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Inhibition of ATPIF1 Ameliorates Severe Mitochondrial Respiratory Chain Dysfunction in Mammalian Cells

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SUMMARY
Mitochondrial respiratory chain disorders are characterized by loss of electron transport chain (ETC) activity. Although the causes of many such diseases are known, there is a lack of effective therapies. To identify genes that confer resistance to severe ETC dysfunction when inactivated, we performed a genome-wide genetic screen in haploid human cells with the mitochondrial complex III inhibitor antimycin. This screen revealed that loss of ATPIF1 strongly protects against antimycin-induced ETC dysfunction and cell death by allowing for the maintenance of mitochondrial membrane potential. ATPIF1 loss protects against other forms of ETC dysfunction and is even essential for the viability of human /C14 cells lacking mitochondrial DNA, a system commonly used for studying ETC dysfunction. Importantly, inhibition of ATPIF1 ameliorates complex III blockade in primary hepatocytes, a cell type afflicted in severe mitochondrial disease. Altogether, these results suggest that inhibition of ATPIF1 can ameliorate severe ETC dysfunction in mitochondrial pathology.

INTRODUCTION
Defects in the activity of the electron transport chain (ETC) are the causative pathology in a diverse family of genetic diseases known as mitochondrial respiratory chain disorders. Patients with these diseases can often present with abnormalities in multiple organ systems (Pfeffer et al., 2012; DiMauro and Schon, 2003; Spinazzola et al., 2006). Although some mitochondrial respiratory chain disorders cause relatively mild abnormalities, such as exercise intolerance, there are severe forms of respiratory chain disorders that can lead to life-threatening loss of tissue parenchyma and organ failure (Morris, 1999; Lee and Sokol, 2007; DiMauro and Schon, 2003; Spinazzola et al., 2006). Yet, despite an extensive characterization of the mechanisms underlying these diseases, there is a paucity of effective therapies to ameliorate severe respiratory chain dysfunction. Indeed, most efforts to date, such as dietary supplementation with small molecules and vitamins that can increase ETC activity or decrease reactive oxygen species, have not demonstrated any clear efficacy across clinical trials, thus underscoring the need for novel therapeutic strategies (Pfeffer et al., 2012; Schon et al., 2010).

Genetic and chemical screens in mammalian cells have previously identified modulators of mitochondrial dynamics (Lefebvre et al., 2013; Kitami et al., 2012; Gohil et al., 2010; Yoon et al., 2010), but genetic screens have not identified gene products in mammalian cells that, when inactivated, increase survival under ETC dysfunction. Beginning with a positive selection screen in human cells using the mitochondrial complex III inhibitor antimycin, we find that loss of ATPIF1 is protective against complex III blockade, as well as a multitude of other insults to the ETC, leading us to propose inhibition of ATPIF1 as a strategy for ameliorating severe mitochondrial respiratory chain disorders.

RESULTS AND DISCUSSION
To identify genes whose products modulate sensitivity to ETC inhibition, we performed a genome-wide, insertion mutagenesis screen in the near-haploid KBM7 human cell line with antimycin, a complex III inhibitor of the ETC. This technology has been used successfully in the past to identify numerous proteins that are essential for the cytotoxicity of microbial factors (Carete et al., 2011b; Guimaraes et al., 2011), as well as transporters for toxic...
small molecules (Birsoy et al., 2013). In brief, we generated a library of mutagenized haploid KBM7 cells harboring approximately 70 million insertions that encompass more than 95% of all genes expressed in KBM7 cells (Carette et al., 2011a). Mutagenized cells were then treated with antimycin for 3 weeks and the surviving cells were expanded and pooled. Insertions in the surviving population were mapped to the human genome using massively parallel sequencing. To identify genomic loci enriched for gene-trap insertions, we performed a proximity index analysis and identified several candidate genes: ATPIF1 (p = 3.04 × 10^{-11}), WT1 (p = 1.78 × 10^{-43}), and TP53 (p = 6.93 × 10^{-8}; Figure 1A). Because both WT1 and TP53 are tumor suppressors (Sher, 2004) and therefore would be less attractive therapeutic targets, we focused our attention on ATPIF1. ATPIF1 is a highly conserved mitochondrial protein that inhibits the ATPase activity of the F1-F0 ATP synthase and has been found to affect a variety of metabolic parameters, such as aerobic glycolysis (Sánchez-Cenizo et al., 2010), ATP synthase dimerization (García et al., 2006), and mitochondrial cristae density (Campanella et al., 2008, 2009).
To investigate the role of ATPIF1 loss in protecting cells against complex III blockade, we isolated a KBM7 clone harboring a gene-trap insertion of ATPIF1 (ATPIF1_KO) and confirmed that it did not express detectable amounts of ATPIF1 protein (Figures 1B and 1C). Consistent with the results of our screen, ATPIF1_KO cells were substantially more resistant to antimycin-induced cell death than their wild-type (WT) counterparts (Figure 1D). Additionally, re-expression of ATPIF1 in ATPIF1_KO cells almost completely restored their sensitivity to antimycin (Figure 1E). As an independent confirmation of our findings, WT KBM7 cells expressing small hairpin RNAs (shRNAs) targeting ATPIF1 also exhibited increased resistance to antimycin (Figure S1).

