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Inhibition of ATP1F1 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 ATP1F1 strongly protects against antimycin-induced ETC dysfunction and cell death by allowing for the maintenance of mitochondrial membrane potential. ATP1F1 loss protects against other forms of ETC dysfunction and is even essential for the viability of human ρ^0 cells lacking mitochondrial DNA, a system commonly used for studying ETC dysfunction. Importantly, inhibition of ATP1F1 ameliorates complex III blockade in primary hepatocytes, a cell type afflicted in severe mitochondrial disease. Altogether, these results suggest that inhibition of ATP1F1 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 res-

piratory 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 ATP1F1 is protective against complex III blockade, as well as a multitude of other insults to the ETC, leading us to propose inhibition of ATP1F1 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, insertional 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 (Carette et al., 2011b; Guimaraes et al., 2011), as well as transporters for toxic

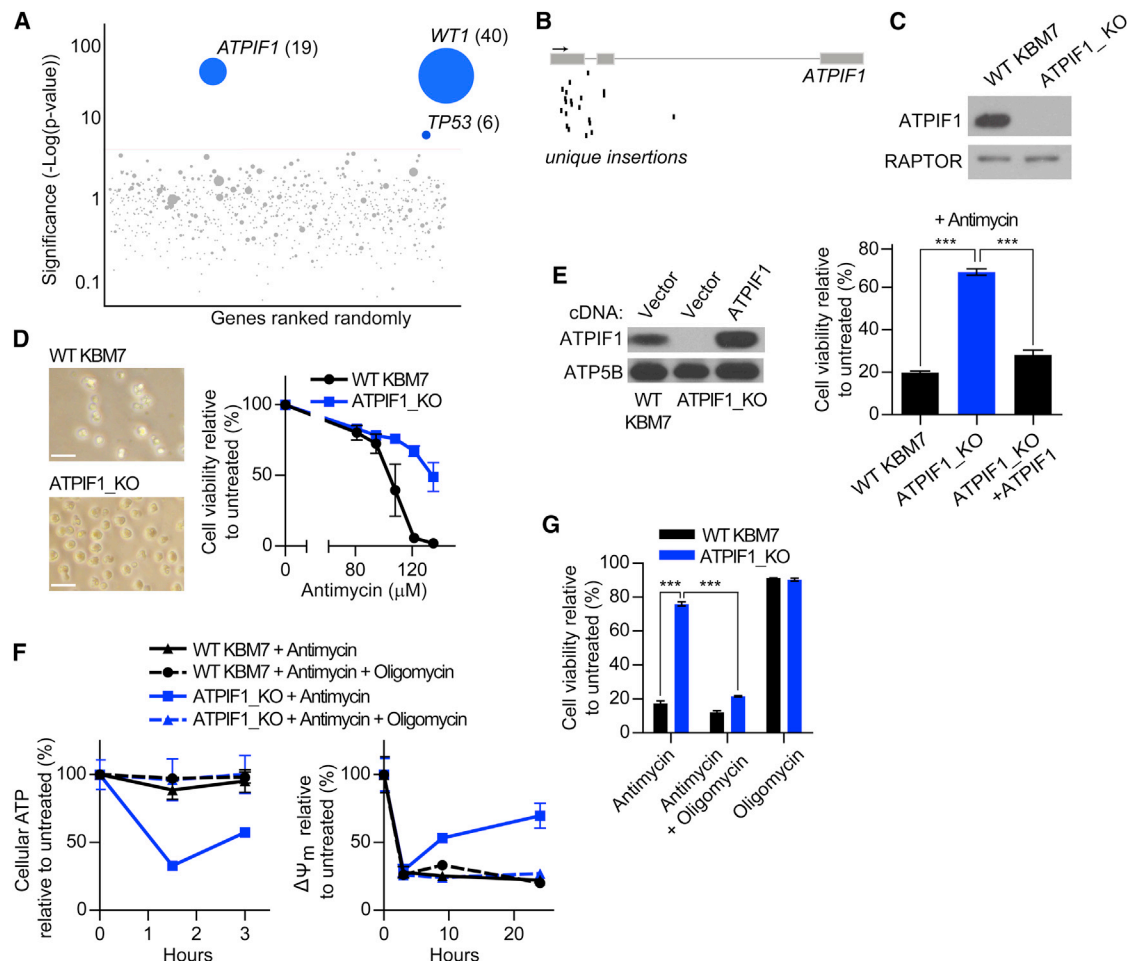


Figure 1. Haploid Genetic Screen Identifies Loss of *ATPIF1* as Protective against Complex III Inhibition

(A) Mutagenized KBM7 cells were treated with antimycin and resistant cells were pooled. Gene-trap insertions were identified by massively parallel sequencing and mapped to the human genome. The y axis represents the statistical significance of a given gene, and the x axis represents the collection of genes with insertions. The red line indicates the cutoff of statistical significance chosen to determine whether a gene scored as a hit in the screen. For *ATPIF1*, *WT1*, and *TP53*, the number of unique insertions per gene is given in parentheses.

