18]. To account for these kinetics, we performed RNASeq on samples at 6h, 24h, 3d and 6d after placing WT K562 cells at three different oxygen tensions – 21%, 5% and 1% oxygen. PCA analysis of all samples reflected 0h, 6h and 24h timepoints as being similar for all oxygen tensions (Fig. 2A). At 3d, 1% and 5% oxygen samples moved along a similar PC axis. By 6d, the 1% oxygen samples were more like the 0h timepoints, corresponding to the negative feedback loop that occurs over time in severely hypoxic conditions. Canonical HIF targets such as the glycolytic enzymes ALDOA, ENO2 and LDHA, all showed a dramatic upregulation in hypoxia, albeit a bit delayed in 5% oxygen relative to 1% oxygen (Fig. 2C). As expected, PHD2, the primarily regulator of HIF degradation was also upregulated at later timepoints. This matched RNASeq dataset demonstrates that the genome-wide screen integrates the role of individual genes in survival across the duration of the screen (9+ days). Thus, it also integrates over the upregulation and then dampening of the hypoxia transcriptional program.

We were interested in comparing the list of oxygen-specific essential genes to the genome-wide transcriptional response in hypoxia. We took the top 300 genes that were selectively essential in 1% or 21% oxygen and looked for whether their gene expression changed as a function of oxygen tension. We assessed the t-test p-value between gene expression at 3d in 1% vs. 21% oxygen of these gene lists (Fig. 2B). If the RNASeq
datasets and essential gene lists were completely independent, we would expect a uniform distribution of p-values. However, the normoxia-sensitive essential genes showed an enrichment for low-p-values (<0.005) of 5-10-fold relative to a random gene list. Interestingly, the hypoxia-sensitive essential genes also showed an enrichment, however less so (2-4 fold). Of note, most normoxia-specific essential genes that also had differential gene expression, showed a transcriptional down-regulation at 1% O₂. This set of genes included mitochondrial ribosomal protein subunits (Fig. 2D). It is possible that downregulation of genes that are less essential in high oxygen is a way of conserving resources and energy, or even actively shifting electron flux away from the mitochondrial respiratory chain.

One might have expected a greater number of canonical HIF targets to be selectively essential in hypoxia. A likely explanation for this lack of overlap, is that many of these genes are essential in both normoxia and hypoxia. For example, glycolytic enzymes are needed across oxygen tensions and any minor relative differences in essentiality would not be picked up in the current design of the genetic screen.

4.3.4. Hypoxia rescues the growth defect of lesions across the respiratory chain, with a complete rescue of Complex 1 mutants

We previously performed a genome-wide screen for therapeutic targets for mitochondrial dysfunction. This led us to develop the hypoxia response and hypoxia itself as a therapeutic for Leigh Syndrome, a pediatric form of mitochondrial disease [16]. Indeed, hypoxia exposure resulted in a 5-fold increase of lifespan in the most accurate mouse model of disease. However, we never characterized the specific type of mitochondrial lesions which could be rescued with hypoxia. We also never demonstrated the mechanism by which hypoxia rescues disease, either in cell culture or in animal models. The current results answer these questions which inform both our understanding of mitochondrial metabolism and hypoxia therapy.

We first plotted the cumulative distribution of hits as a function of positional rank in the screen (1% vs. 5% oxygen) (Fig. 3A). Performing this analysis for complex 1 subunits (NDUFS*) showed a very clear enrichment for hypoxia-rescued genes. Similarly, peroxisome biogenesis genes (PEX*) showed an enrichment for normoxia-rescued
genes. Interestingly, genes that are annotated in KEGG as being oxygen-consuming did not show any enrichment in either direction.

In delving deeper into the hypoxia-rescued genes, we noticed that mitochondrial Complex 1 subunits were the predominant category of hits. Our group recently performed a genome-wide screen of genes which cause mitochondrial dysfunction, using selective apoptosis in glucose-deficient media as a screening phenotype [19]. This screen identified a range of mitochondrial genes as causing electron transport chain-deficiency – complex 1,2,3,4,5 KO cells, as well as other genes associated with respiratory chain function (mtRNA, mtDNA, etc.). However, the hypoxia-rescued genes were predominantly complex 1 subunits (Fig. 3B-C). We compared a manually-tabulated list of mitochondrial disease genes with the hypoxia-rescued genes. Diseases caused by mutations in these genes may serve as ideal candidates for hypoxia therapy. While we focus on mitochondrial genes, it is likely that non-mitochondrial genes that are hypoxia-rescued in this screen, could also be targets for hypoxia therapy.

We generated KO pools corresponding to complex 1-5 subunit dysfunction and subjected them to 1% and 5% oxygen. As expected, hypoxia rescued the growth defect of Complex 1 knockout cells (Fig. 3D-E). Interestingly, Complex 2-5 KO cells also showed an intermediate rescue in cell proliferation by hypoxia exposure. Thus, the rescue mechanisms may not be exclusively limited to Complex 1 dysfunction. However, Complex 2-5 may fulfill additional functions, such as TCA cycle function, nucleotide metabolism, aerobic ATP production, etc. We predict that mixed respiratory chain diseases may show an intermediate rescue with hypoxia exposure, compared to the particularly impressive rescue of complex 1 disease.

4.3.5. Hypoxia protects against respiratory chain dysfunction by shunting pyruvate away from the ETC

The remarkable rescue (partial or complete) of nearly all forms of respiratory chain dysfunction, begs the question – what is the mechanism by which hypoxia is protective during states of mitochondrial dysfunction? A simplistic explanation is that hypoxia upregulates glycolytic flux, which compensates for an ATP-production defect during mitochondrial dysfunction. However, steady-state ATP levels are usually very well
protected in cells. There is also a surprising lack of evidence that ATP deficiency is a primary contributor to pathology in patients with mitochondrial disease.

An initial clue for disease mechanism came from a deeper look at the additional hypoxia-protected gene signatures. Aside from mitochondrial electron transport chain subunits, genes that were involved in ameliorating mitochondrial oxidative stress were also rescued in hypoxia. The KO of mitochondrial superoxide dismutase, SOD2, was completely rescued in 1% oxygen. The cytosolic and extracellular SOD isoforms did not show this signature. Similarly, mitochondrial glutaredoxins were essential in 21% oxygen, but not 1% oxygen. Such results suggested that mitochondrial ROS may be rescued by hypoxia.

Furthermore, there was only one category of gene KOs that showed positive selection under hypoxia – i.e. KO of these genes increased cell proliferation in hypoxia relative to other KO cells. The hits in this category were all related to pyruvate metabolism and carbon entry into mitochondria. Multiple subunits of pyruvate dehydrogenase and even the mitochondrial pyruvate carrier appear to limit growth in hypoxia (e.g. PDHB, MPC1, DLAT, PDHA1). Such hits also pointed at hypoxic adaptations that rerouted carbon flux as being beneficial in hypoxia.

Canonical HIF targets include the pyruvate dehydrogenase kinase enzymes [20]. Upregulation of these kinases results in phosphorylation and inactivation of pyruvate dehydrogenase. This adaptation shunts pyruvate away from the mitochondrial electron transport chain and towards the lactate dehydrogenase reaction. We hypothesized that such shunting away from a broken ETC might be a protective mechanism by which hypoxia alleviates cell proliferation defects. Dichloroacetate is a small molecule that inhibits PDK enzymes, thus forcing the aerobic metabolism of pyruvate. Adding 10mM DCA to complex 1-5 deficient cells blocked the hypoxic rescue of cell proliferation (Fig. 4A). In the presence of DCA, the relative final cell counts (normalized to WT cells) was identical in normoxia and hypoxia treated cells with ETC deficiency. These initial findings highlight that hypoxia may rescue mitochondrial disease by preventing mitochondrial oxidative stress due to a broken ETC. Future work is needed to confirm this finding and determine whether such results apply in vivo.
4.3.6. Peroxisomal plasmalogen synthesis is necessary for survival in low oxygen tensions

The predominant signal in the hypoxia-sensitive side of the oxygen screen was genes involved in peroxisome biogenesis. Peroxisomes are single-membrane bound organelles which contain approximately 100 enzymes involved in lipid metabolism and ROS homeostasis, among a handful of other metabolic functions. Deficiencies in peroxisomal biogenesis genes have devastating effects on human metabolism and physiology [21]. Patients develop severe liver and kidney failure, as well as neurodegeneration, leading to death within the first few years of life.

Loss-of-function of peroxisome biogenesis genes abolishes nearly all metabolic functions of the organelle (Fig. 5A). To discern which specific pathway was essential in hypoxia, but less so in normoxia, we looked up other proteins that were localized to peroxisomes. Of all the peroxisomal matrix proteins, those corresponding to plasmalogen synthesis showed the most striking signature of being selectively essential in low oxygen tensions (Fig. 5B). Plasmalogens are a type of etherphospholipid, where the ester bond in diacyl phospholipids is replaced with an ether bond [22]. While the exact function of plasmalogens remains unknown, it has been postulated that they contribute to membrane fluidity due to their polyunsaturated fatty acid chains. The first three steps in plasmalogen synthesis, including the rate-limiting step, are localized exclusively to peroxisomes. All three of these peroxisomal enzymes showed up as top hits in the oxygen screen – i.e. loss of these genes results in increased cell death at low oxygen tensions.

Initially, we generated pools of KOs corresponding to plasmalogen synthesis genes. However, we did not see a striking sensitivity to growth in hypoxia after 3d. We realized that the oxygen screen lasts over multiple passages and thus at the end of each passage, there may be nutrient limitations which result in cumulative differences in certain KO cells. Thus, we repeated the experiments in media containing 3% FBS or lipoprotein deprived media. Both results recapitulated the increased sensitivity of plasmalogen-deficient cells to low oxygen tensions (Fig. 5C).

A separate lipid-related auxotrophy has recently been reported for hypoxic cells [23]. The oxygen-dependent stearoyl-coA desaturase (SCD1) reaction is responsible for generating the majority of intracellular, unsaturated free fatty acids. More specifically,
Palmitic acid is made by endogenous fatty acid synthesis, elongated to stearic acid and then SCD1 introduces a double-bond to make oleic acid, via an oxygen-dependent reaction. In hypoxia, these reactions do not take place and instead, cells rely on exogenous lysophospholipids for survival. Without external unsaturated lipids, cell death ensues due to a PERK and IRE1-dependent ER stress response [24].

We predict that plasmalogens serve to suppress the lipid saturation-sensitive ER stress response. Unsaturated free fatty acids and plasmalogens may both serve as a brake to this pathway. Loss of one or the other species may be somewhat tolerated, however loss of both brakes may be catastrophic. This would make peroxisome-deficient cells relatively more sensitive to low oxygen tensions. Interestingly, we also observe a depletion of plasmalogens in the plasma of mice that are chronically (~3 weeks) exposed to 11% oxygen, compared to those that are exposed to 21% oxygen (Fig. 5D). Future work is needed to confirm the mechanisms by which plasmalogens are essential in hypoxia, as well as whether their supplementation may protect against hypoxic damage.

