Biochemistry and Chemical Biology

The Role of ATP Synthase Subunit e (ATP5I) in Mediating the Metabolic and Antiproliferative Effects of Biguanides

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eLife Assessment

This **valuable** manuscript describes ATP5I, a subunit of F1Fo-ATP synthase, as a key target of medicinal biguanides, however, it provides **incomplete** evidence of a direct interaction between ATP5I and metformin. The knockout of ATP5I in pancreatic cancer cells mimics biguanide treatment, inducing a metabolic switch from OXPHOS to glycolysis due to a compromised expression of the Complex I protein NDUFB8. This results in a markedly decreased NAD/NADH ratio and decreased cell proliferation. These findings point out ATP5I as a promising mitochondrial target for cancer therapies and contribute to our understanding of metformin's mechanism of action since many of its molecular mechanisms remain poorly understood.

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Abstract

Here we identify the subunit e of F_1F_o-ATP synthase (ATP5I) as a target of medicinal biguanides. ATP5I maintains the stability of F_1F_o-ATP synthase dimers which is crucial for shaping cristae morphology. Although its roles have been mainly studied in yeast models, its function in cellular energy metabolism within the context of cancer remains poorly characterized. In this study, we demonstrate that ATP5I interacts with a biguanide analogue *in vitro* and disabling its expression by CRISPR-Cas9 in pancreatic cancer cells leads to the same phenotype as biguanide treated cells including a decrease in the levels of some respiratory complex subunits, mitochondrial morphology alterations, inhibition of oxidative phosphorylation (OXPHOS) and a compensatory increase in glycolysis. Moreover, ATP5I knockout (KO) cells exhibit resistance to the antiproliferative effects of biguanides, but reintroduction of ATP5I rescues the metabolic and anti-proliferative effects of metformin and phenformin. These findings highlight ATP5I as a significant antineoplastic mitochondrial

target of medicinal biguanides, opening new opportunities for the development of mitochondrial-targeted therapies.

Introduction

Discovering safe and effective therapeutic targets for cancer treatment remains a major challenge in biomedical research[1]. Recently, targeted therapies against complex I of the respiratory chain have garnered considerable attention in pharmacology, offering a promising strategy for cancer treatment due to their potential to disrupt the energy metabolism of tumor cells[2-5]. However, despite initial promises, clinical trials with potent complex I inhibitors, such as IACS-0107596, have been compromised by severe toxicities, raising concerns about their clinical viability. Given the critical role of mitochondria in cancer cells, there is an urgent need for more effective and safer alternatives to target them[6,7]. In this context, medicinal biguanides such as metformin and the more lipophilic phenformin are emerging as attractive alternatives. These biguanides are considered moderate respiratory chain inhibitors and offer a potential avenue for targeting mitochondrial metabolism in cancer[8].

Metformin has been used since the 1950s for its anti-hyperglycemic properties[9]. As such, it has a well-established safety profile and is commonly used for the treatment of type II diabetes[10]. Several epidemiological studies showed that prolonged use of metformin in diabetic patients reduced the incidence of several cancers[11,12], particularly pancreatic cancer[13,14]. However, its clinical use has proven ineffective in patients with advanced pancreatic cancer[15], suggesting that its antitumoral mechanism is more preventive than therapeutic[16,17]. The more potent phenformin[9], which was withdrawn from the market in the 1970s for its anti-hyperglycemic applications due to deaths attributed partly to lactic acidosis[18], is currently undergoing clinical trials for oncology purposes[19].

Unlike other inhibitors of the respiratory chain, the mechanism of action of medicinal biguanides remains elusive[20,21]. However, compelling evidence indicates that energy metabolism plays a pivotal role in their effectiveness[22,23]. At supra-pharmacological doses (1-10 mM), typically 100-1000 times higher doses than those used for anti-hyperglycaemic effects, metformin moderately inhibits the activity of complex I of the respiratory chain in several cellular models[24-26]. This inhibition subsequently decreases OXPHOS activity, leading to reduced cellular respiration and energy production. In response, cells adapt by shifting metabolism to glycolysis and decreasing their anabolic activity through the activation of the energy sensor AMPK[20,22,24]. Nonetheless, to observe a decrease in isolated complex I activity in *in vitro* models, metformin doses of up to 50 mM are required[25-27]. A recent cryo-electron microscopy study revealed that a metformin analogue binds to complex I in a specific conformation with a binding site unique to this analogue[27]. However, further work using *in vivo* models is necessary to validate the biological meaning of this interaction as a critical site of action of metformin[27,28].

Additional targets such as glycerol phosphate dehydrogenase[29], complex IV[30], PEN2[31] and F_1F_o- ATP synthase[25] have also been suggested for metformin. Metformin may interact with various targets in a nonspecific manner[32-35] because of its structural similarity with guanidine, a potent chaotropic agent. These various targets potentially exert pleiotropic effects[20,21] that could act synergistically.

It has been shown that atrazine, a molecule derived from biguanides, binds and inhibits F_1F_o-ATP synthase[36]. Additionally, a study from our group revealed that medicinal biguanides likely disrupt cristae organization[37], a crucial process mediated by the oligomerization of F_1F_o-ATP synthase and resulting in the formation of 'onion-liked structures'[38-41]. It is proposed that complex I and F_1F_o-ATP synthase of the respiratory chain may be particularly sensitive to

biguanide inhibition due to the conformational mobility of their catalytic interfaces[25]. Disrupting the folding of the mitochondrial inner membrane could alter the organization and functions of other respiratory chain complexes[42]. It remains an open question whether biguanides act through multiple targets or a single primary target.

Here, we identify another potential target of biguanides: the e subunit of F_1F_0-ATP synthase (ATP5I), a transmembrane protein involved in the dimerization of this supramolecular complex[38-40]. Although medicinal biguanides are known to accumulate in mitochondria and disrupt the respiratory chain by inhibiting complex I, no direct evidence of their interaction with a subunit of F_1F_0-ATP synthase has been reported until now. CRISPR-Cas9 mediated KO of ATP5I recapitulates many of the effects of metformin in pancreatic cancer cells and reduces metformin sensitivity. In addition, the genetic signature of a cells treated with metformin is more similar to that of cells treated with an F_1F_0-ATP synthase inhibitor than to a complex I inhibitor. Taken together this work adds the F_1F_0-ATP synthase and its subunit ATP5I as a bona fide target of biguanides

Results

Identification of ATP synthase subunit e (ATP5I) as a mitochondrial biguanide binding protein

To identify proteins that bind biguanides, we synthetized a Biotin Functionalized Biguanide or BFB (**Figure 1A**, **Figure 1 – figure supplement 1A-B**) to perform an affinity-based pull-down assay with streptavidin beads[43]. We first assessed the biological activity of BFB comparing it to metformin in the ability to activate AMPK and inhibit cell proliferation in KP-4 pancreatic cancer cells. Similarly to metformin, BFB activated AMPK and its downstream target ACC as indicated by their increased phosphorylation states (**Figure 1B**) and inhibited KP-4 cell growth with an EC_{50} of 1.0 ± 0.2 mM (**Figure 1C**). Additionally, immunofluorescence experiments with fluorophore-conjugated Streptavidin confirmed that BFB accumulates in mitochondria, as shown by colocalization with the mitochondrial protein TOMM20 (**Figures 1D**, **1E**).

After confirming that BFB exhibited activity comparable to that of metformin, we performed pull-down assays using streptavidin immobilized on sepharose beads on mitochondrial-enriched cell extracts to analyse interacting proteins by mass-spectrometry. Given the significant issue of nonspecific binding associated with this technique, we performed multiple parallel pull-down experiments. These included a control biotin-conjugated amine derivative (BFA) possessing the same linker as BFB (**Figure 1 – figure supplement 1C**). Mass spectrometry analysis results identified a total of 69 proteins. Among these, 31 proteins showed specific interaction with BFB under the elution condition with metformin (see Materials and methods). Many of these proteins were cytoskeletal, with several keratin isoforms potentially involved in nonspecific interactions with streptavidin beads. However, several proteins stood out, including glycerol-3-phosphate dehydrogenase, arginase isoform 2 and ATP synthase subunit e (ATP5I). Glycerol-3 phosphate dehydrogenase is a known target of metformin[29], validating our pulldown experiment. Additionally, arginase binds arginine which has a guanidinium group structurally related to metformin[44].

Biguanide pharmacophore interacts specifically with ATP5I

As ATP5I was not previously shown to bind biguanides, we first confirmed this interaction through immunoblot analysis in independent pull-downs experiments. The results indicate that BFB allows specific interaction of ATP5I in streptavidin pull-down while BFA failed to do so. Of note, we observed a nonspecific band (≃11 kDA) in all pull-down conditions with streptavidin beads, possibly associated with the denatured streptavidin monomer in heated SDS buffer (**Figure 1F**).

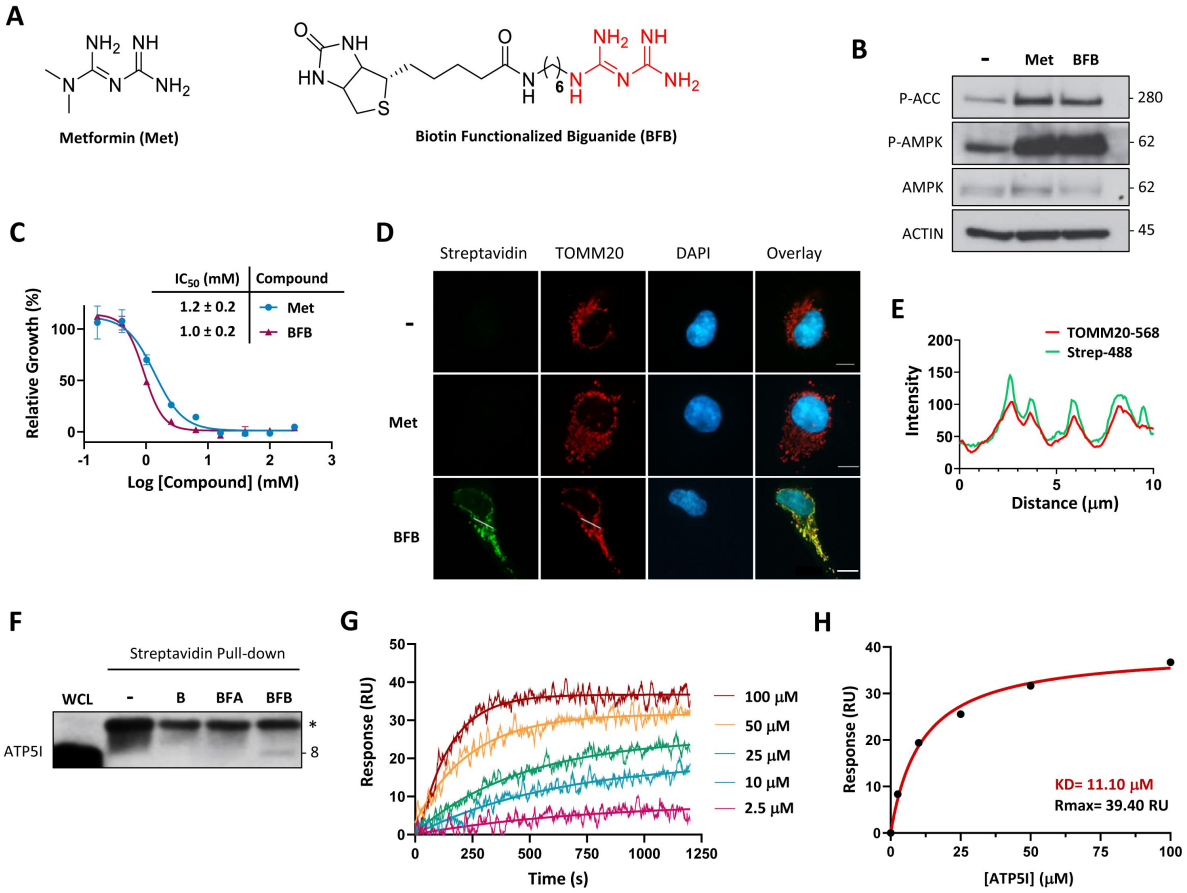

Figure 1.