(B) Map of unique insertions in *ATPIF1* in the resistant cell population. The arrow denotes 5'-3' directionality, boxes represent exons, and black bars indicate insertions.

(C) Immunoblots for the indicated proteins in WT and *ATPIF1_KO* KBM7 cells.

(D) Micrographs (left) and viability (right) of WT and *ATPIF1_KO* KBM7 cells treated with antimycin for 4 days. Error bars are \pm SEM (n = 3). Scale bars, 20 μ m.

(E) Immunoblots for the indicated proteins in WT, *ATPIF1_KO*, and *ATPIF1_KO* KBM7 cells with restored *ATPIF1* expression (left) and viability of cells treated with antimycin (135 μ M) for 2 days (right). Error bars are \pm SEM (n = 3). ***p < 0.001.

(F) Cellular ATP (left) and $\Delta\Psi_m$ (right) in WT and *ATPIF1_KO* KBM7 cells treated with antimycin (135 μ M) and oligomycin (1 μ M). Error bars are \pm SEM (n = 3).

(G) Viability of WT and *ATPIF1_KO* KBM7 cells treated with antimycin (135 μ M) and oligomycin (1 μ M) for 2 days. Error bars are \pm SEM (n = 3). ***p < 0.001.

See also Figures S1–S3 and Table S1.

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* ($p = 3.04 \times$

10^{-43}), *WT1* ($p = 1.78 \times 10^{-40}$), and *TP53* ($p = 6.93 \times 10^{-8}$; Figure 1A). Because both *WT1* and *TP53* are tumor suppressors (Sherr, 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 ATP1F1 loss in protecting cells against complex III blockade, we isolated a KBM7 clone harboring a gene-trap insertion of *ATP1F1* (ATP1F1_KO) and confirmed that it did not express detectable amounts of ATP1F1 protein (Figures 1B and 1C). Consistent with the results of our screen, ATP1F1_KO cells were substantially more resistant to antimycin-induced cell death than their wild-type (WT) counterparts (Figure 1D). Additionally, re-expression of ATP1F1 in ATP1F1_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 ATP1F1 also exhibited increased resistance to antimycin (Figure S1).

To probe the mechanism by which ATP1F1 loss can confer resistance to complex III inhibition, we examined the effects of antimycin on the metabolism and mitochondrial function of WT and ATP1F1_KO KBM7 cells. Upon inhibition of the ETC, the mitochondrial membrane potential ($\Delta\Psi_m$) 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, ATP1F1 dissociates into active dimers upon a large decrease in $\Delta\Psi_m$ and subsequently inhibits reversal of the F1-F0 ATP synthase, an adaptive mechanism to prevent ATP consumption during periods of nutrient and oxygen deprivation (Cabezón et al., 2001; Fujikawa et al., 2012; Lu et al., 2001; Campanella et al., 2008, 2009). In short-term experiments, decreased ATP1F1 activity during ETC dysfunction allows for maintenance of $\Delta\Psi_m$ at the expense of ATP via reversal of the F1-F0 ATP synthase, but it is unclear whether maintenance of $\Delta\Psi_m$ 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 ATP1F1_KO cells had decreased ATP but increased $\Delta\Psi_m$ upon antimycin treatment as compared with WT KBM7 cells (Figure 1F). Metabolite profiling of ATP1F1_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, $\Delta\Psi_m$, 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 ATP1F1_KO cells (Figure 1G). Of note, the addition of oligomycin to antimycin-treated ATP1F1_KO cells decreased $\Delta\Psi_m$ and increased ATP levels, but led to decreased survival, suggesting that maintenance of $\Delta\Psi_m$ is more important than preservation of ATP for ameliorating complex III blockade in KBM7 cells. To rule out any effects of ATP1F1 loss on general mitochondrial metabolism and cellular physiology, we also examined the mitochondrial mass, mtDNA copy number, mitochondrial ultrastructure, and resting $\Delta\Psi_m$, ATP, viability, and oxygen consumption of WT and ATP1F1_KO KBM7 cells, but found no significant differences (Campanella et al., 2008; Figure S3). Collectively, these data demonstrate that ATP1F1 loss confers resistance to complex III blockade through maintenance of $\Delta\Psi_m$ via reversal of the F1-F0 ATP synthase.