To probe the mechanism by which ATPIF1 loss can confer resistance to complex III inhibition, we examined the effects of antimycin on the metabolism and mitochondrial function of WT and ATPIF1_KO KBM7 cells. Upon inhibition of the ETC, the mitochondrial membrane potential (Δψm) decreases and the F1-F0 ATP synthase reverses, consuming ATP to pump protons into the intermembrane space (Campanella et al., 2008, 2009; Lefebvre et al., 2013; Lu et al., 2001). Normally an inactive tetramer, ATPIF1 dissociates into active dimers upon a large decrease in Δψm, and subsequently inhibits reversal of the F1-F0 ATP synthase, an adaptive mechanism to prevent ATP consumption during periods of nutrient and oxygen deprivation (Cabezon et al., 2001; Fujikawa et al., 2012; Lu et al., 2001; Campanella et al., 2008, 2009). In short-term experiments, decreased ATPIF1 activity during ETC dysfunction allows for maintenance of Δψm at the expense of ATP via reversal of the F1-F0 ATP synthase, but it is unclear whether maintenance of Δψm or conservation of ATP is the most important process for survival under ETC dysfunction (Campanella et al., 2008, 2009; Lefebvre et al., 2013). Consistent with the F1-F0 ATP synthase operating in reverse, we observed that ATPIF1 KO cells had decreased ATP but increased Δψm upon antimycin treatment as compared with WT KBM7 cells (Figure 1F). Metabolite profiling of ATPIF1_KO cells under antimycin treatment also revealed a greater depletion of glycolytic intermediates, in agreement with the increased ATP demand under conditions of ETC inhibition (Figure S2; Table S1). Importantly, the differences seen in ATP, Δψm, and overall survival under antimycin could be eliminated by cotreatment with oligomycin, a potent inhibitor of the F1-F0 ATP synthase (Figures 1F and 1G). It is unlikely that the effects of oligomycin on antimycin-treated cells were a result of additive toxicity, because oligomycin itself had no effect on the viability of either WT KBM7 or ATPIF1_KO cells (Figure 1G). Of note, the addition of oligomycin to antimycin-treated ATPIF1_KO cells decreased Δψm and increased ATP levels, but led to decreased survival, suggesting that maintenance of Δψm is more important than preservation of ATP for ameliorating complex III blockade in KBM7 cells. To rule out any effects of ATPIF1 loss on general mitochondrial metabolism and cellular physiology, we also examined the mitochondrial mass, mtDNA copy number, mitochondrial ultrastructure, and resting Δψm, ATP, viability, and oxygen consumption of WT and ATPIF1_KO KBM7 cells, but found no significant differences (Campanella et al., 2008; Figure S3).

Collectively, these data demonstrate that ATPIF1 loss confers resistance to complex III blockade through maintenance of Δψm via reversal of the F1-F0 ATP synthase.

We next sought to determine whether the effects of ATPIF1 loss on KBM7 cells were generalizable to other cell lines and additional forms of ETC dysfunction. Consistent with the results in KBM7 cells, SH-SYSY and HeLa cells expressing an shRNA targeting ATPIF1 were more resistant to antimycin than cells expressing a control hairpin (Figure 2A). In addition, we found that overexpression of ATPIF1 in Malme-3M, a cell line with low endogenous levels of ATPIF1, increased their sensitivity to antimycin (Figure 2B). To investigate whether the protective effect of ATPIF1 loss was limited to only complex III inhibition, we tested a variety of pharmacological and genetic models of ETC dysfunction. ATPIF1_KO KBM7 cells were substantially more resistant to both piericidin, an inhibitor of complex I (Darrouzet et al., 1998), and tigecycline, an inhibitor of mitochondrial translation (Skrtic et al., 2011), when compared with their WT counterparts (Figure 2C). Taken together, these data demonstrate that the levels of ATPIF1 can modulate sensitivity to different forms of ETC dysfunction in various human cell lines.