Biguanide pharmacophore interacts with ATP synthase subunit e (ATP5I).

(A) Design of bio-inspired probe biotin functionalized biguanide (BFB) based on the structure of metformin (Met). **(B)** Immunoblots for the phosphorylation of AMPK (Thr172) and ACC (Ser79) in extracts from KP-4 pancreatic cancer cells treated with 2.5 mM Met or BFB for 16 hours. β-ACTIN was used as loading control. **(C)** Representative quantification of cell viability and growth with corresponding EC_{50} values of 3-day treatments with metformin (Met) or biotin functionalized biguanide (BFB) in KP-4 cells. Values represent the mean ± standard deviation of N=3. **(D)** Representative images of mitochondria and BFB localization in cells as in (B). Cells were treated with 1 mM of metformin (Met) or BFB for 16 hours and mitochondrial signal and BFB localization were analyzed by co-immunofluorescence using Streptavidin fluorophore conjugate and TOMM20 antibody, scale bar= 10 µm. Cells untreated (-) and treated with 1 mM Met were used as negative controls. **(E)** Colocalization between TOMM20 (TOMM20-568) and Streptavidin (Strep-488) fluorophores was analyzed for the BFB condition from (D) through job plot intensity profile. **(F)** Pull-down validation experiments with streptavidin beads alone (-), D-biotin (B), BFA and BFB using antibody followed by immunoblot against ATP5I in cells as in in extracts from HEK-293T embryonic kidney cells. The whole cell lysate (WCL) was added as control. **(G)** Binding interactions studies of BFB with recombinant purified ATP5I (rATP5I) using Surface Plasmons of Resonance (SPR). Representative sensorgrams show affinity kinetics of BFB and rATP5I. BFB was exposed onto streptavidin immobilized sensor chip and several concentrations of rATP5I were added until saturation of the signal. The assay was performed in a running buffer (125 mM NaCl, 5 mM DTT, PBS pH 7.4) at 25°C. RU: Resonance Units. **(H)** Binding affinity curve obtained from each steady state from (F). K_D refers to the dissociation equilibrium constant and Rmax represent the theoretical maximum response.

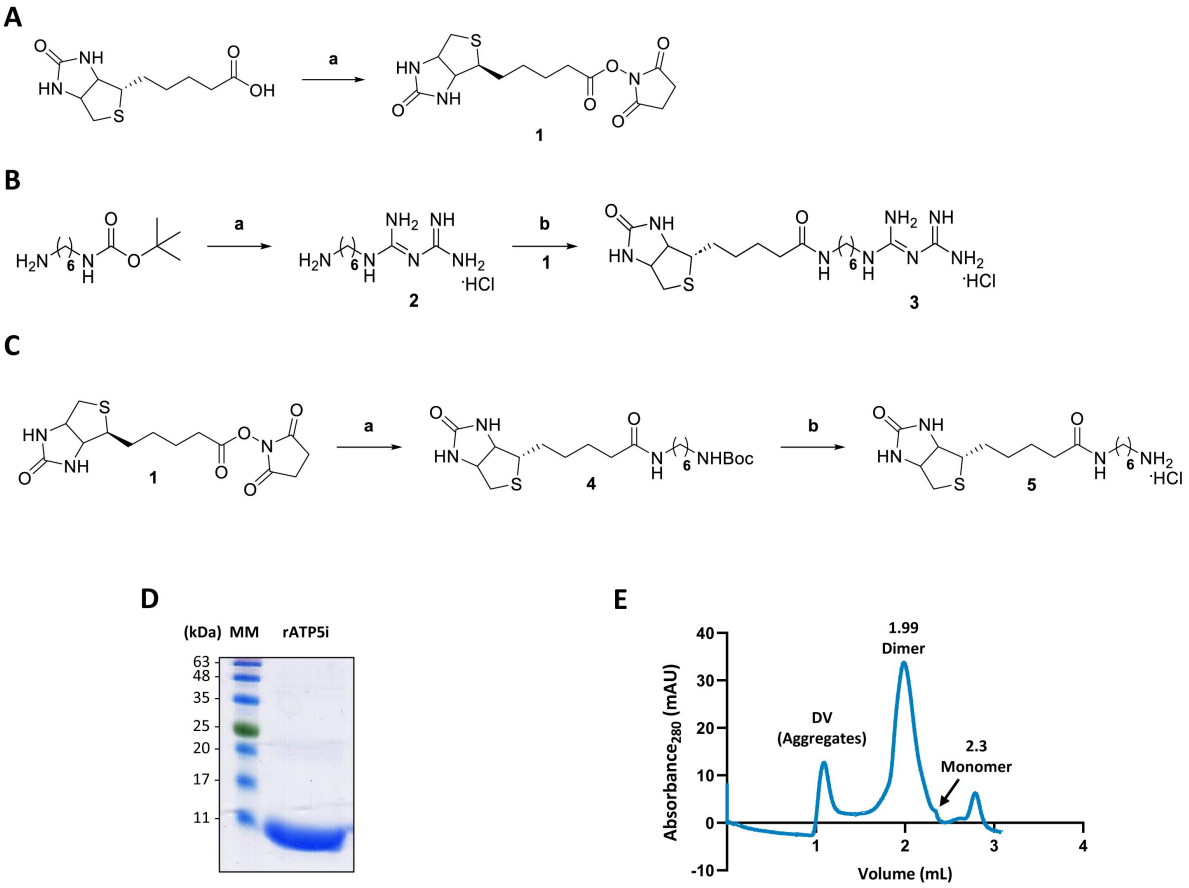

Figure 1 – figure supplement 1.

(A) Synthesis of biotin-NHS (1); (a) NHS, EDC, DMF, room temperature (r.t) overnight (o/n). **(B)** Synthesis of biotin functionalized biguanide (BFB) chloride salt (3); (a) Dicyandiamide, TMSCl, MeCN, 160°C, 3h, 2: 6-aminohexylbiguanide hydrochloride salt; (b) Biotin-NHS, DIPEA, DMF, r.t, o/n. **(C)** Synthesis of biotin functionalized amine (BFA) chloride salt (5); (a) N-Boc-1,6-hexanediamine, DMF, r.t, o/n, 4: biotin functionalized N-Boc-amine; (b) HCl/MeOH, MeOH, r.t, o/n. **(D)** SDS PAGE analysis of recombinant ATP5I (rATP5I) after the purification process. Purity is estimated at ≥ 90 %. MM: molecular weight marker. **(E)** Gel filtration profile analysis of rATP5I using Superose 12 10/300 GL. DV represents column dead volume. mAU: milli-Absorbance Unit.

We then used surface plasmon resonance (SPR) to characterize the binding of BFB and purified recombinant ATP5I. Our purification steps yielded a highly pure recombinant protein, mainly organized in dimeric form (**Figure 1– figure supplement 1D**, **1E**). Although SPR is sensitive[45,46], the size difference between BFB and recombinant ATP5I prompted us to immobilize the small molecule BFB on the gold surface. Adding increasing concentrations of recombinant ATP5I allowed us to estimate an affinity constant (KD ≃ 11.10 µM; Rmax ≃ 39.40 RU) significantly lower than typical metformin concentrations that affect complex I in mM range (**Figures 1G**, **1H**)

ATP5I knockout in pancreatic cancer cells alters the organization of mitochondrial networks

In yeast, inhibition of the subunit e's equivalent impaired mitochondrial inner membrane folding[47]. This structural deficiency in yeast correlates with decreased dimerization of F_1F_o-ATP synthase, highlighting the essential role of ATP5I in dimer stability. However, yeast's subunit e is not essential for the catalytic activity of F_1F_o-ATP synthase[47-51]. In human cells, ATP5I is also crucial for dimer stability[40] but lacks thorough characterization regarding its roles in cellular energy metabolism. To elucidate its function in cancer and its relevance to cells treated with medicinal biguanides, we developed CRISPR-based reagents to inhibit ATP5I expression in KP-4 pancreatic cancer cells.

Initially, two clones each of the two guide (sgATP5I #1 and sgATP5I #2) were isolated, and their mitochondrial phenotype characterized. First, we measured levels of several proteins from the respiratory complexes. The results show that all ATP5I knockout clones did not express ATP5I and exhibited decreased expression of complex I NDUFB8 protein and complex IV COX II protein compared to control clones expressing a guide against GFP (sgGFP) (**Figure 2A**). A moderate to no reduction in mRNA levels encoding these proteins were observed (**Figure 2 – figure supplement 1A**) cannot account for the downregulations at the protein level. Additionally, the mitochondrial/nuclear DNA ratio (**Figure 2 – figure supplement 1B**) indicates no reduction in mitochondrial number, suggesting ATP5I may play a role in maintaining the stability of certain subunits of complexes I and IV. Consistent with this finding, the absence of subunit e in yeast also controls the stability of F_1F_o-ATPase proteins subunit g and subunit k[51].

We then examined mitochondrial morphologies using fluorescence microscopy in ATP5I knockout (KO) cells, revealing a disruption in the mitochondrial network characterized by predominantly punctate mitochondria (**Figure 2B-C** and **Figure 2 – figure supplement 1C**), relative quantification showed that about 70% of ATP5I KO cells exhibited a punctate phenotype, around 25% showed a fragmented phenotype and only a few displayed a filamentous phenotype (**Figure 2C**). These findings confirm that ATP5I plays a crucial role in the organization of mitochondrial network in KP-4 cells. Treatment of the same cell line with medicinal biguanides resulted in similar alterations in mitochondrial network organization[37] reinforcing the hypothesis that ATP5I may be a target of biguanides.