4.4 Discussion

Oxygen availability has driven the evolution of elaborate metabolic and physiological adaptations, many of which remain to be uncovered. Here, we use a systems-level approach to identify pathways which are selectively essential as a function of oxygen tension. Our work demonstrates the crucial role of distinct organelles—mitochondria are essential in relatively normoxic conditions, whereas peroxisomes are essential in relatively hypoxic conditions. While the hypoxia transcriptional response (HIF) has dominated scientific literature for the last two decades, looking forward, we hope to uncover broader adaptive programs that allow animals to cope with changes in oxygen.

We recently demonstrated that continuous hypoxia exposure is protective in models of the pediatric mitochondrial disease, Leigh syndrome. To expand our therapeutic strategy, we asked which additional inborn errors of metabolism could be rescued by hypoxia. Indeed, several hundred genes were essential when cells were cultured in 21% oxygen, but not in 1% oxygen. Focusing on mitochondrial disease genes, we saw that Complex 1 disease genes were particularly rescued by low oxygen tensions. Additional disease candidates include Charcot-Marie-Tooth Disease (MFN2 KO), Leber's Hereditary Optic Neuropathy (OPA1 KO) and Friederich's Ataxia (FXN KO). Of note,
mtDNA-encoded genes were not tested in this screen and may also benefit from therapeutic hypoxia. A sequela of this finding, is that such genetic lesions may also predispose patients to hyperoxia injury with supplemental oxygen.

Conversely, many diseases are associate with pathologically low oxygenation. Common disorders such as stroke, MI and respiratory failure are the most well-appreciated conditions with pathologies of extreme hypoxia or anoxia. However, it is possible that additional genetic disorders show an increased sensitivity to hypoxia. Here, we identify genes associated with peroxisomal biogenesis disorders as being particularly sensitized to low oxygen conditions, likely due to impaired etherphospholipid synthesis, leading to a pathological ER stress response. Future work includes testing peroxisomal disease models for their sensitivity to hypoxia and even the therapeutic benefit of hyperbaric hyperoxia.

Another biomedical application of our findings is targets for tumor hypoxia. Solid tumors often grow beyond their vascular supply, resulting in irregular oxygen and nutrient delivery. Paradoxically, such hypoxic regions also promote metastatic growth and increased resistance to radiation-mediated toxicity. Indeed, solid tumors have previously been shown to have oxygen tensions <1 mmHg. Tumor hypoxia is among the worst prognosis factors for patient survival and treatment with radiation. Thus, genes which are essential in hypoxia may be the ideal targets for killing of hypoxic tumors. This logic has previously been applied to the SREBP pathway [25]. SREBP1 knockout cells are known to be sensitive to low oxygen tensions. Spheroid models and in-vivo xenograft models of tumor hypoxia showed that targeting SREBP1 impaired tumor growth. Similarly, targeting peroxisomal biogenesis genes, or other hypoxia-sensitive genes from our screen may prove to be effective cancer therapeutics.

Interestingly, the rate-limiting enzyme of plasmalogen synthesis, AGPS, is known to be upregulated across aggressive human primary tumors and immortalized cancer cell lines [26]. This results in over-production of etherphospholipids in such tumors. The entire mechanism for this adaptation is not known. It is tempting to speculate that such an increase in plasmalogen synthesis is beneficial for solid tumors that are often anoxic/hypoxic.
In the future, it will be interesting to compare our gene lists to the presence or absence of metabolic pathways across evolutionary time. As an example, it is known that plasmalogen synthesis was present in anaerobic bacteria [27]. As oxygen levels rose in the atmosphere, the metabolic pathways for etherphospholipid synthesis disappeared in facultative and aerobic species. They ultimately reappeared in higher organisms with aerobic respiration. Such evolutionary correlations with ambient oxygen tensions may provide clues as to the role of these hypoxic adaptations.

We believe that this oxygen screen serves as the first step to a systematic study of the role of oxygen in metabolism. Oxygen lies at the crucial intersection of fundamental biochemistry and disease pathophysiology. The genes and metabolic pathways we have identified will likely serve as therapeutic targets for hypoxia and hyperoxia.

4.5 Methods

**Screen Infection:** 120e6 K562 cells were spinfected at a target MOI of 0.3 (~450 cells per guide), using the Brunello, Human CRISPR Knockout Pooled Library. Cells were maintained in DMEM media containing 25mM glucose, Pen/Strep and 5mM glutamine. After 24h, cells were transferred to a spinner flask containing 2ug/ml Puromycin. For more detailed specifications, refer to [16].

**Library Prep and Sequencing:** Stored cell pellets from Day 0, Day 9 and Day 15 were used for RNA extraction, using the Qiagen DNeasy Blood and Tissue Kits. Purified RNA was diluted to 100ug/ml and then used for library prep (Broad Genomics Perturbation Platform (GPP)). Genomic DNA PCR was performed to barcode samples and attach sequencing adapters. The GPP then amplified barcoded oligos, pooled samples and purified products on a 2% agarose gel. The HiSeq 2500 was used for sequencing and alignment was performed using a reference file for known guides in the Brunello library.

**Analysis:** We used MAGeCK software to compile gene level data across individual samples. As input, we initial compared individual guide abundance relative to the Day 0 normoxic samples. However, this analysis confounded noise throughout the screen and showed significant overlap between essential genes at different oxygen tensions. To focus on the primary differences across oxygen tensions, we compared guide abundance across oxygen tensions on Day 15 of the screen. For gene set enrichment analysis, we
used the pre-ranked setting in the GSEA software. For lists of mitochondrial disease genes, we referred to a previously published list [19].

**Single Gene KO Pools:** For follow-up experiments, we created individual pools of KO cells in K562 and HEK293T cell lines. We first annealed oligos corresponding to individual guideRNAs and cloned them into the lentiCRISPRv2 backbone. KO populations were infected using a procedure identical to the spinfection for the original screen. All future experiments were done between 10-21 days after starting puromycin selection.

**DCA experiments:** Sodium dichloroacetate (Sigma) was dissolved in water to 500mM. KO pools corresponding to individual components of the respiratory chain were treated with 10mM DCA or vehicle and placed in 21%, 5% or 1% oxygen.

**Mouse metabolomics:** 5-8 WT mice (mixed genders) age ~50d were placed in 11% or 21% oxygen chambers for three weeks. Mice were then anesthetized with isoflurane and blood was collected via tail vein. Plasma was used for polar and non-polar metabolomics at the Broad Institute Metabolomics platform.

### 4.6 References


Fig 1. Genome-wide CRISPR Screen for Essential Genes in Varying Oxygen Tensions. (A) Diagram of approximate tissue oxygen tensions in the human body, demonstrating the differential role of oxygen in influencing tissue-specific metabolism. (B) Schematic of genome-wide screen for identifying oxygen-dependent essential genes. (C) Principal component analysis (PCA) for guide abundance at Day 0 and Day 15 across oxygen tensions (color scheme same as 1B). (D) Ranking for essential genes (with guideRNAs that are depleted over 15d) in 21% and 1% oxygen. Lines connect rank in 21% to rank in 1%. Genes that are essential in all oxygen tensions are horizontal, whereas genes that are differentially essential are offset. Ranking is from most essential (top) to least essential (bottom). (E) GSEA output for GO categories that are selectively essential in High O₂. (F) GSEA output for GO categories that are selectively essential in Low O₂.
Fig 2. Comparison to Hypoxia Transcriptional Response. (A) PCA analysis for RNASeq data at three different oxygen tensions, from 0h to 6d after starting treatment. (B) Histogram of student t-test p-values for differential gene expression, for the 300 most selectively essential genes in 21% oxygen (top), 1% oxygen (middle) or a randomly-generated list (bottom). (C) Average gene expression (relative to 0h in 21% O₂) over time in 21% (black), 5% (blue) and 1% (red) oxygen for canonical HIF targets. (D) Average gene expression timecourse for mitochondrial ribosomal subunits that are also among the top 300 genes that are selectively essential in 21% oxygen, but not 1% oxygen.
Fig. 3 Mitochondrial electron transport chain subunits are essential in 21% oxygen, but less so in 5% and 1% O2. (A) The cumulative sum of selective essential genes in different gene categories. Peroxisome biogenesis genes are selected based on "PEX" nomenclature. Complex 1 subunit genes are selected based on "NDUF" nomenclature. Mitochondrial disease genes are taken from manually tabulated list and oxygen-consuming genes are based on KEGG reactions. (B) Schematic of stacked bar graph representing percentage of genes that are selectively essential in 21% O2 or selectively essential in glucose-free media and in respective gene categories. Exact proportions will be calculated in future analysis. (C) Guide abundance from 0 to 15d (y-axis) across three different oxygen tensions (21% (red), 5% (light blue), 1% (dark blue)). Validation of findings in KO populations corresponding to complex 1 through complex 5 knockout. Final cell counts normalized to non-targeting guideRNAs after 4d in (D) HEK293T cells and (E) K562 cells.
Fig. 4 Mechanism of hypoxic rescue of mitochondrial dysfunction. (A) Final cell counts normalized to non-targeting guides of Complex 1-5 KO cells in different oxygen tensions. Cells were treated with either vehicle or dichloroacetate (DCA).
Fig. 5 Peroxisomal synthesis of plasmalogens is selectively more essential in low oxygen tensions. (A) Schematic of peroxisome biogenesis pathway. Pex subunits are shown in grey, rankings for genes that are selectively essential in 1% oxygen are in red. Major pathways of peroxisomal metabolism are listed, with detailed schematic showing steps for plasmalogen synthesis to the right. (B) Guide abundance through duration of screen and oxygen tensions for peroxisomal biogenesis genes (top) and plasmalogen synthesis genes (bottom). (C) Final cell counts normalized to non-targeting guides in 21% (left) and 5% (right) oxygen, in media with 3% FBS. (D) Most changing metabolites in the plasma of adult, WT mice exposed to three weeks of continuous 21% or 11% oxygen. All plasmalogens shown in red.
Chapter 5: Organelle Crosstalk Between Mitochondria and Peroxisomes: Metabolic Alterations and Protein Mistargeting in Zellweger Syndrome

5.1 Abstract

Peroxisomes have essential metabolic functions ranging from ROS metabolism to lipid biosynthesis. Dysfunctions in these cellular organelles cause severe, untreatable diseases. Mutations in the biogenesis machinery result in peroxisomal biogenesis disorders (PBD), also known as the Zellweger syndrome spectrum. Interestingly, secondary mitochondrial dysfunction is a repeated finding in patients and animal models with PBDs. Here we investigate metabolic and proteomic crosstalk between peroxisomes and mitochondria. We generate cell lines lacking a subunit of the matrix import machinery, Pex5. We characterize this model, showing impaired mitochondrial and peroxisomal metabolic functions – e.g. etherphospholipid synthesis, fatty acid alpha oxidation, and mitochondrial oxygen consumption. Furthermore, we show extensive metabolic alterations in serine-related phospholipids of Pex5 KO cells. Such data provide candidate biomarkers of disease, which we begin to validate in patient plasma samples. We then go on to perform extensive proteomic experiments in whole cell and isolated mitochondria of our disease model. We uncover remarkable mistargeting of peroxisomal membrane proteins to the mitochondrial outer membrane and demonstrate the Pex3-dependent mechanism of this mistargeting. Future studies will uncover the molecular identities of proteins involved in vesicle formation, budding and merging to form pre-peroxisomes during de novo peroxisome biogenesis. We also identify the fate of peroxisomal matrix proteins, demonstrating the destabilization vs. cytosolic localization of such proteins. This work helps understand pathways of peroxisome biogenesis and the role of organelle crosstalk in Zellweger syndrome.