The observation that ATPIF1_KO KBM7 cells were more resistant to inhibition of complex I, complex III, and mitochondrial protein synthesis raised the possibility that ATPIF1 loss could ameliorate the effects of dysfunction in multiple components of the ETC. To test this genetically, we examined p38+ cells, which are devoid of any mtDNA and consequently have defects in complexes I, III, and IV, resulting in undetectable ETC activity (Jazayeri et al., 2003). To our surprise, we found that HeLa p38+ cells intrinsically possess lower mRNA and protein levels of ATPIF1 compared with their WT counterparts (Figure 2D). Previous work has shown that p38+ cells maintain Δψm by using the electrogenic exchange of ATP and ADP, coupled to ATP hydrolysis by an F1-F0 ATP synthase that is defective in pumping protons, and that this activity is important for cellular health (Buchet and Godinot, 1998; Appleby et al., 1999). We therefore hypothesized that there could be a strong selective pressure to decrease ATPIF1 levels under severe ETC dysfunction in order to facilitate reversal of the F1-F0 ATP synthase. A reduction of ATPIF1 in 143b p38+ cells was observed recently, although the functional significance of this reduction on cell viability was not investigated (Lefebvre et al., 2013). To address this, we overexpressed WT ATPIF1 or a mutant ATPIF1 harboring an E55A substitution that renders the protein unable to interact with the F1-F0 ATP synthase (Ichikawa et al., 2001). Overexpression of WT ATPIF1, but not E55A ATPIF1, strongly impaired proliferation in HeLa p38+ cells, but not in HeLa WT cells (Figure 2E). The differences observed between WT and E55A ATPIF1 were not simply a result of E55A ATPIF1 protein instability, because both variants of ATPIF1 were overexpressed to a similar degree, as seen in the immunoblots of HeLa WT cells (Figure 2E). Intriguingly, at the time of collection, we found that the surviving HeLa p38+ cells infected with virus expressing WT ATPIF1 had lower amounts of ATPIF1 than those infected with virus expressing E55A ATPIF1, which is consistent with a selection against ATPIF1 activity on the F1-F0 ATP synthase in the p38+ state (Figure 2E). Collectively, these data demonstrate that reduced ATPIF1 activity is essential for the viability of human p38+ cells lacking a mitochondrial genome.

Failure to maintain proper amounts of the mitochondrial genome is a distinctive feature of a class of severe respiratory chain disorders known as mtDNA depletion syndromes (Lee...
Because of our interest in ATPIF1 inhibition as a potential strategy for ameliorating severe ETC dysfunction, we asked whether loss of ATPIF1 alone was sufficient to improve cell viability during progressive mtDNA depletion. For this purpose, we cultured WT and ATPIF1_KO KBM7 cells with 2',3'-dideoxyinosine (ddI), an inhibitor of mtDNA replication (Lewis et al., 2003; Walker et al., 2002), for approximately 51 days and monitored cellular behavior at defined time points (Figure 3A; see Experimental Procedures for the exact time points at which phenotypes were assayed). ddI led to an immediate decrease in mtDNA copy number in the initial days of treatment concomitantly with a decrease in cell proliferation that was roughly equivalent between WT and ATPIF1_KO KBM7 cells (Figure 3B). It was previously observed that this amount of mtDNA depletion still allows for residual ETC function (Jazayeri et al., 2003), so it is unlikely that ATPIF1 was maximally activated in the WT KBM7 cells at this point. mtDNA was progressively depleted with each successive week of ddI treatment, and by day 25, both WT and ATPIF1_KO KBM7 cells had trace amounts of mtDNA (Figure 3C). Although both WT and ATPIF1_KO KBM7 cells proliferated more slowly than their untreated counterparts, ATPIF1_KO cells demonstrated a significantly faster rate of proliferation than WT KBM7 cells, consistent with loss of ATPIF1 improving cell viability under conditions of severe ETC dysfunction (Figure 3C). Taken together, these data demonstrate that loss of ATPIF1 is sufficient to improve cell viability during progressive mtDNA depletion.