ATP5I knockout desensitizes pancreatic cancer cells to biguanides

Given the uncertain role of ATP5I in the cellular energy metabolism but considering the changes in global mitochondrial physiology (**Figure 2B**, **2C**), we investigated the effects of ATP5I knockout (KO) on mitochondrial bioenergetics in KP-4 cells. The results reveal that ATP5I deletion significantly decreases the NAD^+/NADH ratio (**Figure 3A**), most significantly affecting NAD^+ concentration (**Figure 3A – figure supplement 1A**, **1B**). Our results also indicate a decrease in the oxygen consumption rate (OCR) to extracellular acidification rate (ECAR) ratio (**Figure 3B** and **Figure 3 – figure supplement 1C**, **1D**) suggesting a reduced respiration associated with a compensatory increase in glycolysis. This metabolic reorganization in ATP5I KO cells renders them up to 6 times more sensitive to glycolysis inhibition with 2-D-deoxyglucose (**Figure 3 – figure**

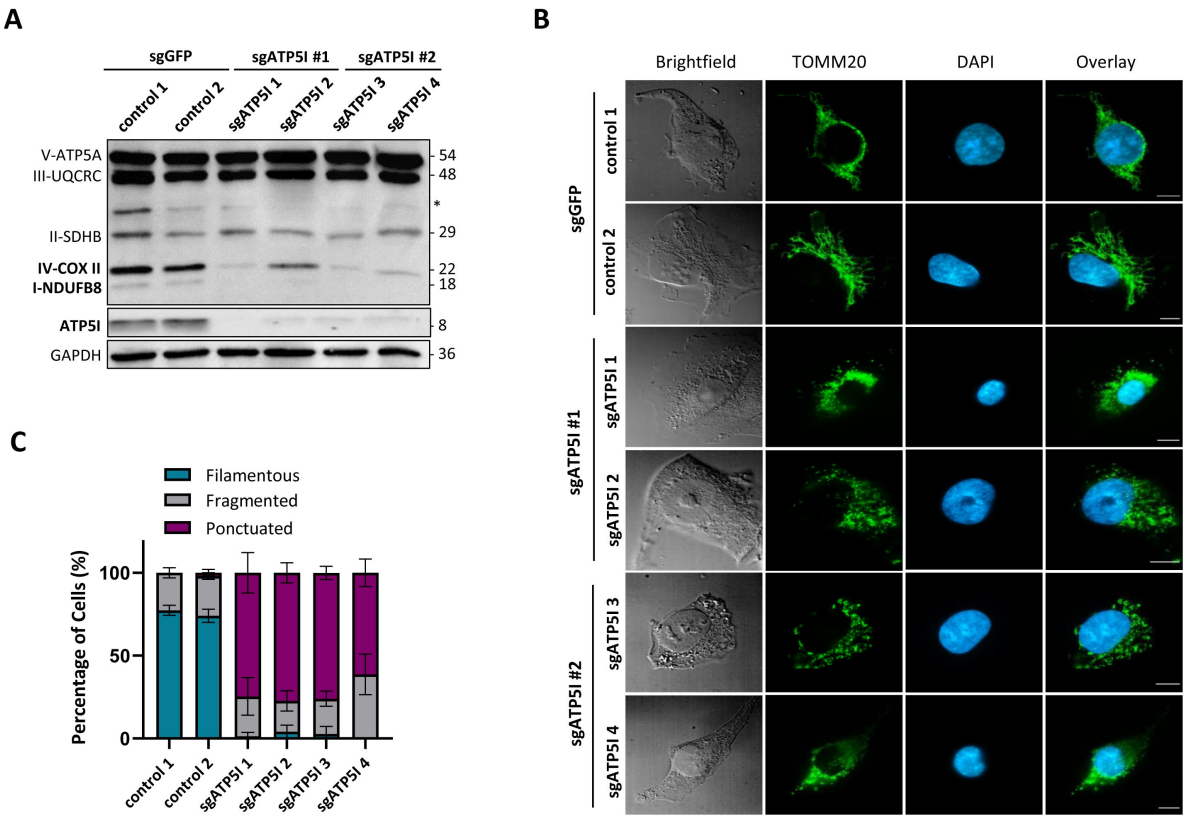

Figure 2.

ATP5I knockout in pancreatic cancer cells alters the organization of the mitochondrial network.

(A) Immunoblot of five proteins from the OXPHOS complexes in extracts from clones of KP-4 cells expressing a control small guide RNA against GFP (sgGFP: control 1 and control 2) or two clones for each of the two different guides targeting ATP5I (sgATP5I #1: sgATP5I 1 and sgATP5I 2, and sgATP5I #2: sgATP5I 3 and sgATP5I 4). GAPDH antibody was used as loading control. **(B)** Representative images of mitochondrial morphologies characterized in cells as in (A). Mitochondrial morphologies were analyzed by immunofluorescence using TOMM20 antibody. DAPI is used as a DNA counter stain. Scale bar= 10 µm. **(C)** Quantification of the percentage of cells exhibiting filamentous, fragmented or punctuated mitochondria from (B). Values represent the mean ± standard deviation of N=3 for each clone, each n= 25-50 cells per clone.

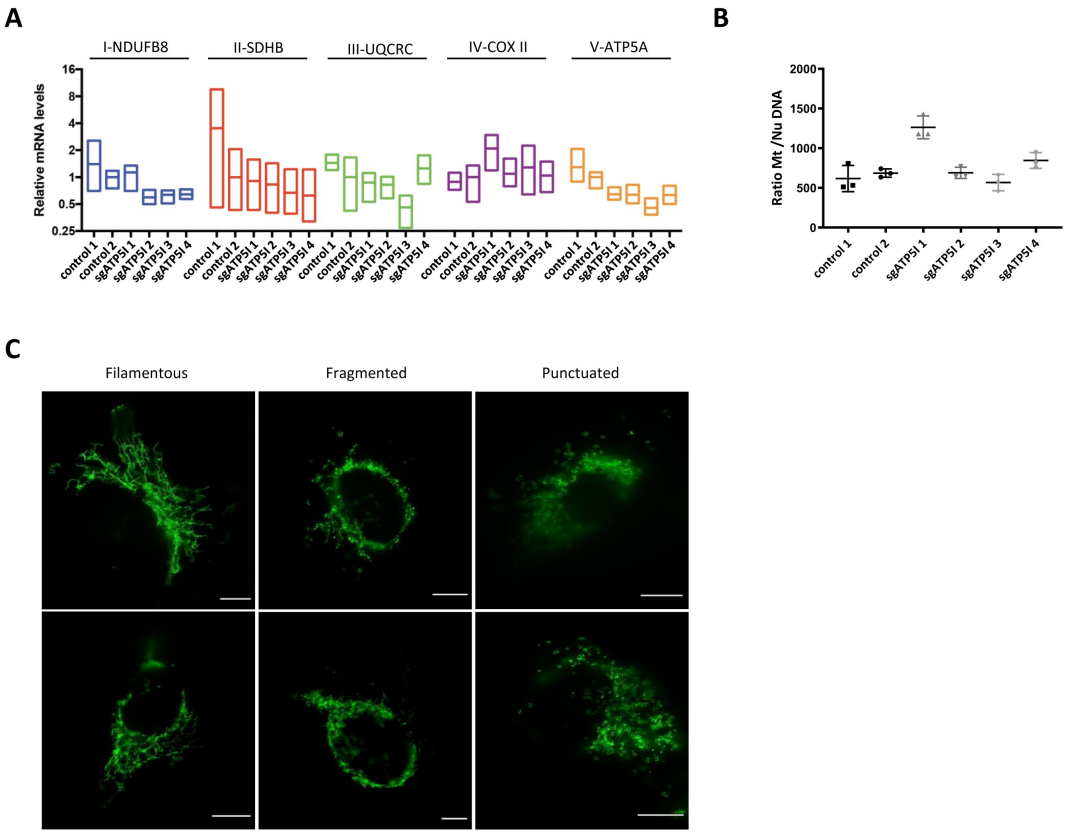

Figure 2 – figure supplement 1.

(A) Relative qPCR quantification of the mRNAs encoding proteins representative of the five OXPHOS complexes (in **Figure 2A**) in clones of KP-4 cells expressing a control small guide RNA against GFP (sgGFP: control 1 and control 2) or two clones for each of the two different guides targeting the knockout of ATP5I (sgATP5I #1: sgATP5I 1 and sgATP5I 2, and sgATP5I #2: sgATP5I 3 and sgATP5I 4). Values represent the mean ± standard deviation of three biological replicates. **(B)** qPCR quantification of mitochondrial genomic DNA (Mt) over cellular nuclear genomic DNA (Nu) in cells as in (A). Values represent the mean ± standard deviation of N=3. **(C)** Representative mitochondrial morphologies of cells exhibiting filamentous, fragmented or punctuated mitochondrial network phenotypes in KP-4 cells of cells as in (A). Mitochondrial morphologies were detected by immunofluorescence using TOMM20 antibody, scale bar= 10 µm.

supplement 1E), similar to control cells treated with 2.5 to 5 mM metformin (**Figure 3 – figure supplement 1F**). These findings confirm that both ATP5I deletion and metformin treatment disrupt respiration conferring a dependency on glycolysis[52].

Moreover, the inhibition of the mitochondrial respiratory chain observed in ATP5I KO cells also induces an energy imbalance, resulting in AMPK activation (**Figure 3C**), similarly to control cells treated with metformin (**Figure 3 – figure supplement 1G**). However, the increased phosphorylation state of AMPK in ATP5I KO cells treated with metformin at these concentrations suggests that metformin can still activate AMPK via interactions with other targets such as PEN2[31].

Energy stress resulting from ATP5I KO in KP-4 cells leads to a noticeable slowdown in cell growth (**Figure 3D**), although this effect can be reversed by supplementing the cell culture medium with pyruvate and uridine (**Figure 3 – figure supplement 1H**). These findings suggest that while ATP5I is not essential for growth in KP-4 cells, its deletion subtly affects energy metabolism, akin to the effects seen in KP-4 cells treated with medicinal biguanides[37].

Seahorse kinetic monitoring of control and ATP5I KO cells treated with 5 mM metformin reveals, as expected, that metformin causes a decrease in oxygen consumption (**Figure 3E**) and an increase in glycolysis (**Figure 3F**) over time. However, these effects are more pronounced in control cells than what is observed in ATP5I cells, particularly in terms of glycolysis activation. The OCR/ECAR ratio also decreases more in control cells upon metformin treatment than in ATP5I KO cells (**Figure 3G**), suggesting reduced sensitivity to metformin in the KO cells. This resistance is also evident in the relative growth of cells under increasing doses of metformin. Indeed, ATP5I KO cells exhibit EC_{50} values up to 9 times higher than those of control cells (**Figure 3H**). Overall, these results suggest that metformin is normally acting partially through ATP5I as indicated by the reduced sensitivity of the KO cells.