We next sought to determine whether the effects of ATP1F1 loss on KBM7 cells were generalizable to other cell lines and additional forms of ETC dysfunction. Consistent with the results in KBM7 cells, SH-SY5Y and HeLa cells expressing an shRNA targeting ATP1F1 were more resistant to antimycin than cells expressing a control hairpin (Figure 2A). In addition, we found that overexpression of ATP1F1 in Malme-3M, a cell line with low endogenous levels of ATP1F1, increased their sensitivity to antimycin (Figure 2B). To investigate whether the protective effect of ATP1F1 loss was limited to only complex III inhibition, we tested a variety of pharmacological and genetic models of ETC dysfunction. ATP1F1_KO KBM7 cells were substantially more resistant to both piericidin, an inhibitor of complex I (Darrrouzet et al., 1998), and tigecycline, an inhibitor of mitochondrial translation (Skrtić et al., 2011), when compared with their WT counterparts (Figure 2C). Taken together, these data demonstrate that the levels of ATP1F1 can modulate sensitivity to different forms of ETC dysfunction in various human cell lines.

The observation that ATP1F1_KO KBM7 cells were more resistant to inhibition of complex I, complex III, and mitochondrial protein synthesis raised the possibility that ATP1F1 loss could ameliorate the effects of dysfunction in multiple components of the ETC. To test this genetically, we examined ρ^0 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 ρ^0 cells intrinsically possess lower mRNA and protein levels of ATP1F1 compared with their WT counterparts (Figure 2D). Previous work has shown that ρ^0 cells maintain $\Delta\Psi_m$ 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 ATP1F1 levels under severe ETC dysfunction in order to facilitate reversal of the F1-F0 ATP synthase. A reduction of ATP1F1 in 143b ρ^0 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 ATP1F1 or a mutant ATP1F1 harboring an E55A substitution that renders the protein unable to interact with the F1-F0 ATP synthase (Ichi-kawa et al., 2001). Overexpression of WT ATP1F1, but not E55A ATP1F1, strongly impaired proliferation in HeLa ρ^0 cells, but not in HeLa WT cells (Figure 2E). The differences observed between WT and E55A ATP1F1 were not simply a result of E55A ATP1F1 protein instability, because both variants of ATP1F1 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 ρ^0 cells infected with virus expressing WT ATP1F1 had lower amounts of ATP1F1 than those infected with virus expressing E55A ATP1F1, which is consistent with a selection against ATP1F1 activity on the F1-F0 ATP synthase in the ρ^0 state (Figure 2E). Collectively, these data demonstrate that reduced ATP1F1 activity is essential for the viability of human ρ^0 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