5.2 Introduction

Peroxisomes are single-membrane bound organelles that are present across eukaryotic species and have a distinct evolutionary origin [1]. Mammalian peroxisomes contain approximately 100 proteins which fall into five general categories: protein import machinery, organelle division, ROS detoxification, lipid metabolism and other (e.g. D-amino acid oxidation, cholesterol synthesis, and even glycolysis in certain species). Many of these metabolic pathways are also distributed across mitochondria and the endoplasmic reticulum [2]. Thus, peroxisomal disorders are an ideal system in which to study organelle crosstalk and how it shapes disease.
Peroxisome protein import is unique in that matrix proteins are imported in their fully-folded form, already bound to cofactors [3]. The Pex genes were first identified in yeast deletion strains as being essential to peroxisome biogenesis. Matrix proteins contain one of two targeting signals, PTS1 or PTS2 [4], [5]. All but 3 matrix proteins encode PTS1, which consists of the three amino acid sequence SKL at the C-terminus. This targeting signal is recognized by the Pex5 protein which transports cargo to the peroxisome membrane, forms a channel and allows for cargo import. Pex7 recognizes the less well-defined PTS2 and transports proteins to the membrane, where they are imported in a Pex5-dependent manner. The logic behind two distinct, but overlapping matrix import pathways is unknown.

The targeting of peroxisomal membrane proteins is even less well understood. In traditional yeast literature, membrane proteins are imported in a Pex19-dependent manner. Through the canonical pathway, Pex19 recognizes a peroxisome membrane signal and shuttles cargo to the peroxisomal membrane, where it docks with Pex3 [6]. Alternatively, a subset of membrane proteins can be inserted into the ER directly and then transported to peroxisomes via vesicular transport. As much of this work has been done in yeast, the specifics in mammalian cells remain a mystery. Intriguingly, a recent study showed that de novo peroxisome biogenesis can occur via insertion of peroxisomal membrane proteins into the ER and mitochondria, followed by vesicular budding and integration to form new pre-peroxisomes [7].

Peroxisomal disorders can result from mutations in individual metabolic enzymes or the protein import machinery [8]. The latter result in peroxisomal biogenesis disorders, the most severe being Zellweger syndrome. In these cases, the peroxisomal matrix import machinery is defective and the majority of peroxisomal functions are disrupted. Patients on the Zellweger syndrome spectrum present with failure to thrive, hepatic dysfunction, seizures, hypotonia, craniofacial dysmorphism and neurodevelopmental delay. There are currently no treatments for these disorders and in the most severe cases, death ensues with the first few years of life.

In one of the earliest reports of these disorders, both peroxisomal and mitochondrial dysfunctions were noted [9]. Peroxisomes were absent in hepatocytes and renal tubules, while mitochondria appeared dysmorphic across tissues. Biochemical
characterization revealed a defective mitochondrial electron transport chain. This secondary mitochondrial dysfunction is a recurrent feature of peroxisome biogenesis disorders [10]. This is recapitulated in the Pex5 KO mouse model which shows mitochondrial hyperproliferation and impaired oxygen consumption in the liver and kidney [11]. However, the cause of secondary mitochondrial dysfunction remains a mystery.

This mitochondrial involvement in peroxisomal disorders brings to light the shared metabolic functions of the two organelles. While the evolutionary origin of the two organelles is thought to be distinct, the fission machinery is shared. Fis1 and Drp1 are required for both mitochondrial and peroxisomal fission [2]. In addition, both organelles house pathways for oxidative metabolism. The mitochondrial electron transport chain oxidizes high energy intermediates for energy production. Peroxisomes are responsible for fatty acid beta oxidation and also produce large amounts of hydrogen peroxide (whereas mitochondrial fatty acid oxidation is coupled to H₂O formation). Thus, both organelles are responsible for much of the cellular ROS production and detoxification. Finally, bile acid synthesis and fatty acid beta oxidation take place in both organelles – very long chain fatty acids (VLCFAs) are oxidized to medium-chain length in peroxisomes and then shuttled to mitochondria for complete oxidation. Such shared pathways highlight the extensive communication between the two organelles, and thus the potential for secondary dysfunction. Here, we investigate the metabolic and proteomic consequences of peroxisome loss. We identify an altered pathway of phosphoserine lipid metabolism and highlight additional biomarkers of disease. We then carefully dissect the fate of the peroxisome proteome in Zellweger syndrome. We demonstrate three potential fates – (1) cytosolic localization and stabilization, (2) degradation or (3) mistargeting to the mitochondrial outer membrane. In the context of recent studies of de novo peroxisome biogenesis, this mitochondrial mistargeting appears to be a way for the cell to be poised to resume peroxisome biogenesis. The set of mistargeted proteins we identify, provide additional clues into the mechanisms of this de novo synthesis pathway.

5.3 Results

5.3.1. Generating a model of Zellweger Syndrome in HEK293T cells
Mutations in any of the Pex genes can disrupt peroxisome biogenesis and lead to peroxisomal biogenesis disorders, or the Zellweger syndrome spectrum. The most frequent mutations lie in Pex1, accounting for 70% of patients [8]. However, Pex5 is directly responsible for the recognition and import of peroxisomal matrix proteins (Fig. 1A). A mouse model of Pex5 dysfunction previously showed significant mitochondrial hyperproliferation and dysfunction. Thus, we created a cell culture model of Pex5-deficient Zellweger syndrome. We assessed the accuracy of the experimental system, assayed canonical peroxisome functions, characterized the extent of secondary mitochondrial dysfunction, and finally studied the metabolic and proteomic changes, especially in the context of organelle crosstalk.

We began by generating single cell clones in HEK293T cells, using guideRNAs targeting Pex5 (Fig. 1B). We obtained three clones, 2 KO populations with single base-pair insertions and one hypomorph, with a 21bp in-frame insertion. We generated a GFP with a peroxisome targeting signal (PTS1) to visualize the import and subcellular location of peroxisomes. As expected, Pex5KO cells lacked the protein, and also showed diffuse, cytosolic localization of the GFP-PTS1 (Fig. 1C-D). WT clones that were transfected with the same guideRNA, but did not have any on-target genome editing, displayed punctate GFP-positive peroxisomes. The hypomorph also formed peroxisomes, albeit to a lesser extent than WT cells. To fully understand the dynamics of peroxisomal loss, division and de-novo formation, it was important to determine whether the peroxisome deficiency could be rescued by exogenous expression of Pex5. Indeed, transfection with FLAG-tagged Pex5 resulted in significant protein expression, which rescued peroxisome formation (Fig. 1E). Thus, we were able to recapitulate the phenomena of de-novo peroxisome formation, as these cells were completely lacking peroxisomes prior to the rescue transfection. Interestingly, WT cells with Pex5 overexpression had a greater number of smaller, punctate structures. The role of Pex5 in regulating peroxisome number has not been previously studied.

5.3.2. Biochemical characterization of peroxisomal function

Before delving into metabolic and proteomic alterations in our model system, we began by characterizing canonical peroxisome functions in our cell lines. The majority of catalase in the cell is localized to peroxisomes, where it is responsible for detoxification
of hydrogen peroxide generated during fatty acid oxidation. Upon loss of peroxisomal protein import, catalase should be found in the cytosol. Immunofluorescence for catalase reflected this mislocalization (Fig. 2A).

Patients suspected of having Zellweger syndrome are subjected to a clinical panel using plasma and skin fibroblasts. We collaborated with the Johns Hopkins Krieger Institute for analogous testing of our cell culture model using the gold standard clinical assays. Total VLCFAs and C26:0 LPC are substrates for peroxisomal beta-oxidation and plasmalogen synthesis respectively (Fig. 2B). PEX5 KO cells showed an accumulation of both types of substrates, as expected. PE plasmalogenes and Lyso PAF (derived from plasmalogens) were both depleted in KOs, due to impaired plasmalogen synthesis. The hypomorph showed an intermediate phenotype in all assays tested.

Direct enzymatic activity was also tested for several canonical peroxisome functions (Fig. 2C). Soluble vs. insoluble catalase activity was directly measured. As expected for cytosolic catalase localization, Pex5KO showed an increased percentage of soluble activity, relative to the hypomorph and WT cells. The first three steps of plasmalogen synthesis take place in the peroxisome, whereas remaining steps take place in microsomes. Thus, peroxisomal incorporation of 14C-hexadecanol versus the microsomal incorporation of the 3H-hexadecyl-glycerol is a proxy for peroxisomal plasmalogen synthesis. This relative activity was impaired in KO cells. Phytanic acid typically undergoes peroxisomal alpha-oxidation to generate pristanic acid. This activity was also defective KO cells. Thus, all clinical assays used to diagnose Zellweger syndrome confirmed the accurate disease phenotype of our model system.

5.3.3 Bioenergetic characterization of mitochondrial function

The initial reports by Goldfischer et al. in the 1970s describing Zellweger syndrome commented equally on peroxisomal and mitochondrial dysfunction [9]. Secondary mitochondrial dysfunction has been noted in subsequent studies in both animal models and patient fibroblasts. It is tempting to speculate that the disease presentation of Zellweger patients can be partly attributed to this mitochondrial dysfunction. We thus studied basic mitochondrial bioenergetics in our experimental system. Basal and uncoupled oxygen consumption was decreased by nearly half in KOs and the hypomorph.
relative to WT cells (Fig. 2D). Furthermore, mitochondrial membrane potential was decreased across cell lines with peroxisomal dysfunction (Fig. 2E).

In the Pex5 KO mice and in the kidney/liver of human patients, mitochondria appear dysmorphic and hyperproliferative. We used mitochondrially-targeted APEX to label cells for electron microscopy [12]. The hypomorph samples had clearly dysmorphic mitochondria with abnormal cristae and membrane structure (Fig. 2F). Further studies would be needed to quantify and characterize this feature.

This universal presentation of secondary mitochondrial dysfunction begs the questions: What is the mechanism leading to bioenergetic failure? Would treating the secondary mitochondrial dysfunction alleviate any of the disease symptoms of Zellweger syndrome patients?