Similar trends where obtained, when using 100 µM phenformin in seahorse kinetic with more pronounced changes in both respiratory activity decline (**Figure 3I**) and glycolysis activation (**Figure 3J**). These differences appear earlier but over a shorter time compared to metformin, possibly due to phenformin's pharmacokinetic profile[53]. Again, phenformin affects both control and ATP5I KO cells but it does so less efficiently in ATP5I KO cells. The difference in OCR/ECAR ratio between control cells and the two ATP5I KO cell lines at 3 hours is significantly less impacted (**Figure 3K**), indicating lesser sensitivity of ATP5I KO cells to phenformin. Resistance to phenformin is also evident in cell growth, with ATP5I KO cells showing EC_{50} up to 4 times higher than those of control cells (**Figure 3L**). These results suggest that ATP5I mediates the metabolic and antiproliferative effects of biguanides in KP-4 cells.

Re-expression of ATP5I normalizes mitochondrial morphology, metabolic profile and resensitizes ATP5I knockout pancreatic cancer cells to biguanides

To validate our previous hypotheses regarding the mechanism of action of ATP5I, we reintroduced wild-type ATP5I in ATP5I KO KP-4 clones. Immunoblot analysis results confirmed that exogenous ATP5I could be re-expressed in ATP5I KO cells, albeit at slightly reduced levels compared to control cells. Reintroducing ATP5I also restored the protein levels of NDUFB8 and COX II in KP-4 ATP5I KO cells (**Figure 4A**). Furthermore, exogenous ATP5I colocalized with TOMM20, indicating its successful mitochondrial localization and led to a reorganization of the mitochondrial networks (**Figure 4 – figure supplement 1**). Indeed, quantitative analysis showed that about 60% of ATP5I KO cells with exoATP5I exhibited an intermediate fragmented phenotype between punctate and filamentous forms, while around 34% showed a filamentous phenotype and only few displayed a punctate phenotype (**Figure 4C**).

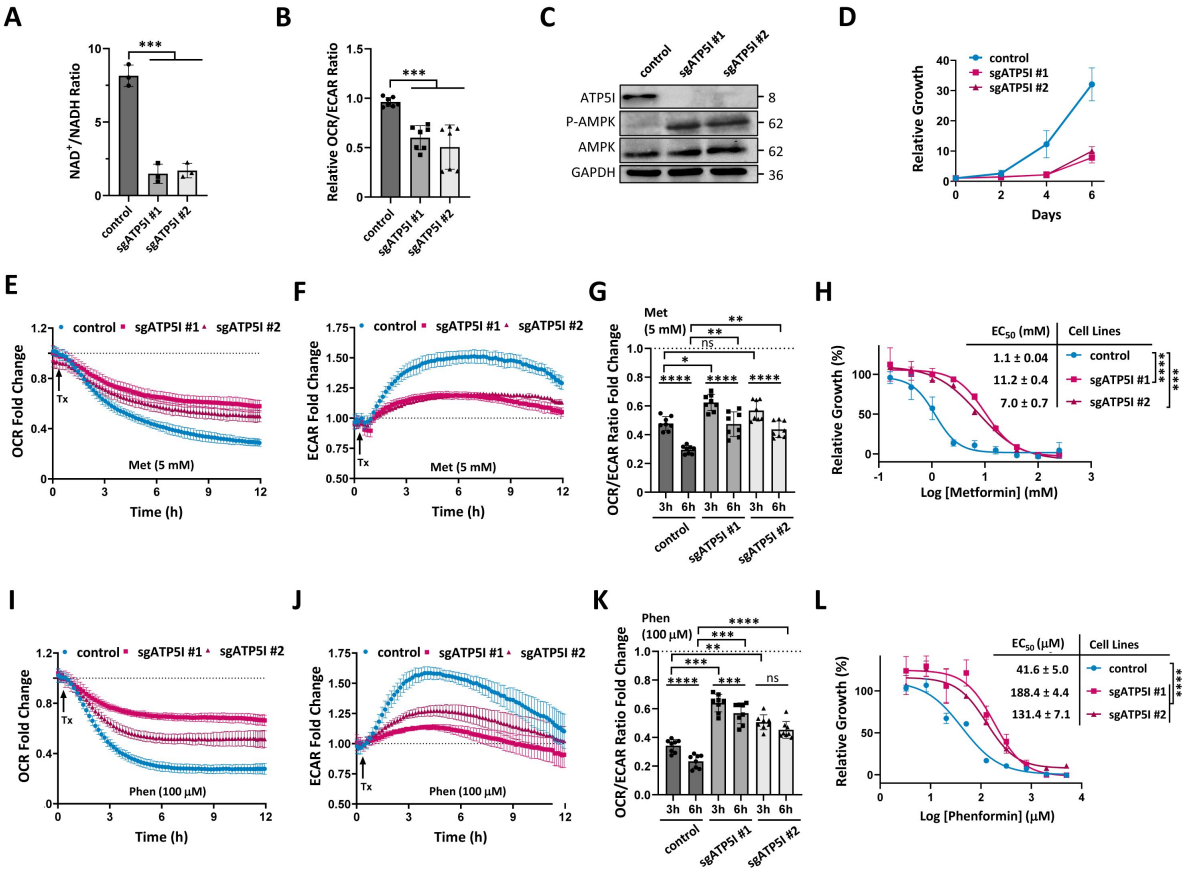

Figure 3.

ATP5I knockout desensitizes pancreatic cancer cells to biguanides.

(A) Quantification of NAD^+/NADH ratio in KP-4 cells expressing a control small guide RNA against GFP (sgGFP) or a representative clone of two different guides targeting ATP5I (sgATP5I #1 or sgATP5I #2). Values represent the mean ± standard deviation of N=3. (***) P < 0.001 using an unpaired Student's *t*-test. **(B)** Relative quantification of oxygen consumption rate (OCR) over extracellular acidification rate (ECAR) by Seahorse analysis in cells as in (A). Values represent the mean ± standard deviation of at least N=3. (***) P < 0.001 using a paired Student's *t*-test. **(C)** Immunoblot for total and phosphorylated levels of AMPK (Thr172) protein in extracts from cells as in (A). ATP5I confirms loss of expression in KO and GAPDH was used as loading control. **(D)** Growth curves of cells as in (A) measuring the relative number of cells over 6 days. Media was changed every two days. **(E)** Representative kinetic curves of OCR in cells as in (A) treated with 5 mM of metformin (Met) relative to control treated cells (dashed line) using Seahorse. **(F)** Representative kinetic curves of ECAR in cells as in (A) treated with 5 mM metformin (Met) relative to control treated cells (dashed line) using Seahorse. **(G)** Quantification of OCR/ECAR ratio fold change at 3 and at 6 hours from kinetic curves (E-F). Values represent the mean ± standard deviation of N=3. (ns) not significative, (*) P < 0.05, (**) P < 0.01, (****) P < 0.0001 using a repeated measures (RM) one-way ANOVA with Sidak's multiple comparison test. **(H)** Representative growth of cells as in (A) exposed to different concentrations of metformin (Met) for three days with corresponding EC_{50} values of metformin. Values represent the mean ± standard deviation of N=3. (***) P < 0.001 and (****) P < 0.0001 using an unpaired Student's *t*-test. **(I)** Representative kinetic curves of OCR in cells as in (A) treated with 100 µM of phenformin (Phen) relative to control treated cells (dashed line) using Seahorse. **(J)** Representative kinetic curves of ECAR in cells as in (A) treated with 100 µM of phenformin (Phen) relative to control treated cells (dashed line) using Seahorse. **(K)** Quantification of OCR/ECAR ratio fold change at 3 and at 6 hours from kinetic curves (I-J). Values represent the mean ± standard deviation of at least three biological replicates. ns: not significative, (**) P < 0.01, (***) P < 0.001, (****) P < 0.0001 using a RM one-way ANOVA with Sidak's multiple comparison test. **(L)** Representative growth of cells as in (A) exposed to different concentrations of phenformin (Phen) for three days with corresponding EC_{50} values. Values represent the mean ± standard deviation of N=3. (****) P < 0.0001 using an unpaired Student's *t*-test.

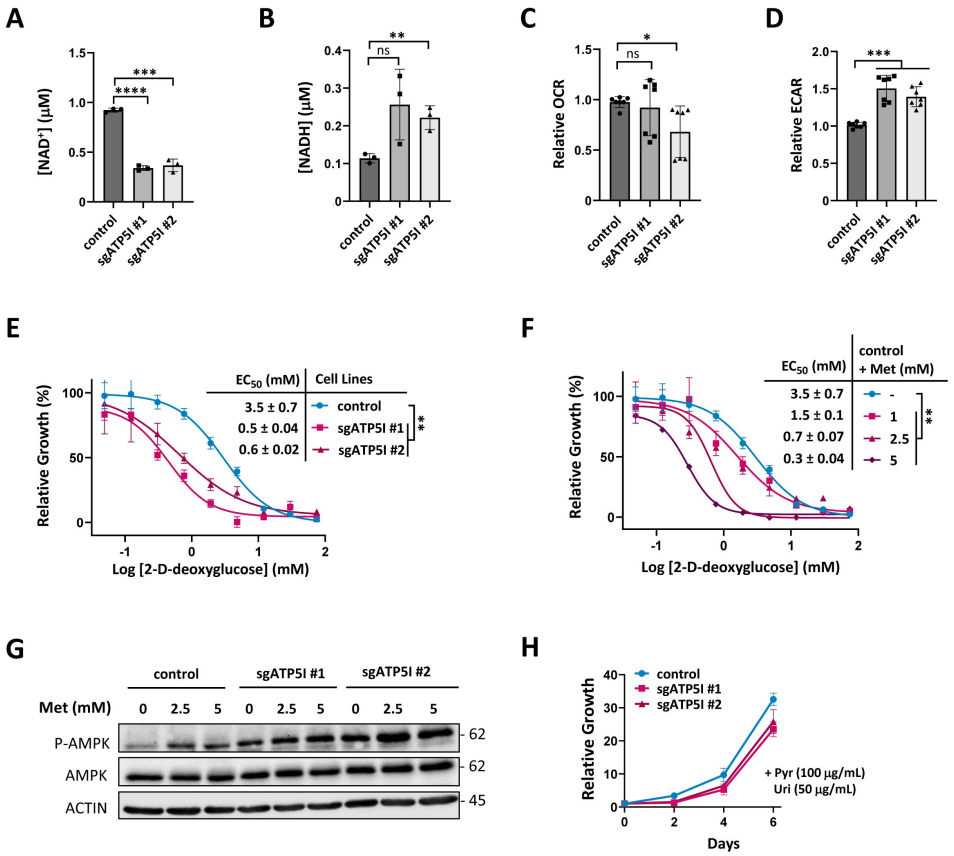

Figure 3 – figure supplement 1.