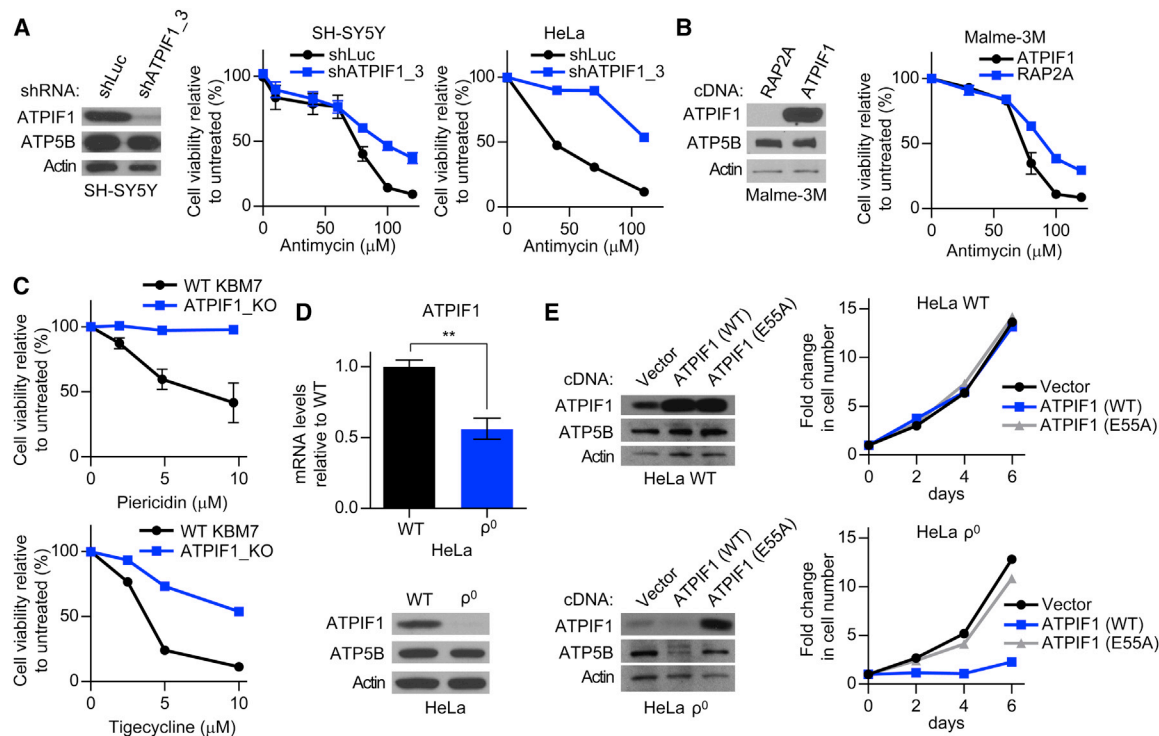


Figure 2. Loss of ATPIF1 Is Beneficial in Both Pharmacological and Genetic Models of ETC Dysfunction

(A) Left: immunoblots for the indicated proteins in SH-SY5Y cells expressing a control shRNA against Luciferase (shLuc) or an shRNA against ATPIF1 (shATPIF1_3). Middle and right: viability of SH-SY5Y (middle) and HeLa (right) cells treated with antimycin for 4 days. Error bars are \pm SEM ($n = 3$).

(B) Left: immunoblots for the indicated proteins in Malme-3M cells overexpressing control RAP2A or ATPIF1. Right: viability of Malme-3M cells treated with antimycin for 4 days. Error bars are \pm SEM ($n = 3$).

(C) Viability of WT and ATPIF1_KO KBM7 cells treated with piericidin (top) or tigecycline (bottom) for 4 days. Error bars are \pm SEM ($n = 3$).

(D) Relative ATPIF1 mRNA levels (top) and immunoblots for the indicated proteins (bottom) in HeLa WT and ρ^0 cells. Error bars are \pm SEM ($n = 3$). ** $p < 0.01$.

(E) Immunoblots (left) and relative proliferation (right) of HeLa WT and ρ^0 cells transduced with control vector, ATPIF1 (WT), or ATPIF1 (E55A) constructs. Error bars are \pm SEM ($n = 3$).

and Sokol, 2007). 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'-di-deoxyinosine (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 pro-

liferation 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 ρ^0 cells, we hypothesized that WT ρ^0 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., ρ^0 state) at the end of the time course (Figures 3B–3D). Furthermore, WT ρ^0 KBM7 cells with reduced ATPIF1 expression proliferated to a similar extent as ATPIF1_KO ρ^0 KBM7 cells, suggesting that complete loss of ATPIF1 activity had no additional benefit for cell proliferation in the terminal ρ^0 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^{-/-} mice were obtained

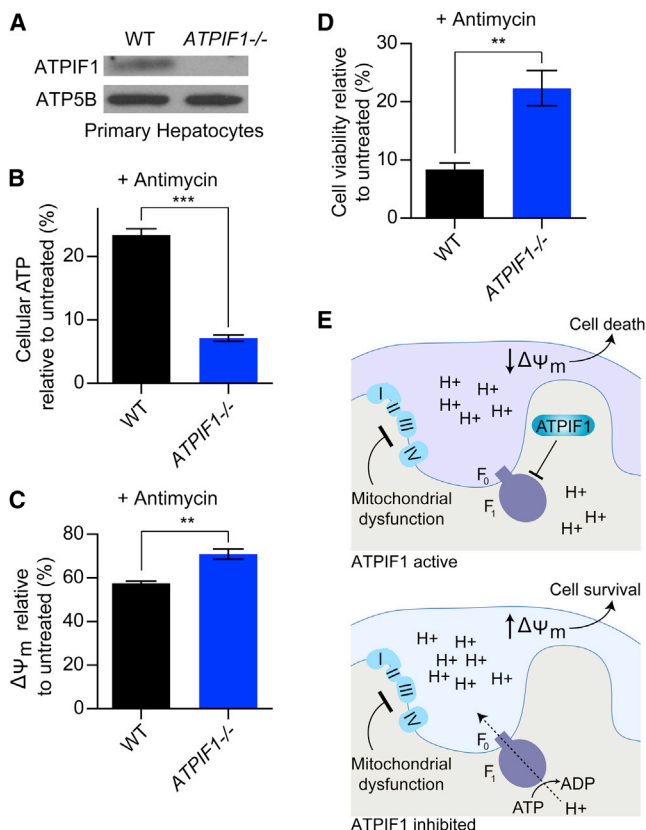


Figure 4. Inhibition of ATP1F1 Ameliorates the Effects of Complex III Blockade in Primary Hepatocytes