5.3.4 **Metabolic characterization of Pex5-deficient cells**

Altered lipid composition is a hallmark of peroxisome dysfunction. Changes in etherphospholipids and other peroxisomal substrates have been well-noted for several decades and are the gold-standard biomarkers for disease. However, a general characterization of metabolic alterations has not been performed for these disorders. Remaining questions include: (1) What are the specific lipid species that are altered? (2) Are there compensatory changes in other lipids? (3) Do changes in protein localization of peroxisomal proteins result in an altered metabolic profile (due to enzymatic activity in other cellular compartments)? (4) What are the broad changes in polar metabolites for these disorders and can they serve as more specific biomarkers?

To answer these questions, we performed metabolomics for polar and non-polar metabolites, on cell pellets and spent media. As expected, the majority of plasmalogen species were significantly depleted in cell pellets of PEX5 KO cells (> 100-fold) (Fig. 3A). However, this trend was only true for plasmalogens with phosphethanolamine and phosphocholine headgroups, but not for phosphoserine headgroups. PS plasmalogens were unaltered in KO vs. WT cells. Thus, PS plasmalogens may be generated in an independent pathway than PE and PC plasmalogens. Alternatively, the substrate for PS plasmalogens may be significantly higher and thus push the equilibrium of plasmalogen synthesis towards this particular head group. Either way, PS plasmalogens may play a
compensatory role in peroxisome-deficient cells. Future studies are needed to dissect the
mechanism of this selective alteration in plasmalogen subtypes.

Interestingly, diacylphospholipids displayed much less variation between WT and
Pex5 KO cells (Fig. 3B). However, the two most abundant PS diacyl lipids (PS 34:0 and
PS 32:0) were almost 100-fold elevated in KO cells. More minor PS species were not as
drastically altered between groups, but may be below the threshold of linear detection.
Such changes in serine-related lipids, led us to carefully profile polar metabolites in these
samples. Intriguingly, multiple steps of the serine and PS lipid synthesis pathway were
altered in cell pellets and media of Pex5 KO cells. Thus, it is likely that a build-up of
upstream metabolites (e.g. phosphoserine, glutamate, serine) has a ricochet effect
leading to a build-up of PS lipids (Fig. 3C). The exact mechanistic details and implications
for these changes remain unknown.

In profiling across samples, we identified a putative list of plasma biomarkers (Fig.
3D). As current biomarkers do not distinguish between subsets of peroxisomal disorders,
additional metabolites may be beneficial for pinpointing specific biochemical defects. We
were able to recapitulate known polar metabolites which serve as biomarkers (e.g.
docosahexanoic acid and HETE molecules, which are breakdown products of arachidonic
acid). To determine the validity of these biomarkers, we measured polar metabolites in
the plasma of 18 patient plasma samples of peroxisomal disorders (1 PEX10, 1 PEX16,
2 PEX26, 2 PEX6, 11 PEX1 or unidentified and 1 DBF) and age-matched healthy controls
(Fig. 3E). Several metabolic alterations from cell pellets and media were recapitulated in
the plasma samples, such as glutamate and a putative phosphoserine peak. More
complete metabolomics on these patient samples would be needed to provide a
comprehensive list of biomarkers.

5.3.5. Fate of Peroxisomal Proteins

Peroxisomal biogenesis disorders result in a general loss of peroxisomal protein
import. A natural question that arises is: what is the fate of these once-peroxisomal
proteins? In theory, there are at least three possibilities: (1) Proteins can remain in the
cytosol and continue to function, depending on the cofactor and substrate availability. (2)
Peroxisomal proteins may localize to the cytosol, but be dysfunctional or degraded due
to insufficient binding partners. (3) A third possibility is that peroxisomal proteins may be
mistargeted to other cellular compartments, where they may or may not continue to function.

Previous reports have described dual-targeting of a subset of proteins, such as the fission machinery, to mitochondria and peroxisomes. There are also a few examples of peroxisomal proteins being mistargeted to mitochondria in the setting of peroxisome loss. The presence of secondary mitochondrial dysfunction, as well as such known mistargeting, led us to perform proteomics on isolated mitochondria and whole cells with and without peroxisome dysfunction (Fig. 4A).

We first ensured that isolated mitochondria were sufficiently devoid of cytoplasmic markers and enriched for mitochondrial proteins. Markers for mitochondrial matrix, inner membrane and outer membrane were all dramatically enriched in isolated mitochondria, whereas actin and catalase (peroxisomal marker) were enriched in whole cells (Fig. 4B).

In our first iTRAQ experiment, we compared the abundance of all detectable proteins in mitochondria of Pex5 KO and WT cell lines. We saw a dramatic enrichment for peroxisomal proteins in both selectively enriched and selectively depleted samples (KO vs. WT mitochondria) (Fig. 4C-E). 47 peroxisomal proteins (of ~100) were detected in a total of ~8000 proteins. As the mitochondrial proteome only consist of ~1500 proteins, the remainder must be extramitochondrial contamination that can be detected due to the extreme sensitivity of our proteomic methods. Of the top 30 most enriched proteins in Pex5 KO mitochondria vs. WT mitochondria, 12 were annotated peroxisomal proteins. Of the 30 most depleted proteins, 14 were known to be peroxisomal. The remaining ~20 peroxisomal proteins were present in similar amounts in both samples. Upon closer inspection, we noticed that all enriched proteins were recognized by the Pex19 protein and were membrane proteins (Fig. 4E). On the other hand, all depleted proteins were annotated as being peroxisomal matrix proteins and recognized by either Pex5 or Pex7.

One possibility was that enriched or depleted proteins reflected whole cell trends that were just manifesting in our isolated mitochondria samples due to contamination during mitochondrial purification. We performed a second iTRAQ experiment to assay whole cell proteomic differences. Peroxisomal proteins were particularly depleted in KO cells relative to WT cells. However, peroxisomal proteins were not enriched in whole cells, as was the case in mitochondrial samples. A final iTRAQ with both whole cell and
mitochondria from both KO and WT samples allowed for a clear relative comparison across samples. We measured the relative presence of a given protein in mitochondria vs. whole cell pellets (Fig. 4F). We then looked at this delta value in KO vs. WT samples. If proteomic changes were a consequence of whole cell contamination, proteins would fall along the x-y axis. If there was selective mistargeting or depletion in mitochondria, they would fall off-the-diagonal. Both categories of proteins were noted in our samples. A closer look showed that all peroxisomal membrane proteins that were enriched in KO mitochondria were a true signal (enrichment in mitochondria, but not in whole cell) (Fig. 4F). However, the peroxisomal matrix proteins were depleted in both mitochondria and whole cell, reflecting a general instability of these proteins in the KO cells. Thus, all three predicted categories of localization/function were taking place in peroxisome-deficient cells (Fig. 4G). The extensive mitochondrial mistargeting of membrane proteins could have a profound impact on mitochondrial function and compartment-specific metabolism.

We went on to confirm this mitochondrial mistargeting for a subset of membrane proteins.

5.3.6. Validation and Mechanism of Mistargeting

We performed immunofluorescence for a peroxisomal membrane protein, ACBD5, that appeared to be mistargeted in our proteomics datasets. In WT cells, it formed punctate structures as expected for peroxisomal localization (Fig. 5A). However, in Pex5 KO cells, ACBD5 was localized to mitochondria. Pex3 is the protein which recruits Pex19-bound membrane proteins and also appeared to be mistargeted to mitochondria in our datasets. While this work was underway, another group showed that Pex3 is involved in mitochondrial targeting of such proteins during peroxisome-loss. We validated this finding by showing that ACBD5 did not localize to any membrane in Pex3 KO cells. Furthermore, high magnification imaging of three such mistargeted proteins demonstrated localization to the mitochondrial outer membrane (Fig. 5B). Pex16 was recently implicated in forming ER-derived vesicles which then merge with mitochondrial-derived vesicles to form pre-peroxisomes. We showed that Pex16-deficient patient fibroblasts still mistargeted peroxisomal membrane proteins to mitochondria demonstrating the independent regulation of these two vesicle subtypes (Fig. 5C). Important questions that remain are: (1) What is the mechanism of vesicle budding from mitochondria and which enzymes are responsible? (2) What is the mechanism by which mitochondria-derived vesicle identify
and merge with ER-derived vesicles? (3) Is this extensive mistargeting of peroxisomal proteins to mitochondria responsible for secondary mitochondrial dysfunction? (4) Are any of the metabolic changes in peroxisome-deficient cells due to the aberrant activity of mistargeted proteins? We hope that the complete list of mistargeted proteins and metabolomic findings will provide further insights into these phenomena.

5.4 Discussion

Subcellular compartmentalization is arguably the key distinguishing factors of eukaryotes and a key driver of increased biological complexity. The existence of organelles with distinct metabolic compositions allows otherwise incompatible reactions to proceed. However, this advantage comes with the added responsibility of correctly targeting proteins and intermediate metabolites that allow for organelle crosstalk. The plasticity of such targeting is evident in the evolutionary history of protein localization. Individual proteins were annotated as “co-occurring” if they either existed in different organelles across distinct species, or were dual-localized within a species [13]. Such an analysis revealed that a remarkable 64% of peroxisomal proteins in mammals display this co-occurrence with mitochondria. This surpasses all pairwise comparisons between organelles except the nuclear envelope and the endomembrane system. A prime example of this feature is the targeting of alanine-glyoxylate transferase (AGXT) [14]. This enzyme is localized to mitochondria in carnivores, peroxisomes in herbivores and both in omnivores. AGXT is responsible for the detoxification of glyoxylate, preventing the accumulation of oxalate crystals. Interestingly, glyoxylate is derived from either glycolate (in peroxisomes) or hydroxyproline (in mitochondria) in herbivores vs carnivores due to differences in diet composition. Mutations in humans that lead to preferential targeting to mitochondria, instead of peroxisomes, cause excessive oxalate crystallization and kidney stones.

Such examples highlight both the importance and risk of metabolic compartmentalization. Thus, it is crucial to understand how such organelle crosstalk is regulated in health, as well as perturbed during disease. How do lesions in each organelle affect the structure and function of other organelles? How does this contribute to metabolic alterations and ultimately, disease phenotypes?
We addressed such questions of crosstalk between peroxisomes and mitochondria, in the context of peroxisomal biogenesis disorders. We generated a cell culture model of Zellweger syndrome and validated metabolic alterations in peroxisomal and mitochondrial functions. We then demonstrated extensive metabolic rewiring of phosphoserine lipid metabolism and proposed additional biomarkers of disease. Finally, we showed remarkable mistargeting of peroxisome membrane proteins to the mitochondrial outer membrane.

We demonstrated that nearly a third of detectable peroxisomal proteins were destabilized in Pex5KO cells. These proteins were all matrix enzymes and involved in metabolic functions ranging from plasmalogen synthesis to beta-oxidation. Thus, dysfunction of such metabolic pathways is a consequence of enzyme degradation. It has previously been noted that AGPS and DHAPAT stabilize each other in the peroxisome matrix [15]. Loss of this co-localization leads to instability and enzyme degradation. Such known examples validate our experimental approach. Alternatively, peroxisomal membrane proteins appear to largely mislocalize to the mitochondrial outer membrane. Based on recently published studies, it appears that this localization is a means to anticipate de novo peroxisome biogenesis. Our list of mislocalized proteins give clues as to the molecular identities of proteins responsible for budding of pre-peroxisomal vesicles and fusion with ER-derived vesicles.