(A) Quantification of NAD^+ concentration in KP-4 cells expressing a control small guide RNA against GFP (sgGFP) or a representative clone of two different guides targeting ATP5I (sgATP5I #1 or sgATP5I #2). Values represent the mean ± standard deviation of N=3. (***) P < 0.001, (****), P < 0.0001 using an unpaired Student's *t*-test. **(B)** Quantification of NADH concentration in cells as in (A). Values represent the mean ± standard deviation of N=3. (ns) not significative, (**) P < 0.01 using an unpaired Student's *t*-test. **(C)** Relative quantification of oxygen consumption rate (OCR) by Seahorse analysis in cells as in (A). Values represent the mean ± standard deviation of at least N=3. (ns) not significative, (*) P < 0.05 using a paired Student's *t*-test. **(D)** Relative quantification of extracellular acidification rate (ECAR) by Seahorse analysis in cells as in (A). Values represent the mean ± standard deviation of at least N=3. (***) P < 0.001 using a paired Student's *t*-test. **(E)** Representative cell growth of cells as in (A) treated with different concentrations of 2-D-deoxyglucose with corresponding EC_{50} values of 2-D-deoxyglucose treatment in cells as in (A). Values represent the mean ± standard deviation of N=3. (**) P < 0.01 using an unpaired Student's *t*-test. **(F)** Representative cell viability curves with corresponding EC_{50} values of treatments of 2-D-deoxyglucose in combination without (-) and with different concentrations (1, 2.5 and 5 mM) of metformin (Met) in KP-4 cells expressing control sgGFP. Values represent the mean ± standard deviation of three biological replicates. (**) P < 0.01 using an unpaired Student's *t*-test. **(G)** Total and phosphorylated levels of AMPK protein extracts from cells as in (A) treated with 2.5 mM or 5 mM of Met for 16 hours. β-ACTIN antibody was used for loading control. **(H)** Growth curves of cells as in (A) supplemented with 100 µg/mL sodium pyruvate (Pyr) and 50 µg/mL uridine (Uri) by measuring the percentage of confluency over 6 days. Media was changed every two days.

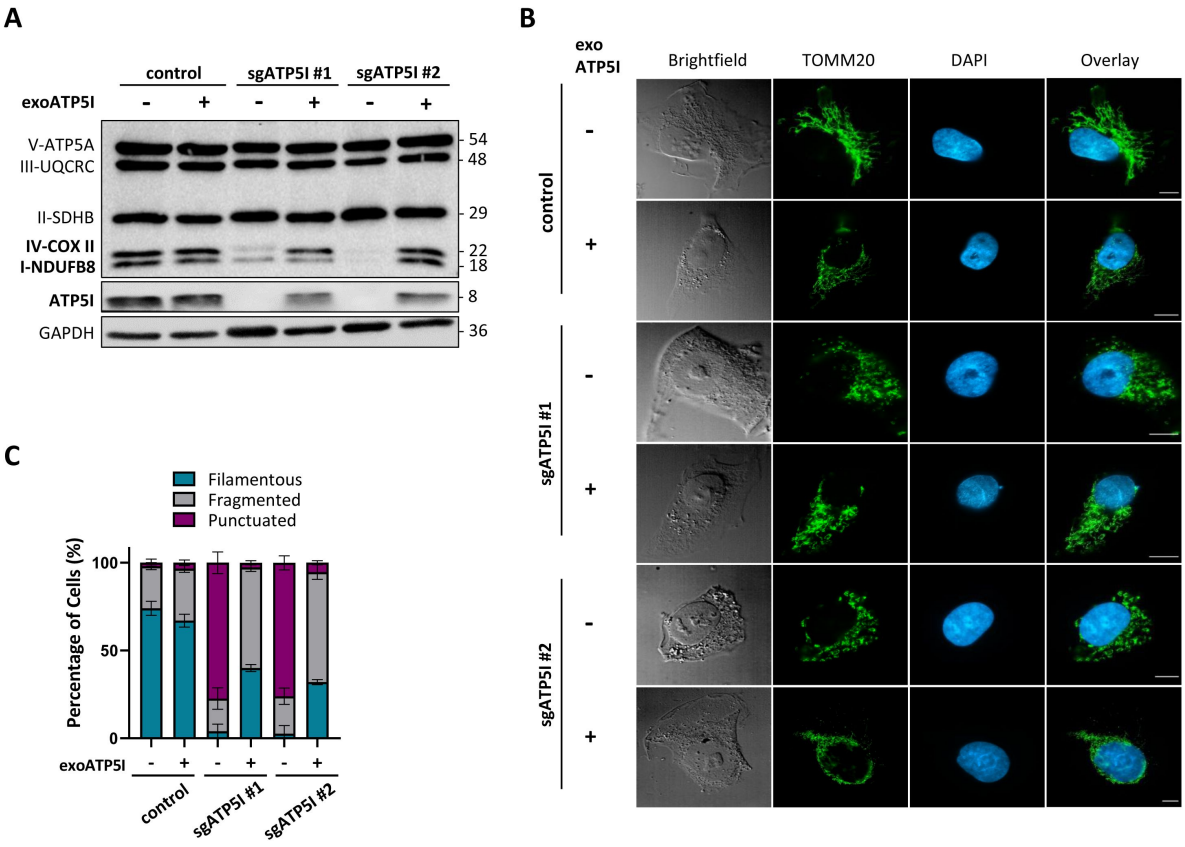

Figure 4.

Exogenous ATP5I enable the reorganization of mitochondrial network in ATP5I knockout pancreatic cancer cells.

(A) A representative immunoblot of the five OXPHOS complexes in KP-4 cells expressing exogenous ATP5I (exoATP5I: +) in control cells expressing a small guide RNA against GFP (sgGFP) or in ATP5I KO cells (clones of two different small guide RNAs: sgATP5I #1 sgATP5I #2) compared with the same cell lines without expression of exogenous ATP5I (-). GAPDH antibody was used as loading control. **(B)** Representative images of mitochondrial morphologies characterized in cells as in (A). Mitochondrial morphologies were analysed by immunofluorescence using TOMM20 antibody, scale bar= 10 µm. DAPI was used as DNA counterstain. **(C)** Quantification of the percentage of cells exhibiting filamentous, fragmented or punctuated mitochondria from (B). Values represent the mean ± standard deviation of N=3 for each clone, n= 50-100 cells per clone.

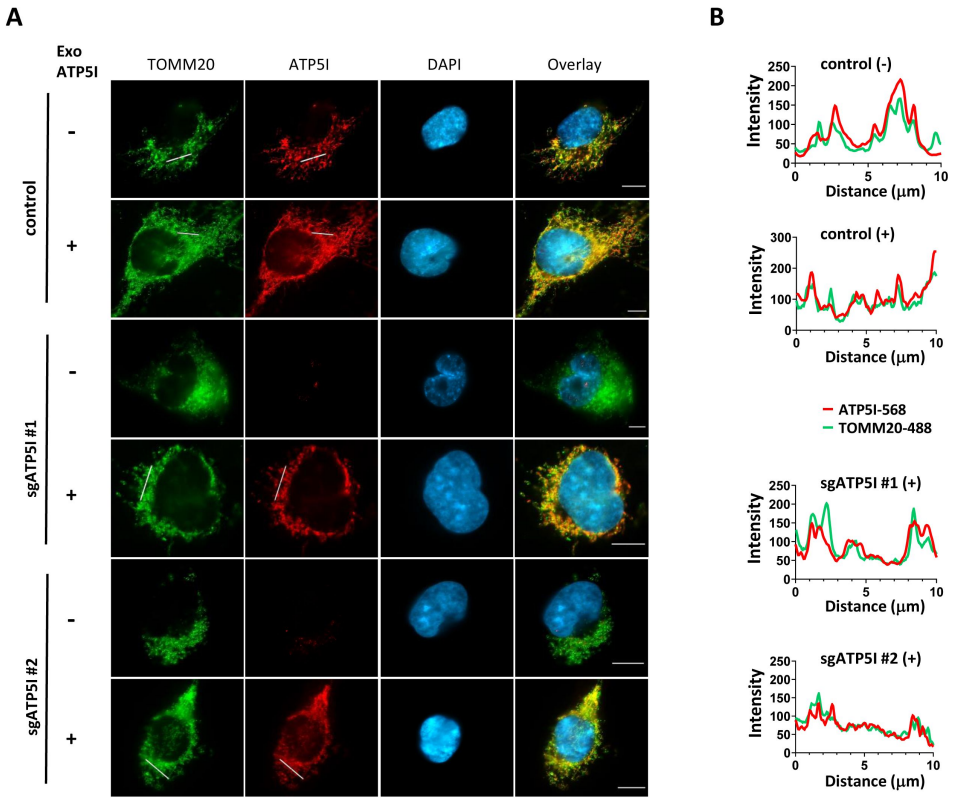

Figure 4 – figure supplement 1.

(A) Representative images of mitochondrial localization of ATP5I in KP-4 cells expressing exogenous ATP5I (exoATP5I: +) in control (clones with sgGFP) or a representative clone of each of two small guide RNA against ATP5I (sgATP5I #1 or sgATP5I #2) compared with the same cell lines without expression of exogenous ATP5I (-). Mitochondrial localization was analyzed by co-immunofluorescence using ATP5I and TOMM20 antibodies, scale bar= 10 μm. DAPI was used as a DNA counter stain. **(B)** Colocalization between TOMM20 (TOMM20-568) and ATP5I (ATP5I-488) signals was analyzed with job plot intensity profile.

Similarly, ATP5I KO cells expressing exogenous ATP5I showed increased NAD^+/NADH ratios (**Figure 5A**) by restoring NAD^+ concentration (**Figure 5 – figure supplement 1A**, **1B**), increased OCR/ECAR ratio (**Figure 5B**), enhancing respiration and reducing compensatory glycolysis (**Figure 5 – figure supplement 1C**, **1D**) to levels comparable to control cells. This metabolic reorganisation made these cells up to 3 times less sensitive to 2-D-deoxyglucose (**Figure 5 – figure supplement 1E**) compared to ATP5I KO cells, thereby alleviating energy stress as seen with less phosphorylated AMPK (**Figure 5C**) and enabling increased cell growth (**Figure 5D**). Restoring the ATP5I expression is also correlated with increased sensitivity to the metabolic effects induced by metformin and phenformin on mitochondrial respiration and glycolysis and decreased OCR/ECAR ratios (**Figure 5 – figure supplement 2**). The rescue also enhanced the antiproliferative effects of metformin, with EC_{50} values up to 5-fold lower than those of ATP5I KO cells (**Figure 5E**), as well as those of phenformin, with EC_{50} values up to 3-fold lower than those of ATP5I KO cells (**Figure 5F**).