(A) Immunoblots for the indicated proteins of primary hepatocytes derived from WT and *ATP1F1*^{-/-} mice.
(B) Cellular ATP of WT and *ATP1F1*^{-/-} primary hepatocytes treated with antimycin (0.625 μ M) for 1.5 hr. Error bars are \pm SEM (n = 3). ***p < 0.001.
(C) $\Delta\Psi_m$ of WT and *ATP1F1*^{-/-} primary hepatocytes treated with antimycin (10 μ M) for 1.5 hr. Error bars are \pm SEM (n = 3). **p < 0.01.
(D) Viability of WT and *ATP1F1*^{-/-} primary hepatocytes treated with antimycin (1.25 μ M) for 2 days. Error bars are \pm SEM (n = 3). **p < 0.01.
(E) Schematic diagramming the behavior of cells with ETC dysfunction under conditions in which ATP1F1 is active or inhibited. Inhibition of ATP1F1 is depicted by absence of the protein, but represents any strategy to block ATP1F1 activity on the F₁-F₀ ATP synthase.
See also Figure S4.

In conclusion, we have used a positive selection screening method in human cells to identify loss of ATP1F1 as protective against complex III blockade. We have further shown that ATP1F1 inhibition protects different cell types against numerous insults to the ETC. In particular, our work demonstrates that loss of ATP1F1 activity is essential for the viability of human ρ^0 cells, a widely used system to study mitochondrial dysfunction, and that inhibition of ATP1F1 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 ATP1F1 is a promising approach that warrants further investigation.

EXPERIMENTAL PROCEDURES

Fluorescence-Activated Cell Sorting Assays

For fluorescence-activated cell sorting (FACS) assays, measurements of $\Delta\Psi_m$ were obtained by incubating 100,000 cells with tetramethylrhodamine methyl ester (TMRM, 25 nM) and the indicated amounts of drugs for the indicated amounts of time before collection. For measurements of mitochondrial mass, 100,000 cells were incubated with MitoTracker Green FM (50 nM) for 1 hr. For primary hepatocytes, cells were assayed in suspension immediately after they were harvested from the liver, and incubated with verapamil (20 μ M) to facilitate retention of TMRM and MitoTracker Green FM signals. Afterward, cells were collected by centrifugation, washed once with PBS, and resuspended in PBS with 7-AAD (2 μ g/ml) for analysis. KBM7 cells and primary hepatocytes were centrifuged at 2,000 rpm for 5 min and 500 rpm for 5 min at 4°C, respectively.

Cell Viability Assays

For KBM7 cells, 100,000 cells were seeded and treated with the indicated drugs for the indicated amounts of time. They were then analyzed by 7-AAD staining and FACS (BD Pharmingen) according to the manufacturer's instructions unless indicated otherwise. For HeLa, SH-SY5Y, and Malme-3M cells, 500–2,000 cells were seeded per well of white, clear-bottom, 96-well plates (Greiner Bio-One), treated with drugs, and then analyzed using CellTiter-Glo (Promega) according to the manufacturer's instructions. For primary hepatocytes, 100,000 cells were seeded per well of a 24-well TPP plate (Light Labs), treated with antimycin, and then analyzed using CellTiter-Glo.

Cell Proliferation Assays

For KBM7 cells, cell proliferation was assessed with a Beckman Z2 Coulter Counter using a size range of 8–30 μ m. For all other cell lines, cell proliferation was assessed using CellTiter-Glo according to the manufacturer's instructions and by normalizing all readings to initial values measured at the start of the experiment.

Long-Term Exposure to ddl

mtDNA copy number analysis was performed on cells treated with ddl for 8, 26, and 41 days. Rates of proliferation were measured for cells treated with ddl for 5, 31, and 51 days. Immunoblot analysis was done on cells treated with ddl for 5, 11, and 50 days.

Statistical Analysis

All data are expressed as mean \pm SEM. Significance was determined with a two-tailed unpaired Student's t test. Differences with a p value less than 0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.02.046>.

AUTHOR CONTRIBUTIONS

W.W.C., K.B., and D.M.S. conceived the project. W.W.C. and K.B. designed and performed most experiments and data analyses with input from D.M.S. M.M.M., B.Y., and E.C.B. assisted with experiments. J.E.C. and T.R.B. assisted with haploid genetic screening. C.B.C. performed metabolite profiling and analysis. H.S., I.S., and D.D.S. performed electron microscopy analysis. W.W.C., K.B., and D.M.S. wrote and edited the manuscript.

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