Given that low ATPIF1 levels were necessary for viability in HeLa p<sub>e</sub> cells, we hypothesized that WT p<sub>e</sub> KBM7 cells would express low amounts of ATPIF1 as well. In accordance with this, we observed a gradual decrease in ATPIF1 over 50 days of ddI treatment, with WT KBM7 cells exhibiting substantially reduced amounts of ATPIF1 and undetectable quantities of mtDNA (i.e., p<sub>e</sub> state) at the end of the time course (Figures 3B–3D). Furthermore, WT p<sub>e</sub> KBM7 cells with reduced ATPIF1 expression proliferated to a similar extent as ATPIF1_KO p<sub>e</sub> KBM7 cells, suggesting that complete loss of ATPIF1 activity had no additional benefit for cell proliferation in the terminal p<sub>e</sub> state (Figure 3D).

Because severe forms of mitochondrial respiratory chain disorders can lead to cell death and loss of tissue parenchyma in organs such as the liver (Morris, 1999; Lee and Sokol, 2007), we transitioned to a more physiological context and asked whether loss of ATPIF1 in hepatocytes could ameliorate ETC dysfunction and improve cell viability. WT and ATPIF1<sup>−/−</sup> mice were obtained...
from the International Knockout Mouse Consortium (Brown and Moore, 2012; Figures S4A and S4B), and primary hepatocytes isolated from ATPIF1−/− mice had undetectable amounts of ATPIF1 (Figure 4A). Consistent with the results obtained in cell lines, antimycin treatment led to a greater decrease in cellular ATP (Figure 4B) and a greater increase in δΨm (Figure 4C) in ATPIF1−/− hepatocytes than in WT hepatocytes, indicating that there was greater reversal of the F1-F0 ATP synthase in ATPIF1−/− hepatocytes. Importantly, ATPIF1−/− hepatocytes had increased cell viability relative to WT hepatocytes following treatment with antimycin (Figure 4D). This demonstrates that the beneficial effects of ATPIF1 loss under severe ETC dysfunction are not limited to rapidly proliferating cancer cell lines and can also occur in postmitotic, differentiated cells that better recapitulate the metabolism of tissues affected in severe mitochondrial respiratory chain disorders (Vander Heiden et al., 2009). The smaller effects of ATPIF1 loss on hepatocyte viability during antimycin treatment, as compared with KBM7 cells, are partially due to the fritality of primary mouse hepatocytes when cultured ex vivo (Edwards et al., 2013; Klaunig et al., 1981). Taken together, these data demonstrate that ATPIF1 loss in primary hepatocytes can ameliorate the effects of complex III blockade.

Our results suggest that ATPIF1 inhibition can be a strategy for ameliorating severe ETC dysfunction in mitochondrial respiratory chain disorders. Impaired ETC function in the presence of normal ATPIF1 activity leads to a persistent loss of δΨm, which hinders mitochondrial import of proteins (Neupert, 1997) and eventually promotes apoptosis (Gottlieb et al., 2003). However, upon ATPIF1 inhibition, increased reversal of the F1-F0 ATP synthase can bolster δΨm and improve mitochondrial and cellular health (Figure 4E), although it remains to be seen whether ATPIF1 loss is beneficial to all cell types in vivo since the ratio of ATPIF1 to F1-F0 ATP synthase expression, the amount of glycolysis, and the consumption of ATP can vary substantially among different tissues. Regardless, because several mitochondrial respiratory chain disorders, such as Alpers-Huttenlocher syndrome and Pearson’s syndrome, lead to progressive liver failure (Lee and Sokol, 2007), our findings in primary hepatocytes at least suggest that hepatic delivery of RNAi constructs targeting ATPIF1 either via adeno-associated virus or lipid nanoparticles, both of which have shown clinical efficacy in gene therapy of the liver, may have therapeutic value (Nathwani et al., 2011; Fitzgerald et al., 2014). Notably, ATPIF1−/− mice appear phenotypically normal and their hepatocytes exhibit no significant alterations in ATP synthase activity or mitochondrial structure (Nakamura et al., 2013). In agreement with these findings, we did not observe any significant differences in the mitochondrial mass of WT and ATPIF1−/− primary hepatocytes (Figure S4C). Altogether, these data suggest that ATPIF1 inhibition is relatively well tolerated.
In conclusion, we have used a positive selection screening method in human cells to identify loss of ATPF1 as protective against complex III blockade. We have further shown that ATPF1 inhibition protects different cell types against numerous insults to the ETC. In particular, our work demonstrates that loss of ATPF1 activity is essential for the viability of human ρ0 cells, a widely used system to study mitochondrial dysfunction, and that inhibition of ATPF1 can ameliorate the effects of complex III blockade in primary hepatocytes, a cell type that is often affected in severe respiratory chain disorders. Given the lack of therapies for severe mitochondrial respiratory chain disorders, we thus believe that inhibition of ATPF1 is a promising approach that warrants further investigation.
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