A natural follow-up question is whether these mislocalized membrane proteins are still functional in mitochondria and whether this mislocalization contributes to the secondary mitochondrial dysfunction. The dramatic increase in phosphoserine-related lipids in KO cells may be a consequence of such mistargeting. Of note, phosphatidylserine is typically considered an apoptosis signal, where membrane flipping leads to its extracellular exposure and begins the apoptotic cascade. The exact localization of PS lipids and their effect on intracellular signaling warrants further study in the context of peroxisome loss.

Another interesting concept is the relative dosage of mitochondria and peroxisomes, especially in the context of their shared metabolic functions. Fis1 is involved in membrane fission for both organelles in WT cells. In Pex5 KO cells, this protein appears enriched in mitochondrial membranes. It is possible that this affects mitochondrial function
and abundance, perhaps as a compensatory mechanism. Future studies will be needed to extend our findings of organelle crosstalk to disease pathogenesis and physiologically-relevant phenomena, such as pexophagy and rapid peroxisome proliferation in response to external cues.

5.5 Methods

**Generation of KO cells**: Pex5 KO cells were generated using guideRNAs targeting exon 3. 1e6 HEK293T cells were transfected with 1ug of Cas9-expressing plasmid and 200ng of U6 PCR product. Transfected pools were diluted to generate single-cell clones. Rather than screening individual colonies for Pex5 genome editing, we transfected single cell colonies with PTS1-GFP and chose colonies which displayed diffuse GFP localization, rather than punctate patterns. Using this approach, we isolated 3 unedited cell lines (referred to as WT) and 3 edited cell lines (2 KO and 1 hypomorph). In order to determine the exact genome editing in the Pex5 locus, we amplified the surrounding regions (F-primer: AGCAATCAGAGGAGAAGC and R-primer: AAACTGTCCATACTCCTTTTCAC) and sequenced the inserts.

**Cloning for PTS1-GFP**: To generate a peroxisome-targeted GFP construct, we began by using a mitochondrially-targeted GFP plasmid (pLYS labeled Flag5 mitoGFP HA, Addgene: 50057). The MTS-GFP was digested with AgeI and EcoRI. PTS1 was cloned into the C-terminus of the digested plasmid (F-primer: CCGGGATGGTGAGCAAGGGCA and R-primer: GAATTCTTATAATTTGGACTTGTACAGCTCGTCCATG).

**Enzymatic assays for peroxisomal function**:

**Lipid measurements**: C26:0 LPC, Lyso PAF, total VLCFA and PE plasmalogens were measured using either capillary gas chromatography or LC-MSMS.

**Soluble catalase activity**: Digitonin titration was used to isolate soluble vs. particle-bound catalase, corresponding to cytosolic vs. peroxisomal localization. Catalase activity was then measured across samples, by measuring oxygen production polarographically.

**Plasmalogen synthesis**: Plasmalogen biosynthesis takes place across multiple organelles. The first two reactions take place in peroxisomes, and subsequent steps in microsomes. To determine whether the peroxisomal steps are impaired, we use a use the double-substrate, double isotope method which compares the incorporation of 14C-hexadecanol into peroxisomes vs. 3H-hexadecyl-glycerol into microsomes.
Phytanic acid oxidation: fatty acid alpha oxidation was assessed by measuring the amount of $^{14}\text{CO}_2$ produced from [1-$^{14}$C] phytanic acid. Labeled CO$_2$ was collected in KOH-soaked cloth by O/N shaking. This was then transferred to a scintillation vial and radioactivity was measured.

**Oxygen consumption measurements:** Cells were seeded in XF24 24-well cell culture microplates in 200µl of normal growth media (DMEM, Life Technologies-11965, 3.7g/L NaHCO3 and 10% FBS). Plates were maintained in an incubator containing 5% CO$_2$ at 37C. OCR was measured at cycles of mixing (2m), waiting (2m) and measurement (4m). Measurements were performed at baseline, or with small molecule RC inhibitors (antimycin, piericidin, oligomycin or CCP).

**Mitochondrial membrane potential measurements:** Mitochondrial membrane potential measurements were made by soaking cells in DMEM media without phenol red, but containing ~10nM TMRM. Cells were then analyzed by the Envision plate reader. Values were normalized to cell number.

**Electron microscopy:** Cells were transfected with plasmid for MTS-APEX and then processed for EM using standard techniques.

**Metabolomics:** KO, WT and hypomorph cell lines were seeded at 8e5 cells/ml in 10cm plates. Cells were placed in normal DMEM media (10% FBS, P/S) and incubated for 3d. Spent media was collected and frozen at -80C after removing cell debris by pelleting. Cells were pelleted and washed with PBS, before being frozen at -80C.

**Proteomics:** We performed three separate iTRAQ experiments as described in the text. Material was taken from either cell pellets or isolated mitochondria. Samples were labeled with iTRAQ Reagent 114, 115, 116 or 117 and processed by the Broad proteomics platform.

5.6 References:


Acknowledgments: We would like to thank Ann B. Moser and the Krieger Institute for providing us with de-identified patient plasma samples. Peroxisomal Disorder Patient Fibroblasts (GM13266, GM13268, GM06231) were obtained from the NIGMS Human Genetic Cell Repository at Coriell Cell Repositories.
Fig 1. Generating a cell culture model of Zellweger syndrome. (A) Schematic of the import pathway for peroxisomal matrix proteins. Pex5 recognizes the PTS1-containing cargo and forms the core of the transport channel. (B) Specific mutations in cell lines with disrupted Pex5. (C) KO cell lines lack Pex5, whereas transfection with FLAG-tagged Pex5 results in significant overexpression. (D) Transfection with GFP-PTS1 revealed intact peroxisomes in WT clones, lack of peroxisomes in KO clones and an intermediate presence in the hypomorph. (E) Overexpression of Pex5 rescued peroxisome formation, implying de novo formation.
Fig 2. Mitochondrial and peroxisomal functions in Pex5 KO cells. (A) IF for catalase in KO and WT cells. (B) Abundance of key substrates and products of peroxisome metabolism across cell lines. (C) Catalase activity in soluble vs. insoluble fractions in KO and WT cells, relative to known patient and healthy controls. Similarly, plasmalogen synthesis and pristanic acid synthesis were impaired in KO cells. (D) Basal oxygen consumption and uncoupled respiration across cell lines. (E) Mitochondrial membrane potential is lower in KO and HM cell lines. (F) Aberrant mitochondrial morphology in hypomorph relative to WT cells.
Fig 3. Rewiring of metabolism and phosphoserine lipids in Pex5 KO cells. (A) Volcano plot of all changing lipids, with plasmalogens (PE/PC vs. PS) highlighted. (B) Volcano plot of all changing lipids, with diacyl lipids (PE/PC vs. PS) highlighted. (C) Schematic of metabolic pathways involved in synthesis of PS lipids. (D) Most changing metabolites in spent media of KO vs WT cells. (E) Targeted profiling of changing metabolites in the plasma of patients with peroxisomal disorders vs. age-matched controls.
Fig 4. Localization of peroxisomal proteins during loss of peroxisomal protein import. (A) Experimental design for proteomics of mitochondrial and whole cell samples in Pex5KO and WT cells. (B) Western blot for organelle-specific markers for isolated mitochondria and whole cells. (C) Relative abundance of organelle proteins in mitochondria of KO vs. WT in duplicate sample sets. (D) Histogram of relative enrichment for all organelle proteins, mitochondrially-annotated proteins and peroxisomally-annotated proteins. (E) The 30 most enriched and depleted proteins in mitochondria of KO vs. WT cells, as well as their known targeting signals. (F) Summary of iTRAQ3 comparing the abundance of proteins in mitochondria vs. whole cell samples in WT and KO cells (left). Ranks in iTRAQ 1 and iTRAQ 2 for proteins that were depleted or enriched in isolated mitochondria (right). (G) Schematic of potential fates of peroxisomal proteins in KO cells.
Fig 5. Validation and mechanism of mitochondrial targeting of peroxisomal proteins. (A) Immunofluorescence for mitochondrial outer membrane protein Tim23 (red) and peroxisomal membrane protein ACBD5 (green) in WT, Pex5KO and Pex3KO cells. Mistargeting appears to be dependent on presence of Pex3. (B) Higher magnification images of GFP-tagged peroxisomal membrane proteins and mitochondrial dye (red) in Pex5 KO cell lines. (C) GFP-tagged peroxisomal membrane proteins in WT and Pex16-deficient patient-fibroblasts. Peroxisomal proteins are still mislocalized to mitochondria in cells lacking Pex16.
Chapter 6: Implications and Future Directions
6.1 Implications of Thesis Work

6.1.1 Human Clinical Trials: Since our initial reporting of hypoxia therapy for mitochondrial disease, the members of the Mootha and Zapol labs have been working tirelessly to translate our findings to humans. Hypoxia physiology research has a rich history, often spearheaded by anesthesiologists, cardiologists and respiratory physiologists. A more recent study in the New England Journal of Medicine, focused on a gradual ascent to Mt. Everest with continuous blood gas and oxygen content measurements [1]. Analogous studies have been performed in high altitude vs. sea-level natives – looking at pulmonary hypertension, polycythemia, blood glucose regulation, child birth, among other phenomena [2], [3]. Finally, epidemiological data from army expeditions to high altitude and intermittent hypoxia exposure in coal miners provide clues to the safety and effects of human hypoxia exposure [4]–[6]. To design and assess the safety of chronic hypoxia in a controlled environment, it is important to replicate such findings in-house. Based on our initial findings of hypoxia therapy for mitochondrial disease, the first healthy human clinical trials are already under way to evaluate the safety of controlled hypoxia exposure for one week. This work will provide assurance that such approaches can be practically applied in the hospital setting.

6.1.2 The Use of Supplemental Oxygen: While the use and testing of hypoxia therapy will be a gradual process, a more immediate application of our work is restraint in the use of supplemental oxygen without clinical indication. Previous studies have alluded to the toxic effects of supplemental oxygen to human health [7], [8]. Our work demonstrates this concept in animal models of Leigh syndrome and shows that pulmonary edema caused by 100% oxygen in WT animals, occurs with moderate hyperoxia (55% oxygen) in our disease model. Anecdotal reports of mitochondrial disease patients exposed to high oxygen have supported this concept. Thus, a direct clinical application of our findings is more discretion in the use of supplemental oxygen. Undoubtedly, supplemental oxygen is beneficial in many disease settings and should be continued to be used where there is a clear benefit.