In conclusion, these findings collectively demonstrate that the re-expression of ATP5I in ATP5I KO clones rescues mitochondrial morphology, metabolic profile and sensitivity to biguanides, which supports the concept that ATP5I mediates the metabolic and antiproliferative effects of these compounds in KP-4 cells.

Chemogenomic screening of metformin reveals an imprint on F_1F_O-ATP synthase

To obtain an unbiased observation of biological processes affected by metformin, we performed a genome-wide pooled CRISPR/Cas9 KO screen in NALM-6 cells cultured in the presence of metformin at a concentration affecting growth (16 mM). Over the 8-day period of screening, cells had 1.47 population doublings whereas untreated controls had 7.5. Based on the sgRNA frequency changes observed afterward between treated vs untreated samples, we generated enrichment/depletion scores for each genes using the CRANKS algorithm. Many gene KO were then predicted together to potentiate (sgRNA depletion, KO creates sensitivity, negative CRANKS scores) or to suppress (sgRNA enrichment, KO creates resistance, positive CRANKS scores) metformin-induced growth inhibition (**Figure 6A**). Inactivation of the amino acid transporter SLC7A6 that plays a role in arginine export[54] enhanced metformin activity, likely because this protein may also export metformin. Indeed, the end of the R group is very similar chemically to metformin. In addition, disabling the transcription factors SOX4, TCF4, SPI1 and the histone H3K79 methyltransferase DOT1L also enhanced metformin activity perhaps because they promote gene expression programs that compensate the mitochondrial dysfunction associated to metformin action. For example, SOX4 and SPI1 can increase glycolysis by promoting HK2 expression[55,56] while TCF4 increases glycolysis by inducing the expression of the glucose transporter GLUT3[57]. Moreover, metformin can increase DOT1L mediated H3K79 methylation promoting the expression of SIRT3, a mitochondrial deacetylase that stimulates mitochondrial health and biogenesis[58]. On the other hand, many genes were found to be required for metformin-mediated growth inhibition. Of note, DDX3X, an RNA helicase required for the translation of interferon response genes[59], suggests a role for the interferon pathway in the antiproliferative activity of metformin. The actions of metformin were also suppressed by inactivation of the OMA1-DELE1-HRI pathway. OMA1, is a protease localized to the inner mitochondrial membrane activated by mitochondrial stress. DELE1 is a target of OMA1 that interacts with and activates EIF2AKI (also known as heme-regulated inhibitor of HRI). HRI catalyzes eIF2a phosphorylation which in turns activates the translation of ATF4[60] which is also stimulated by DDX3[61]. ATF4 together with ATF3 regulates the expression of prop-apoptotic genes in response to both ER and mitochondrial stress[60,62]. Finally, inactivation of VDAC2, BAK1, HCCS (which makes Cytochrome C), Cytochrome C (CYCS) and APAF1 also conferred resistance to metformin, suggesting that metformin induces the classical mitochondria-related intrinsic apoptosis in treated cells. We compared the pattern of genetic interactions of metformin with that of rotenone, a complex I inhibitor (**Figure 6B**) and oligomycin A, a complex V inhibitor (**Figure 6C**). Interestingly, there were more similarities

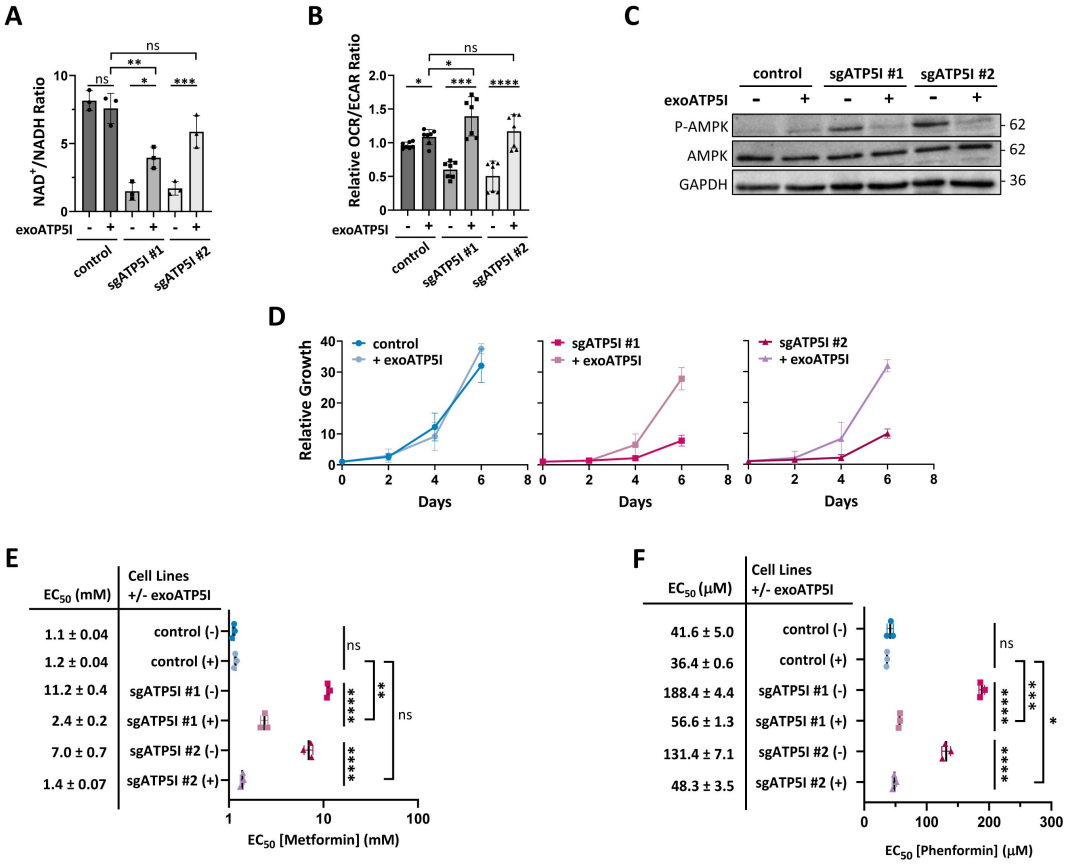

Figure 5.

Re-expression of ATP5I rescues metabolic profile and resensitizes ATP5I knockout pancreatic cancer cells to biguanides.

(A) Quantification of NAD^+/NADH ratio in KP-4 cells expressing exogenous ATP5I (exoATP5I: +) in control sgGFP or a representative clone of two different small guide RNAs (sgATP5I #1 and sgATP5I #2) compared with the same cell lines without expression of exogenous ATP5I (-). Values represent the mean ± standard deviation of three biological replicates. (ns) not significative, (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ using an ordinary one-way ANOVA with Sidak's multiple comparison test. **(B)** Relative quantification of oxygen consumption rate (OCR) over extracellular acidification rate (ECAR) by Seahorse analysis in cells as in (A). Values represent the mean ± standard deviation of at least three biological replicates. (ns) not significative, (*) $P < 0.05$, (***) $P < 0.001$, (****) $P < 0.0001$ using a repeated measures (RM) one-way ANOVA with Sidak's multiple comparison test. **(C)** Immunoblot of total and phosphorylated levels of AMPK (Thr172) protein in extracts from cells as in (A). GAPDH antibody was used as loading control. **(D)** Growth curves of cells as in (A) by measuring the relative number of cells over 6 days. Media was changed every two days. **(E)** EC_{50} values of metformin (Met) treatments in cells as in (A). Values represent the mean ± standard deviation of N=3. (ns) not significative, (**) $P < 0.01$, (****) $P < 0.0001$ using an ordinary one-way ANOVA with Sidak's multiple comparison test. **(F)** EC_{50} values of phenformin (Phen) treatment in cells as in (A). Values represent the mean ± standard deviation of three biological replicates. (ns) not significative, (*) $P < 0.05$, (***) $P < 0.001$, (****) $P < 0.0001$ using an ordinary one-way ANOVA with Sidak's multiple comparison test.

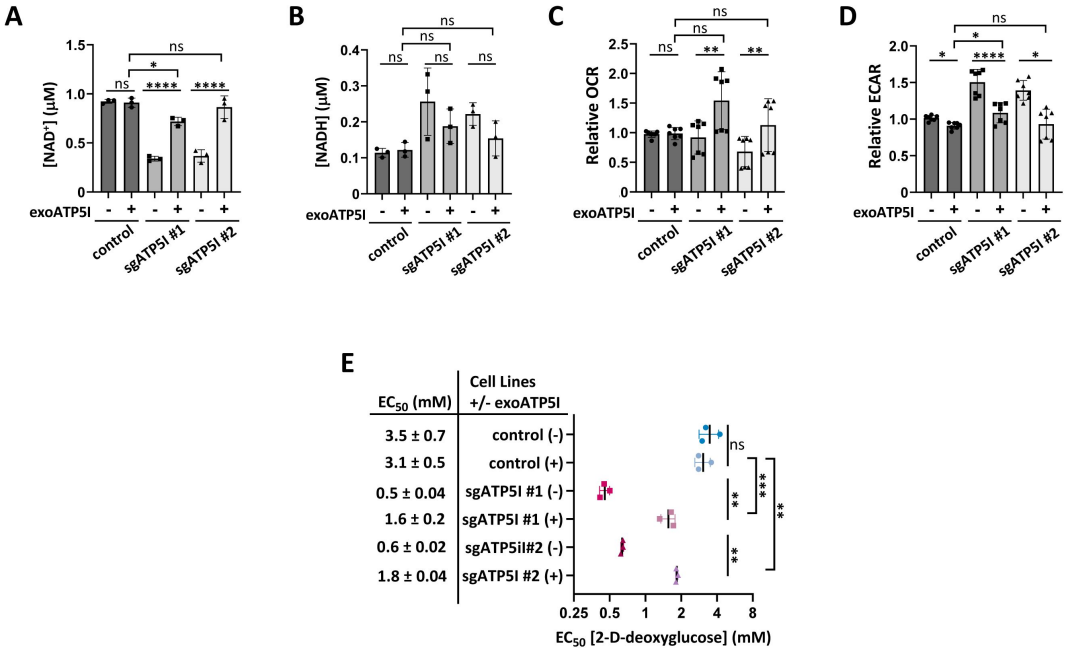

Figure 5 – figure supplement 1.

