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

Walter W. Chen,1,2,3,4,9 Kıvanç Birsoy,1,2,3,4,9 Maria M. Mihaylova,1,2,3,4 Harriet Snitkin,6 Ivona Stasinski,6 Burcu Yucel,1,2,3,4 Erol C. Bayraktar,1,2,3,4 Jan E. Carette,7 Clary B. Clish,3 Thijn R. Brummelkamp,8 David D. Sabatini,6 and David M. Sabatini 1,2,3,4,5,*

1Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142, USA
2Department of Biology, Massachusetts Institute of Technology (MIT), Cambridge, MA 02139, USA
3Broad Institute, Seven Cambridge Center, Cambridge, MA 02142, USA
4David H. Koch Institute for Integrative Cancer Research at MIT, 77 Massachusetts Avenue, Cambridge, MA 02139, USA
5Howard Hughes Medical Institute, MIT, Cambridge, MA 02139, USA
6Department of Cell Biology, New York University School of Medicine, New York, NY 10016, USA
7Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305, USA
8Department of Biochemistry, Netherlands Cancer Institute, Plesmanlaan 121 1066 CX, Amsterdam, the Netherlands
9These authors contributed equally to this work
*Correspondence: sabatini@wi.mit.edu

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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 \( \rho^0 \) 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, 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...
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 x 10^-3; Carette et al., 2011a), WT1 (p = 1.78 x 10^-4), and TP53 (p = 6.93 x 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).

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) Immunobots 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 ± SEM (n = 3). Scale bars, 20 μm.
(E) Immunobots 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 μM) for 2 days (right). Error bars are ± SEM (n = 3). ***p < 0.001.
(F) Cellular ATP (left) and ΔΨm (right) in WT and ATPIF1_KO KBM7 cells treated with antimycin (135 μM) and oligomycin (1 μM). Error bars are ± SEM (n = 3).
(G) Viability of WT and ATPIF1_KO KBM7 cells treated with antimycin (135 μM) and oligomycin (1 μM) for 2 days. Error bars are ± SEM (n = 3). ***p < 0.001.

See also Figures S1–S3 and Table S1.
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 (Cabezón 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 (Skrüć 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 p53 WT cells, which are devoid of any mtDNA and consequently have defects in complexes I, III, and IV, and resulting in undetectable ETC activity (Jazayeri et al., 2003). To our surprise, we found that HeLa p53 cells intrinsically possess lower mRNA and protein levels of ATPIF1 compared with their WT counterparts (Figure 2D). Previous work has shown that p53 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 p53 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 p53 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 p53 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 p53 state (Figure 2E). Collectively, these data demonstrate that reduced ATPIF1 activity is essential for the viability of human p53 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.

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 and cultured with antimycin for 4 days. Error bars are ± SEM (n = 3).

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 ± 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 ± 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 ± SEM (n = 3).
(D) Relative ATPIF1 mRNA levels (top) and immunoblots for the indicated proteins (bottom) in HeLa WT and p0 cells. Error bars are ± SEM (n = 3). **p < 0.01.
(E) Immunoblots (left) and relative proliferation (right) of HeLa WT and p0 cells transduced with control vector, ATPIF1 (WT), or ATPIF1 (E55A) constructs. Error bars are ± SEM (n = 3).
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, 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.

Figure 3. Loss of ATPIF1 Improves Cell Viability during Progressive mtDNA Depletion

(A) Schematic depicting the experimental paradigm for long-term treatment of WT and ATPIF1_KO KBM7 cells with ddI. Early (days 0–10), middle (days 10–40), and late (days 40+) periods of ddI treatment are indicated and demarcated by dotted lines. During each period, mtDNA copy number, cell proliferation over 4 days, and ATPIF1 expression were analyzed. (B–D) mtDNA copy number (left), cell proliferation (middle), and immunoblots for the indicated proteins (right) of WT and ATPIF1_KO KBM7 cells treated with ddI during the early (B), middle (C), and late (D) periods. Error bars are ± SEM (n = 3). ***p < 0.001. The smaller effects of ATPIF1 loss on hepatocyte viability during antimycin treatment, as compared with KBM7 cells, are partially due to the frailty 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
In conclusion, we have used a positive selection screening method in human cells to identify loss of ATPIF1 as protective against complex III blockade. We have further shown that ATPIF1 inhibition protects different cell types against numerous insults to the ETC. In particular, our work demonstrates that loss of ATPIF1 activity is essential for the viability of human rho− cells, a widely used system to study mitochondrial dysfunction, and that inhibition of ATPIF1 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 ATPIF1 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 (TMRE, 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 μM) to facilitate retention of TMRE and MitoTracker Green FM signals. Afterward, cells were collected by centrifugation, washed once with PBS, and resuspended in PBS with 7-AAD (2 μg/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-SYSY, and Malme-3M cells, 500–2,000 cells were seeded per well of 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 μm. 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 ddI **
mtDNA copy number analysis was performed on cells treated with ddI for 8, 26, and 41 days. Rates of proliferation were measured for cells treated with ddI for 5, 31, and 51 days. Immunoblot analysis was done on cells treated with ddI for 5, 11, and 50 days.

** Statistical Analysis **
All data are expressed as mean ± 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 **

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