6.1.3 Potential Clinical Regimens for Hypoxia Therapy: Our preclinical studies of tissue pathologies and clinical regimens will pave the path for the most practical, yet effective forms of hypoxia therapy. We found that mice with mitochondrial disease
develop left ventricular dysfunction and arrhythmias over time. Furthermore, hyperoxia leads to pulmonary edema and respiratory distress. This knowledge will allow clinicians to continuously evaluate such pathologies in mitochondrial disease patients exposed to varying oxygen tensions. Furthermore, the finding that intermittent and moderate hypoxia regimens do not alleviate disease is just as important for outlining the restrictions of hypoxia therapy. Even more so, removal of diseased mice from chronic hypoxia to normoxia leads to their rapid decline. These limitations will guide the first human studies to optimize chances of success. Finally, the finding that hypoxia can not only prevent, but even reverse disease offers hope to the countless patients that suffer a severe metabolic crisis, prior to diagnosis with mitochondrial disease. A caveat of our work is that our studies have only been conducted in the Ndufs4 mouse model. Future work will determine whether our findings generalize to other mitochondrial disease models and common diseases.

6.1.4 Additional Targets for Hypoxia and Hyperoxia Treatment: By performing a genome-wide screen for essential genes at different oxygen tensions, we have created a ranked list of potential diseases which may benefit from modulating oxygen exposure. We have identified the subset of mitochondrial disorders which are the most likely to benefit from hypoxia therapy, as well as loss-of-function mutations which may benefit from hyperoxia exposure. We have also highlighted potential therapeutic targets for tumor hypoxia. We can now systematically investigate how low and high oxygen can affect disease more generally.

6.1.5 Understanding Oxygen Sensing and Adaptation: The ability of organisms to sense and adapt to oxygen has intrigued scientists for centuries. Our work identifies novel metabolic pathways involved in such adaptations. We highlight the role of entire organelles in allowing for survival at varying oxygen tensions. Such a detailed understanding will help us to better understand diseases of impaired oxygenation: stroke, MI, cancer and vascular disease, to name a few.

6.1.6 Peroxisome Metabolism and Biogenesis: Christian de Duve identified peroxisomes as distinct organelles approximately fifty years ago. Since then, we have come to appreciate the devastating effects of peroxisome loss on human health. Yet, there are still no treatments for peroxisomal disorders. Furthermore, a very basic
understanding of organelle biogenesis, metabolism and protein targeting is lacking. We characterize a cellular model of Zellweger syndrome and identify systematic perturbations in lipid metabolism. We also comprehensively characterize the fate of peroxisomal proteins in such diseases. We propose potential mechanisms by which peroxisomes are formed de novo. This work furthers our basic understanding of metabolic compartmentalization and organelle biogenesis.

6.1.7 Scientific Approach: The work reported in my PhD thesis is the culmination of four years of invaluable training in the Mootha and Zapol labs, the closest of collaborations, endless hours in the TC and mouse facilities and rich discussions within and outside of the lab. This work demonstrates the power of applying systems and quantitative approaches to biomedical problems, banding together a team of passionate scientists, physicians and engineers. I am fortunate that my PhD training has spanned these boundaries. My coursework has been a collection of graduate courses in computer science/statistics, combined with the first year of medical school classes. Similarly, my training has spanned the guidance and environment of a systems metabolism lab and an animal physiology lab. Together, this marriage between approaches, framework, and thought have allowed us to develop novel therapies and an in-depth understanding of metabolic disorders. We hope that this work will also serve as a template for future studies using systems approaches to understand disease.

6.2 Future Directions
6.2.1 Alternative Hypoxia Regimens: Our recent work demonstrates the need for chronic hypoxia of ~14% or lower oxygen levels for effective treatment of Leigh syndrome. Such treatment regimens can be accomplished using equipment that was originally developed for sports training. Nitrogen generators can be used to make entire rooms hypoxic. However, this produces severe limitations on the mobility and quality of life of patients. Therefore, we are simultaneously exploring alternative ways to make patients hypoxic, either by directly impacting oxygen content or oxygen delivery.

6.2.2 Mechanistic Studies of Hypoxia Rescue: My ongoing work is focused on deciphering the mechanism by which hypoxia is alleviating mitochondrial disease. By understanding the mechanism of rescue, we will also be able to identify the patient population that is most likely to benefit. Our efforts are several-fold. We are performing
genetic crosses with known members of HIF pathway. We are also testing additional mouse models of mitochondrial disease and oxidative stress. This is coupled with extensive metabolic characterization of WT and KO mice, exposed to different oxygen tensions. It is our hope that clues from the oxygen CRISPR screen and in-vivo work will arrive at a similar answer as to why hypoxia therapy is alleviating disease.

6.2.3 Follow-up on Additional Hits from the Oxygen Screen: The oxygen screen lays the foundation for (1) oxygen-based therapeutic strategies and (2) a more thorough understanding of oxygen sensing and adaptive pathways. Future work over the coming years will involve testing additional metabolic disorders for hypoxia/hyperoxia therapy, based on therapeutic targets identified in this screen. We are particularly interested in extending our findings to aging and age-associated disorders. Furthermore, many genes that are not directly associated with the mitochondrial electron transport chain or peroxisome biogenesis pathways are showing differential essentiality in hypoxia vs. hyperoxia. Understanding the mechanistic basis for these findings will further the fields of oxygen biology and disease applications.

6.3 References:


Supplemental Information
Supplement (for Ch. 2)

Materials and Methods:

1. Genome-wide Cas9-mediated Knockout Screen
   a. Virus Production

The genome-scale CRISPR knockout (GeCKO) library v1 was generously provided by the Zhang Lab. Library details have previously been published (10). For library lentivirus production, 1.2x10^7 cells were placed in each of 25, T225 flasks in 50ml of full DMEM (Life Technologies 11995) media (containing Pen/Strep, 10% FBS). 18h later, media in each flask was replaced with 13ml of DMEM (no Pen/Strep, 10% FBS) and 2h later, media was replaced with 20ml OptiMEM (Life Technologies 31985-070; no serum, no Pen/Strep). A transfection mastermix was made by combining individually prepared mastermix A (94ml of OptiMEM, 2.4ml of Lipofectamine 2000 (Life Technologies)) and mastermix B (94ml OptiMEM, 2.1ml of PLUS Reagent (Life Technologies), 240µg of pVSVg plasmid, 360µg of psPAX2 plasmid and 480µg of GeCKO plasmid library). Mastermixes A and B were combined for 20m. 8ml of the final mastermix was added to each T225 flask of HEK293T cells. After 6h, the media was changed to 30ml of DMEM media (Life Technologies 11995 w/ 1% BSA (Sigma)) and cells were incubated for 48-72h, before virus-containing supernatant was collected. Virus was concentrated by centrifugation for 2h at 24,000 rpm in a SW32Ti rotor. Virus was resuspended overnight at 4C and stored at -80C prior to use.

b. Knockout Screen

K562 cells were obtained from ATCC and maintained in full DMEM media (10% FBS, Pen/Strep). K562 cells were grown in 1L spinner flasks (Matrical) on magnetic stir plates (Bellco). Cells were always passaged before reaching confluency (1x10^6/ml) and subcultured at a concentration of 1x10^5/ml.

i. Virus Infection
2.5x10^8 K562 cells were resuspended to a concentration of 1.5x10^6 cells/ml. Polybrene (Sigma) was added to 120ml of the K562 cell suspension at a final concentration of 4µg/µl. 2ml of this cell suspension was placed in each well of 5, 12-well plates. 10ul of virus was added to each well for a target MOI of 0.3, ensuring that most cells incorporated 1 or 0 lentivirus particles. Plates were centrifuged for 2h at 1000g and placed in an incubator for 1h, after which media was aspirated. 2ml of full DMEM media (10% FBS, Pen/Strep) was placed in each well and cells were resuspended. 12h later, all wells were pooled into a spinner flask with 800ml of full DMEM media. A sample was taken for virus titration to ensure that the target MOI was obtained. 24h after the infection, Puromycin (Invitrogen) was added at a final concentration of 2µg/ml to begin selection for infected cells. Two independent infections (Infection 1 and Infection 2) were performed to control for variability in library infection.

ii. Passaging
Infected cells were passaged before reaching 1x10^6/ml and maintained in Puromycin-containing conditions for one week after infection. At this point, 7x10^7 cells were pelleted and stored as pre-treatment (Early) samples for each infection replicate.

iii. Experimental Selection
After 1 week of Puromycin selection, cells from each infection replicate were transferred to experimental conditions of (a) untreated cells, (b) moderate disease and (c) severe disease. 7x10^7 cells were pelleted and resuspended in media corresponding to each experimental condition. The untreated condition was defined as DMEM media (11965-092) with 1mM pyruvate (Invitrogen) added. The moderate disease condition was defined as DMEM media with 100nM antimycin (Sigma) and 1mM pyruvate. The severe disease condition was defined as DMEM media with 100nM antimycin, without pyruvate. All media also contained 200µM uridine (12).
iv. **Passaging in Experimental Conditions**

Cells were subcultured at $1 \times 10^5$/ml and passaged before reaching $1 \times 10^6$/ml. At each passage, $7 \times 10^7$ cells were pelleted and stored for intermediate screen time points.

c. **Library Prep**

Sequencing libraries were prepared as previously described (10). Briefly, DNA was extracted using the Qiagen Blood and Cell Culture DNA Maxi Kit from $7 \times 10^7$ cells per experimental condition, for each infection replicate. DNA was then purified using Micro Bio-Spin columns (BioRad 732-6224). 25 PCR reactions were performed using Herculase II Fusion DNA Polymerase (Agilent) to amplify the single guideRNAs (sgRNAs) from genomic DNA, at a minimum coverage of 450x per guideRNA. 30µl from the first pooled PCR samples were used as input for the second PCR reaction, allowing for attachment of barcodes and sequencing adapters. Barcode replicates were included for the moderate disease condition to ensure that PCR errors did not substantially affect results. The final PCR products were run on an agarose gel and the correct size PCR products were gel extracted and sequenced on an Illumina HiSeq 2500.

d. **Analysis**

i. **Processing of sgRNA Reads**

Custom Python and Matlab scripts were written for processing of sequencing reads. Reads were trimmed to remove barcodes and sequences corresponding to the GeCKO library backbone. A custom bowtie library was created for mapping between sgRNA sequences and guide/gene names. Bowtie alignment was performed, allowing for single mismatches. Finally, guide abundance was compiled for each experimental condition and a matrix mapping guide name to abundance for all samples was created.

ii. **Identification of Enriched and Depleted sgRNAs**

1. **Cell Viability Screen**
In order to identify genes which are essential to cell viability, guide abundance was first normalized to total number of reads per sample. The fold-enrichment was calculated for untreated samples (day 21 after experimental selection) relative to pre-treatment conditions (immediately before experimental selection), for both infection replicates. As infection replicates were very well correlated ($r^2 > 0.8$), the top 500 most depleted genes (by RIGER (41) analysis) were found for each infection replicate. The 500 most essential genes were determined for both infection replicates and compared to the known list of mitochondrial proteins, or MitoCarta to identify essential mitochondrial genes (42, 13). Genes without entrez id mappings were excluded (< 3% of list). RIGER output was generated for each infection replicate (untreated relative to pre-treated conditions) and used for Gene Set Enrichment Analysis (GSEA) (43).