(A) Quantification of NAD^+ concentration in KP-4 cells expressing exogenous ATP5I (exoATP5I: +) in control sgGFP clones or a representative clone of two different small guide RNAs against ATP5I (sgATP5I #1 and sgATP5I #2) compared with the same cell lines without expression of exogenous ATP5I (-). Values represent the mean ± standard deviation of three biological replicates. (ns) not significative, (*) $P < 0.05$, (****) $P < 0.0001$ using an ordinary one-way ANOVA with Sidak's multiple comparison test. **(B)** Quantification of NADH concentration in cells as in (A). Values represent the mean ± standard deviation of three biological replicates. (ns) not significative using an ordinary one-way ANOVA with Sidak's multiple comparison test. **(C)** Relative quantification of oxygen consumption rate (OCR) by Seahorse analysis in cells as in (A). Values represent the mean ± standard deviation of at least three biological replicates. (ns) not significative, (**) $P < 0.01$ using a repeated measures (RM) one-way ANOVA with Sidak's multiple comparison test. **(D)** Relative extracellular acidification rate (ECAR) by Seahorse analysis in cells as in (A). Values represent the mean ± standard deviation of at least N=3. (ns) not significative, (*) $P < 0.05$, (****) $P < 0.0001$ using a RM one-way ANOVA with Sidak's multiple comparison test. **(E)** EC_{50} values of 2-D-deoxyglucose treatment in cells as in (A). Values represent the mean ± standard deviation of N=3. (**) $P < 0.01$, (***) $P < 0.001$ using an ordinary one-way ANOVA with Sidak's multiple comparison test.

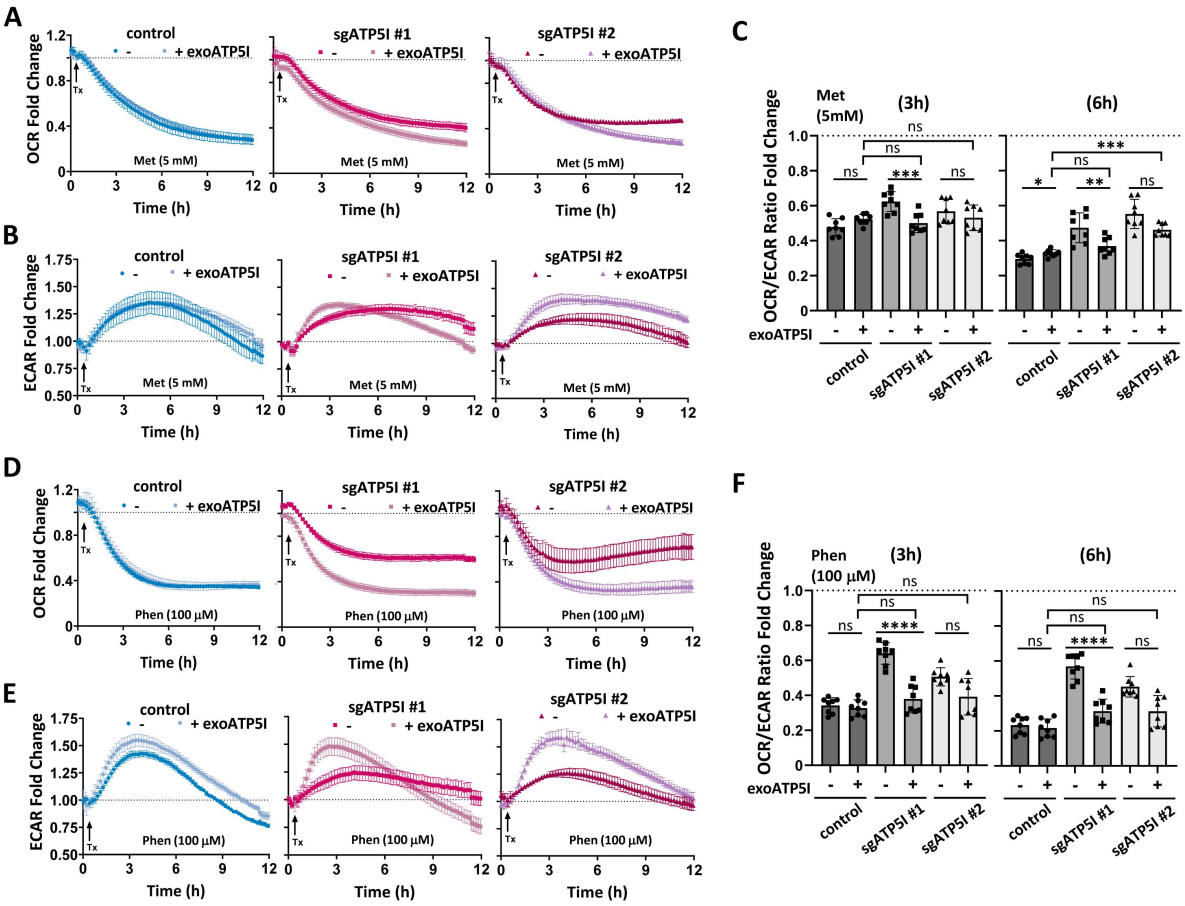

Figure 5 – figure supplement 2.

(A) Representative kinetic curves of oxygen consumption rate (OCR) fold change in KP-4 cells expressing exogenous ATP5I (exoATP5I: +) in control sgGFP clones or a representative clone of each of two small guide RNA against ATP5I (sgATP5I #1 and sgATP5I #2) compared with the same cell lines without expression of exogenous ATP5I (-) treated with 5 mM metformin (Met) using Seahorse. **(b)** Representative kinetic curves of extracellular acidification rate (ECAR) fold change in cells as in (A) treated with 5 mM Met using Seahorse. **(C)** Quantification of OCR/ECAR ratio fold change at 3 and at 6 hours from kinetic curves in (A) and (B). Values represent the mean ± standard deviation of at least three biological replicates. (ns) not significative, (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ using a repeated measures (RM) one-way ANOVA with Sidak's multiple comparison test. **(D)** Representative kinetic curves of OCR fold change in cells as in (A) but treated with 100 µM phenformin (Phen) using Seahorse. **(E)** Representative kinetic curves of ECAR fold change in cells as in (A) but treated with 100 µM Phen using Seahorse. **(F)** Quantification of OCR/ECAR ratio fold change at 3 and at 6 hours from kinetic curves (D) and (E). Values represent the mean ± standard deviation of at least three biological replicates. (ns) not significative, (****) $P < 0.0001$ using a RM one-way ANOVA with Sidak's multiple comparison test.

between oligomycin A and metformin (16 genes in common) than between rotenone and metformin (4 genes in common). Notably, inactivation of the mitochondrial apoptotic pathway through OMA1-DELE1-HRI as well as glycolytic pathway through SOX4 and TCF4 significantly affected both oligomycin A and metformin but not rotenone. On the other hand, DDX3X was required for the actions of all three drugs. Taken together, this genetic experiment shows that the OMA1-DEL1-HRI pathway mediates the antiproliferative activity of both biguanides and the F1ATPase inhibitor oligomycin (**Figure 6D**) while a compensatory glycolysis protects the cells.

Discussion

Using a biologically active biotin functionalized biguanide (BFB), we identified and characterized subunit e of F_1F_0-ATP synthase (ATP5I) as a potential antineoplastic mitochondrial target of medicinal biguanides. Knockdown of ATP5I in KP-4 human pancreatic cancer cells specifically altered the expression of NDUFB8 (complex I) and COX II (complex IV) proteins of the mitochondrial electron transport chain without affecting their mRNA levels, suggesting ATP5I as a role in maintaining OXPHOS protein stability. Thus, metformin binding to ATP5I can directly alter complex V activity and indirectly affect other respiratory complexes.

Interestingly, NDUFB8, a protein we found poorly expressed in ATP5I KO cells, is a target of the mitochondrial protease ClpP, whose activation can inhibit pancreatic adenocarcinoma growth[63]. Our work shows that another protease, OMA1, is required for the action of metformin, suggesting that mitochondrial proteases can have potent antitumor activities. Other studies, using mass spectrometry, revealed interactions between ATP5I and essential components of mitochondrial protein import, such as TIMMDC1, involved in complex I's import and assembly[64,65]. These findings suggest that ATP5I is integral to respirasome assembly and mitochondrial proteome stability. Moreover, recent evidence implicates transmembrane proteins TMEM70 and TMEM242 in complex I and F_1F_0-ATP synthase assembly or subunit expression regulation, linking the ATP synthase complex to complex I[66]. Therefore, biguanides may inhibit complex I activity by binding to and disrupting either complex I or the ATP synthase complex.

Similar to medicinal biguanides effects on KP-4 cells[37], ATP5I depletion leads to mitochondrial structural changes such as mitochondrial fragmentation. Additionally, ATP5I deletion disrupts energy metabolism, decreasing complex I mediated NADH reoxidation and respiration, promoting glycolysis and AMPK activation, and impairing cell growth. Furthermore, ATP5I KO cells exhibit resistance to metformin and phenformin growth effects, indicating its pivotal role in mediating their metabolic and antiproliferative effects. Reintroducing wild-type ATP5I in ATP5I KO cells partially restored their sensitivity to biguanides, consistent with the proposed role of ATP5I being a biguanide target. Of note, the phenotypes observed in the absence of subunit g (ATP5L)[67], the direct partner of ATP5I, and subunit f (ATP5K)[68] in HeLa cells are similar to those of ATP5I mutants and metformin treated cells. Further work is needed to determine how metformin affects the interactions between ATP5I, ATP5L and ATP5K. Together these three proteins are located at the base of F_1F_0-ATP synthase peripheral stalk which interact with OSCP (oligomycin sensitivity conferring protein) located in the F1 portion of the enzyme [69]. ATP5I's movement within F_1F_0-ATP synthase[69] may regulate and transfer information between the F1 and the F0 rotating complex [42,64-66]. These structural connections could underpin the results reported here that metformin and oligomycin share a common genetic signature in a CRISPR screening in NALM-6 cells. Our findings, combined with recent published results, strengthen the correlation between mitochondrial ultrastructure and respiratory activity[70], suggesting that medicinal biguanides partially affect mitochondrial respiratory chain activity by indirectly inhibiting complex I of OXPHOS through ATP5I binding, although the exact complete molecular mechanism remains unclear.