2. Enrichment Screen

In order to identify gene knockouts which allow cells to cope with mitochondrial dysfunction, fold-enrichment was calculated for moderate or severe disease conditions relative to pre-treatment (early) conditions. The most enriched genes were then individually checked for their overall effect on cell viability (untreated relative to pre-treatment conditions). Alternatively, fold enrichment was also calculated for untreated vs. disease conditions. However, this form of analysis confounds genes which are enriched in disease conditions or selectively depleted in untreated conditions. The top hit was found using either approach.

iii. RIGER Analysis

RIGER analysis (41) was used to generate a summary statistic by combining information corresponding to all sgRNAs for a given gene.
sgRNAs were pre-ranked by fold-enrichment between two conditions. The Kolmogorov-Smirnov method was used with 1000 permutations. Gene scores were not adjusted for the number of sgRNAs corresponding to a given gene.

iv. GSEA Analysis
Output from RIGER analysis was used for gene set enrichment analysis (43). All curated gene sets were used during analysis in GseaPreranked mode. All other parameters were set to default values.

2. Lentiviral Cas9 Vector
Individual sgRNAs targeting VHL from the GeCKO library were cloned into the lentiviral Cas9 vector and used for follow-up experiments. Additionally, “dummy” non-targeting sgRNAs were generated as controls. The following oligonucleotides were used for lentivirus sgRNA cloning:

Oligo_1: 5’ – CACCG <20bp sgRNA sequence> -3’
Oligo_2: 5’ AAAC <complimentary 20bp sgRNA sequence> - C – 3’

<table>
<thead>
<tr>
<th>Oligo Name</th>
<th>Oligo Seq (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lenti_VHL_1_F</td>
<td>CACCGAGTACCTGGCAGTGTGATAT</td>
</tr>
<tr>
<td>Lenti_VHL_1_R</td>
<td>AAACATATCACACTGCCAGGTACTC</td>
</tr>
<tr>
<td>Lenti_VHL_2_F</td>
<td>CACCGCAGGTCGCTCTACGAAGATC</td>
</tr>
<tr>
<td>Lenti_VHL_2_R</td>
<td>AAACGATCTTTCTAGAGCGACCTGC</td>
</tr>
<tr>
<td>Lenti_VHL_3_F</td>
<td>CACCGGCAGCCTGCGTCGTCTGCCCGTA</td>
</tr>
<tr>
<td>Lenti_VHL_3_R</td>
<td>AAACGACTGGAAGCGACCCAGCGGCCACSACAGC</td>
</tr>
<tr>
<td>Lenti_VHL_4_F</td>
<td>CACCGGTGCAATCTCTGATCTGAGAGGAGAGA</td>
</tr>
<tr>
<td>Lenti_VHL_4_R</td>
<td>AAACCTCAACATTAGAGATGGCACC</td>
</tr>
<tr>
<td>Lenti_VHL_5_F</td>
<td>CACCGTGCGTCAACATTAGAGAAGA</td>
</tr>
<tr>
<td>Lenti_VHL_5_R</td>
<td>AAACCTCCTCAATGTTGACGGACAC</td>
</tr>
<tr>
<td>Lenti_DUMMY_1_F</td>
<td>CACCGACTTCCCACCTTTAGGTTG</td>
</tr>
<tr>
<td>Lenti_DUMMY_1_R</td>
<td>AAACCAACCTAAGAAGTGGAAGTC</td>
</tr>
<tr>
<td>Lenti_DUMMY_2_F</td>
<td>CACCGTGAGGCGACGCGAAAAGAA</td>
</tr>
</tbody>
</table>
The lentiCRISPRv1 plasmid was digested with BsmBI, gel-purified and phosphorylated by T4 PNK. Above oligonucleotides were separately annealed for each sgRNA construct. The annealed product was ligated into the lentiCRISPRv1 plasmid and transformed into Stbl3 competent cells.

3. Growth Curves

All growth curves were performed in either K562, HT29 or HEK293T cells (ATCC). All conditions were counted in duplicate wells for each growth curve time point. Each growth curve was performed in independent experimental set-ups a minimum of two times in each cell type. Representative growth curves are shown in Fig 2.

a. Lentivirus VHL Growth Curves

K562 cells were infected using the lentivirus CRISPR construct containing sgRNAs targeting VHL or dummy guides, following the same protocol as used in the original GeCKO screen. Puromycin selection was started the day following the infection. Cells were allowed to grow in Puromycin-selection conditions for at least one week, before beginning growth curves. The VHL-edited cells and dummy-guide infected cells were grown at a starting concentration of 2.5x10^4 or 5x10^4 cells/ml in 24-well plates. Cells were placed in DMEM media (Life Technologies 11995, 10% FBS, Pen/Strep) with (a) DMSO, (b) 100nM antimycin (Sigma), (c) 100nM oligomycin (Sigma) or (d) 1μM piericidin (Santa Cruz). All conditions for growth curves contained 1mM pyruvate and 200μM uridine, reflecting the moderate disease condition. Cells for each condition were counted in duplicate wells every 24h for at least 3 days.

b. FG-4592 Growth Curves

FG-4592 was purchased from MedChem Express (HY-13426) and Cayman Chemicals (CAS 808118-40-3). Drug stocks were made in DMSO at
150 mM. Cells were pre-treated with 40-75 μM FG-4592 for at least 24h before beginning growth curves. This allowed for the HIF transcriptional response to begin prior to treatment with respiratory chain inhibitors. All cells were resuspended at a concentration of 2.5x10^4/ml in 24-well plates and placed in (a) DMSO, (b) 100 nM antimycin, (c) 100 nM oligomycin or (d) 1 μM piericidin. Cells were counted daily (HEK293T and K562) or every other day (HT-29). Cells in all conditions were counted in duplicate or triplicate wells. At least two independent growth curves were performed with independent cell stocks for each growth curve presented in Fig 2.

4. HIF1α Immunoblotting
Cells were exposed to DMSO, 100 nM antimycin or 100 nM oligomycin, ± 50 μM FG-4592, in 6-well plates. Plates were then placed in normoxic (21% O₂) or hypoxic (1% O₂) conditions for 12-18h. Air tanks corresponding to 1% O₂ (balance nitrogen) or 21% O₂ were purchased from Airgas. Cell culture plates were placed in hypoxic chambers (Billups-Rothenberg), sealed and flushed with air corresponding to different conditions (normoxia or hypoxia) for 7-10m. After overnight treatment, hypoxia chambers were opened and cells were collected using standard methods and placed in RIPA buffer (Boston BioProducts). As the normoxic half-life of HIF proteins is less than 5 minutes at room temperature, all samples and buffers were handled at 4°C. 30 μg of protein were loaded onto an 8% SDS-PAGE gel (Invitrogen). HIF1α was detected using Bethyl A300-286A and the loading control, β-tubulin was detected using CST 2128S.

5. qPCR
24h prior to RNA collection, 2.5x10^6 HEK293T or HT-29 cells were seeded per well in a 6-well plate in 3ml of DMEM (Life Technologies (11995), 10% FBS). Cells were incubated in 50 μM FG-4592, 100 nM antimycin, 1 μM piericidin as indicated. Total RNA was extracted using an RNeasy Mini Kit (Invitrogen). cDNA, generated by Superscript III Reverse Transcriptase (Life Technologies), was combined with Taqman Fast Advanced Master Mix and assayed by quantitative PCR. All CT values for genes of interest were normalized against the HPRT gene (probe/control). The ratio (probe/control) was set to 1 for vehicle-treated
cells. Oligonucleotide primer pairs for GLUT1, HK2, LDHA and PDK1 were purchased from Life Technologies.

6. Lactic Acid Measurements

Cells were placed in 6-well plates at a concentration of 1x10^5/ml and pre-treated with 50µM FG-4592 or DMSO for at least 24h. After pre-treatment, media was replaced so that it contained either (a) DMSO, (b) 100nM antimycin or (c) 100nM oligomycin ± 50 µM FG-4592. Cells were incubated in given conditions for 8h. Media was then collected, spun down to remove cell debris and subsequently used for lactic acid measurements. Lactic acid concentrations were measured using the YSI 2900 Biochemistry Analyzer. Samples were run in duplicate or triplicate for each set of conditions. Each set of conditions was tested in at least two independent experiments. A representative experimental set is shown in Fig 3.

7. Oxygen Consumption Measurements

48h prior to measurement, cells were seeded at 3-4x10^4 per well in XF24 24-well cell culture microplates in 200µl of normal growth media (DMEM (Life Technologies, 11965), 3.7g/L NaHCO3 and 10% FBS) and were incubated at 37°C in a 5% CO2 incubator. 12h after seeding, 800µL of normal growth medium containing either FG-4592 or DMSO was added to each well, resulting in final drug concentration of 50µM. 15m prior to input of cell plate in XF24 Extracellular Flux Analyzer, media was replaced with 850µL of assay medium -DMEM (US Biological, D9800), 25mM glucose, 1mM pyruvate, 15.9 mg/L phenol red, 10% FBS and 25mM HEPES-KOH, pH 7.4. Assay medium contained either 50µM FG-4592 or DMSO. Measurements were performed at consecutive intervals of mixing (2m), waiting (2m) and measurement (4m). Basal measurements were collected 4 times. OCR measurements normalized to cell number were averaged from three independent trials.

8. Zebrafish FG-4592 Treatment and Reporter Fish Imaging

Fish were raised and treated according to institutional protocols and regulations on an approved IACUC protocol, HMS 04626. Transgenic reporter embryos [Tg(phd3::EGFP)] and wild-type sibling controls were exposed to 2.5µM FG-4592 in 0.1% DMSO at 96hpf. Fluorescence was assessed in vivo using a Zeiss
Discovery V.8 fluorescent stereoscope equipped with an AxioCam MRc digital camera.

9. **Zebrafish Antimycin Survival Curves**
Wild-type zebrafish embryos (TU strain) were raised to 96hpf, and then exposed to 2.5nM Antimycin ± 2.5μM FG-4592. Embryonic death was assessed over the next 72 hours.

10. **VHL -/- Zebrafish Survival Curves**
*vlh+/−* were incrossed, and resultant embryos were raised to 48hpf (since *vlh−/−* show lethality at later time points) and then exposed to 2.5nM antimycin. Fish were then scored as *vlh−/−* or WT and heterozygotes. Embryonic death was assessed over the next 24h.

11. **VHL in-situ hybridizations**
In situ hybridization for *glut1* and *ldha1a* were performed on zebrafish embryos fixed in 4% paraformaldehyde at 120hpf, after 24h of treatment with 2.5μM FG-4592. In situ hybridization was conducted using standard protocols [http://zfin.org/ZFIN/Methods/ThisseProtocol.html](http://zfin.org/ZFIN/Methods/ThisseProtocol.html).

12. **Breedings and General Animal Care for Mouse Work**
The Palmiter lab at University of Washington generously provided us with *Ndufs4* +/- mice. We then expanded our colony and bred sufficient *Ndufs4* +/- and control (*Ndufs4* +/- and WT) mice for experiments included in this manuscript. Pups were weaned and genotyped at ~25d after birth. All cages were provided with food and gel, as well as water bottles. Food and gel were replaced three times a week and cages were changed once a week. Mice were humanely euthanized if they lost more than 20% of peak body weight. *Ndufs4* +/- and WT mice are indistinguishable in all assays we tested and were therefore combined to serve as control samples. All animals were cared for under the guidelines of Partners Healthcare. All the animal studies were approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital, Boston, MA.

13. **Chronic Hypoxic, Normoxic and Mild-Hyperoxic Exposures**
Wild type and *Ndufs4* KO mice were exposed to chronic hypoxia (11% O₂), normoxia (21% O₂) or mild hyperoxia (55% O₂) at ambient sea-level pressure. Mice
were placed in 60 liter plexiglass chambers that were given a gas mixture of compressed air and 100% N₂, compressed air alone or compressed air and 100% O₂ (Airgas Inc.). The gas flow rates were measured and controlled with rotameters and valves. Oxygen concentrations were measured several times each day at the outlet of the chambers using an oxygen analyzer (MiniOx I Oxygen Analyzer, Ohio Medical Corporation) and the flow rates of air, nitrogen and oxygen were modified if necessary in order to obtain a stable oxygen concentration of 11% in the hypoxic chamber and 55% in the mild-hyperoxia chamber. Soda lime (Sodasorb, Smiths Medical) (approximately 250g), was placed on the floor of each chamber to scavenge carbon dioxide (CO₂) produced by the animals and replaced every 3 days. The CO₂ concentration in each chamber as well as the temperature and the humidity were monitored continuously using a dedicated infrared CO₂ analyzer, thermometer and humidity meter (Extech CO200 Monitor, Extech Instruments). The total flow of fresh gas flushing each chamber was adjusted between 5 and 10 L/min to maintain the chamber CO₂ level below 0.4% and the relative humidity between 30% and 70%. Mice were exposed to gas treatment continuously for 24 hours per day, 7 days a week. The chambers were briefly opened three times a week to weigh the mice, evaluate their neurological status, clean the cages and add water and food.

Due to the extremely long duration of the experiment, several accidental deaths were observed in the different conditions due to severe infection (retinal conjunctivitis), blunt trauma and accidental CO₂ accumulation. As each of these were isolated incidents and unrelated to the experimental question, this data was not incorporated into the manuscript.

14. Blood and Tissue Collection
Mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine (120 mg kg⁻¹) and fentanyl (0.09 mg kg⁻¹). Following tracheostomy, rocuronium (1 mg kg⁻¹) was injected i.p. to induce muscle relaxation. Volume-controlled ventilation was provided at a respiratory rate of 90 breaths min⁻¹, a tidal volume of 10 ml kg⁻¹, PEEP 1 cmH₂O and inspired O₂ fraction (FI0₂) of 0.21 (Mini Vent 845; Harvard Apparatus). Blood was collected by cardiac puncture after opening the chest via
median sternotomy and tissues were collected and immediately frozen in liquid nitrogen.

15. **Rotarod Activity Measurements**

A rotarod machine (Ugo Basile) was used to measure the ability of mice to stay on an accelerating, rotating rod. Rotarod parameters were as follows: acceleration of 5rpm/m and a maximum speed of 40rpm. On each measurement day, three trials were performed, with individual trials at least 10m apart to allow mice to recuperate. The median time on rotarod is reported. If mice used their body to grasp the rod (rather than walking on it) for more than 10s, this time was recorded as time of fall. Age of measurements is +/- 5 days for practical purposes without any age bias between groups.

16. **Spontaneous Locomotor Activity Measurements**

The open field instrument (Med Associates Inc.) was used to measure spontaneous locomotor activity. Mice were blindly chosen for a given day and placed in open field chambers for 1h. Spontaneous locomotor activity was measured based on beam breaks and recorded by the instrument. The traces shown in Fig. 5E are representative of a sick, Ndufs4 -/- mouse exposed to 21% O₂ (age of such sickness varies slightly between mice) and age-matched KO and WT mice exposed to 11% O₂, and WT mice exposed to 21% O₂. Age of measurements is +/- 5 days for practical purposes without any age bias between groups.

17. **Histology**

Mice were anesthetized as during tissue collection (see above). The chest cavity was opened and a catheter was placed in the left ventricle. The whole body was perfused with ice cold PBS and then with 4% PFA. The brain was dissected out, stored overnight in 4% PFA and then placed in 30% sucrose (in PBS) for two days. Formalin-perfused brains were sectioned parasagittally at the olfactory lobes. Two transverse sections of cerebellum and brainstem were also collected: a rostral section with subjacent pons, and a more caudal section with medulla oblongata. Immunohistochemistry was performed on adjacent tissue sections using an
antibody recognizing the microglial marker Iba-1 (Wako; 2μg/ml) according to methods described elsewhere (40).

18. Complex I Activity Assay

Complex I Activity was measured in cerebellum tissue from mice. Tissue was homogenized in 1ml of ice-cold PBS using a Qiagen TissueLyser II. Approximately 50-100mg of tissue was used as input material for the Complex I Enzyme Activity Microplate Assay Kit (ab109721, Abcam). Absorbance was read at 450nm wavelength and recorded every 30s for 135 total measurements. Background signal was not subtracted in data shown.

19. Erythropoietin (Epo) Measurements

Mice were exposed to 21% O₂ or 8.5% O₂ for 6h. Blood was collected as described above in EDTA-containing tubes. Plasma was then used for detection of Epo using the Mouse Erythropoietin Quantikine ELISA Kit (EP00B, R&D Systems).

20. Plasma Metabolomics

Lactate and α-hydroxybutyrate were quantified in mouse plasma by spiking in each corresponding isotope labeled standard (CDN isotope). A series of standard solutions of metabolites at 7 different concentrations were prepared in a surrogate matrix buffer (PBS buffer with 30g/L human serum albumin). 30μL of the mouse plasma sample were combined with 20μL of isotope labeled internal standard, vortexed for 10s and spun down for 10s. Metabolite extractions were performed using 70% acetonitrile. A Q Exactive Plus Orbitrap Mass Spectrometer coupled to a Dionex UltiMate 3000 UHPLC system (Thermo Fisher Scientific) was used for LC-MS. The Xbridge amide HILIC column (2.1 X100 mm, 2.5 μM particle size, from Waters 186006091) was used to separate metabolites and MS was acquired under the negative ionization mode. The column was maintained at 27°C during runs. The mobile phase A was 20mM ammonium acetate, 0.25 % ammonium hydroxide pH adjusted to 9. The mobile phase B was 100% acetonitrile. The MS data acquisition was full scan mode in a range of 70–1000 m/z, with the resolution set at 140,000, the AGC target at 3E6, and the maximum injection time at 400 msec.
Figure S1. Essential genes and gene categories are depleted in screen over time. Gene set enrichment analysis (GSEA) was performed using the depletion of sgRNAs over time in untreated conditions (untreated relative to early (pre-treatment)). Shown are GSEA plots for the curated gene sets that were the most depleted over three weeks of growth. Each plot reflects a single gene category. Black lines reflect ranks of individual genes within gene set. Most depleted gene sets correspond to essential cell functions.
Figure S2. sgRNA enrichment vs. rank for all experimental conditions and infection replicates, relative to pre-treatment time point. sgRNA enrichment magnitude vs. sgRNA enrichment rank for each of ~65,000 sgRNAs included in the screen. sgRNAs corresponding to VHL are shown in red. Data is shown for both infection replicates for untreated vs. early conditions (top row), moderate disease vs. early conditions (middle row), and severe disease vs. early conditions (bottom row).
Figure S3. sgRNA abundance for all experimental conditions and infection replicates, relative to pre-treatment time point. sgRNA abundance in pre-treatment conditions (Infection 1 vs. Infection 2) shown in grey, reflecting experimental noise. Guide abundance in a given condition vs. early time point is shown in black, with VHL sgRNAs shown in red. Each dot represents an individual sgRNA. Data is shown for both infection replicates for untreated vs. early conditions (top row), moderate disease vs. early conditions (middle row), and severe disease vs. early conditions (bottom row).
Figure S4. Abundance of sgRNAs targeting VHL across all samples. sgRNA abundance is shown for each of five, VHL sgRNAs. Data is shown for each individual infection replicate for the early time point, untreated conditions, moderate disease conditions and severe disease conditions. All sgRNA abundance values were normalized to total number of sequencing reads for a given condition.

E = Early
U = Untreated
M = Moderate Disease
S = Severe Disease
* Data shown for both infection replicates
Figure S5. Rescue size of VHL sgRNAs, in presence of RC inhibition, is correlated with relative enrichment in screen. Growth curves for lentiviral VHL sgRNAs and dummy sgRNAs in untreated (left) or antimycin (right) conditions. sgRNAs are colored by their relative enrichment in the CRISPR screen. The strongest guides are shown in darker blue, and weaker guides are in light blue. Dummy guides are shown in black. All experiments were performed in duplicate wells. Growth curves are representative of at least two independent experiments.
Figure S6. FG-4592 causes a dose-dependent rescue of the growth defect caused by multiple forms of respiratory chain inhibition. Growth curves are shown for increasing concentrations of FG-4592 in untreated or RC inhibition conditions in HEK293T cells. Untreated growth curve is shown in black. Growth with RC inhibition, but without FG-4592 is shown in red. Increasing doses of FG-4592 shown in blue, reflecting increasing rescue during respiratory chain inhibition.
Figure S7. FG-4592 treatment activates transcription of HIF-responsive genes, whereas RC inhibition alone does not. Normalized transcript levels for the known HIF targets glucose transporter 1 (GLUT1), hexokinase 2 (HK2), lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1) +/- RC inhibition, +/- FG-4592. All data is averaged over three independent experiments (Mean ± S.E.) and normalized so vehicle-treated expression (probe/control) is set to 1. Data is shown for two additional cell types (K562 and HEK293T cells).
Condition | Mean      | STE
--- | --- | ---
WT (21% O₂) | 56 pg/ml | +/- 17 pg/ml
WT (11% O₂) | 2775 pg/ml | +/- 1070 pg/ml
KO (21% O₂) | 77 pg/ml | +/- 37 pg/ml
KO (11% O₂) | 2788 pg/ml | +/- 324 pg/ml

Figure S8. WT and Ndufs4 KO mice are able to increase Erythropoietin levels after acute hypoxia exposure. 3 mice per group were exposed to 8.5% or 21% O₂ for 6h. Plasma erythropoietin levels were measured (Mean ± S.E.)
Figure S9. Spontaneous activity was measured in WT and Ndufs4 mice exposed to normoxia or hypoxia. Distance travelled and jump counts within 1h are shown (Mean ± S.E.). n = 7, 5, 9, 9 for WT (21% O₂), WT (11% O₂), KO (21% O₂), KO (11% O₂) respectively.