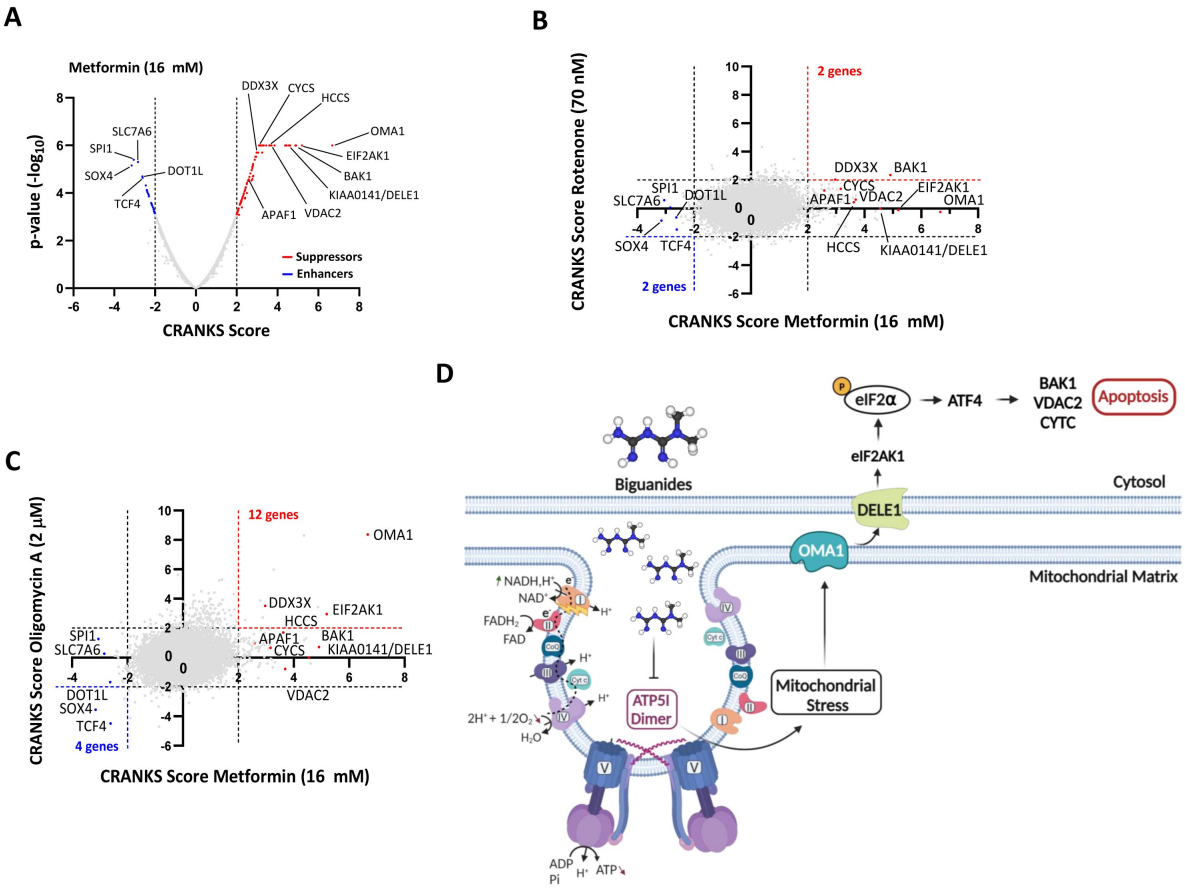

Figure 6.

Chemogenomic screening of metformin reveals an imprint on F_1F_o-ATP synthase.

A) Results of the pooled genome-wide CRISPR/Cas9 KO screen made in NALM-6 cells treated with 16 mM metformin or control. Data are represented as a Volcano plot of gene enrichment/depletion scores vs p-values from using the CRANKS algorithm. Some genes of interest are labeled. Enhancers of metformin growth inhibition with negative CRANKS scores below 2.5 (dashed line) are labeled blue, while suppressors with positive CRANKS scores above 2.5 (dashed line) are labeled red. **B-C)** Pairwise comparison of gene CRANKS scores obtained from screening metformin 16 mM in NALM-6 cells against that from screening either 70 nM rotenone or **(C)** 2 µM oligomycin A. D) Model for metformin action triggering the OMA1-DELE1-HRI pathwat.

The upregulation of ATP5I observed in hypoxia[71], DNA damage[72], liver cancer[73], and low-fat diet responses[74,75] underscores a regulatory role still poorly understood. In another study, RNA-seq data form A549 cells also suggest ATP5I levels influence metformin sensitivity[76], highlighting its potential clinical relevance. Identifying ATP5I as a potential antineoplastic target for medicinal biguanides provides insights into the mechanisms underlying their anticancer effects and opens new avenues for targeting mitochondrial metabolism. These findings also underscore the need for further exploration of ATP5I's functions within F_1F_o-ATP synthase and its connections with other OXPHOS complexes, potentially leading to novel therapeutic strategies for cancer treatment.

Acknowledgements

We wish to express our deepest gratitude to Professor Omichinski, for his expert guidance and unwavering support in the purification of proteins, and also to Professor Masson and Affinité Instrument for their generosity in providing access to the SPR system.

Author contributions

The manuscript was drafted by G.L., with all authors approving the final version. The project was initially launched by M.C.R. and F.M., who conducted the first pull-down experiment and BFB biosynthesis, respectively (**Fig. 1F** and supporting information). V.B. generated the stable ATP5I knockout cells and performed all qPCR analyses (**Fig. 2 – figure supplement 1A**, **1B**). E.L. conducted the Seahorse experiments (**Fig. 3B**, **3E**, **3F**, **3G**, **3I**, **3J**, **3K**, **5B**, **Fig. 3 – figure supplement 1C**, **1D**, and **Fig. 5 – figure supplement 2**). T.B. and M.K. carried out the chemogenomic CRISPR/Cas9 knockout screen (**Fig. 6**). G.L. completed the remaining work. A.R.S., G.F., and S.P.G. supervised the research and finalized the manuscript. All authors declare no conflict of interest.

Materials and methods

Drugs

Metformin hydrochloride was purchased from Combi-Blocks (#ST-9194; San Diego, CA, USA), phenformin hydrochloride was purchased from Sigma Aldrich (#P7045; Oakville, On, Canada), 2-D-deoxyglucose was purchased from Bioshop (#DXG498.5; Burlington, On, Canada) and D-biotin was purchased from Invitrogen/Thermo Fisher Scientific (#B1595; Waltham, MA, USA).

Synthesis

All chemicals were purchased from Sigma Aldrich, Oakwood Chemicals (Estill, SC, USA) and Combi-Blocks in their highest purity and were used without further purification. Deuterated dimethylsulfoxide (DMSO-*d*6), deuterated methanol (MeOD) and deuterium oxide (D_2O) were purchased from CDN Isotopes (Pointe-Claire, Qc, Canada). NMR spectra were recorded on Bruker avance 400 and Bruker avance 500 spectrometers. Coupling constants (J) were reported in hertz (Hz), chemical shifts were reported in parts per million (ppm, δ) and multiplicities were reported as singlet (s), doublet (d), triplet (t) and multiplet (m). ^1H and ^{13}C chemical shifts are relative to the solvents: δH 2.50 δC 39.5 for DMSO-*d*6, δH 3.35, 4.78 and δC (49.3) for MeOD and δH 4.65 for D_2O. Final compounds were purified using a preparative liquid chromatography (prepLC-MS-Quadrupole, Waters) equipped with a C18 reverse phase column (2.1 × 100 mm, 3 µm, Atlantis) with HPLC-grade solvents (Sigma Aldrich). High-resolution mass spectra (HRMS) and LC-MS purity

analysis were collected using LC-TOF with ESI ionisation source (Agilent Technologies, Santa Clara, CA, USA) by the regional mass spectrometry center of University of Montreal. After prep HPLC, all final compounds were subsequently freeze-dried and aliquots of the monochloride salt powder were carefully weighed.

Cell culture

Human pancreatic ductal carcinoma cell line KP-4 was as a kind gift from Dr. N. Bardeesy (Massachusetts General Hospital, Boston). Human embryonic kidney HEK-293T cells were obtained from Thermo Fisher Scientific. Phoenix ampho packaging cells was as a kind gift from Dr. S.W. Lowe (MSK, New York). NALM6 cells were a gift from Stephen Elledge (Harvard, USA). Except for NALM-6 cells which were cultured in RPMI medium (#350-035; Wisent, All cells were cultured in Dulbecco's modified Eagle medium (DMEM #319-015; Wisent, St-Jean-Batiste, Qc, Canada) supplemented with 10% fetal bovine serum (FBS, Wisent), 2 mmol/L L-glutamine (Wisent) and 1% penicillin/streptomycin sulfate (Wisent) in a humidified incubator at 37 °C with 5% CO_2.

Immunoblotting

For immunoblotting, proteins extracts were obtained from 6-well cell culture dishes at 80-90 % confluence. Cells were treated with the corresponding drugs or vehicle 16 hours after seeding. As previously described[77], cells were washed twice with ice-cold phosphate-buffered saline solution (PBS), then the residual PBS was removed by aspiration. Cells were lysed in 250 µL of 2x Laemmli buffer (4% SDS, 20% glycerol, 120 mM Tris-HCl pH 6.8), recovered using a cell scraper and transferred into Eppendorf tubes for a mild sonication of 20s followed by heating for 5 min at 95 °C. Protein extracts were cooled to room temperature (rt) and quantified by measuring absorbance at 280 nm using Nanodrop microvolume spectrophotometer (2000c, Thermo Fisher Scientific). Samples were then diluted to a final concentration (2 mg/mL) using a modified Laemmli Buffer (10% β-mercapthoethanol, 0.1% bromophenol, 2x Laemmli) and were kept at - 20 °C until use. A quantity of 25 to 40 µg of protein extracts were loaded into SDS-PAGE (12-15% for resolving gels, 4% for stacking gels) and run in Tris-Glycine SDS buffer (192 mM glycine, 0.1% SDS, 25 mM Tris-Base) at 90 V. SDS-PAGE were then transferred on a nitrocellulose membrane (0.45 µm, Bio-Rad, Mississauga, On, Canada) in Tris-Glycine buffer (96 mM glycine, 10 mM Tris-Base) for 1h30 at 120 V. Membranes were blocked in a modified Tris-buffered saline + 0.05% Tween-20 (TBST) solution with 5% skim milk for 1h at rt, washed with TBST 3x 5 min at rt and were then incubated with an appropriate diluted solution of primary antibody (in 0.1% BSA, 0.02% sodium azide, PBS pH 7.4) overnight (o/n) at 4°C. After primary antibody incubation, membranes were washed with TBST 3x 5 min and incubate with a diluted (1:3000 in 1% skim milk/TBST) secondary antibody coupled HRP for 1 hour at rt. After secondary antibody incubation, membranes were washed with TBST 3x 5 min. The signal was revealed using enhanced chemiluminescence reagent (ECL), (#RPN2106, GE Healthcare Life Sciences, Chicago, IL, USA) and images were acquired by exposition with autoradiographic films or using ChemiDoc Imaging Sytems (XRS+; Bio-Rad). FroggaBio BLUelf Prestained Protein ladder (FroggaBio, Concord, Om, Canada) was used to estimate protein molecular weight. Of note, most membranes were cut into pieces and were incubated with several antibodies. The following primary antibodies were used: anti-phospho-ACC S79 (Rabbit, 1:1000, #3661S, Cell signaling, Danvers, MA, USA), anti-AMPK (Rabbit, 1:1000, #2532, Cell signaling), anti-phospho-AMPK T172 (Rabbit, 1:1000, #2531, Cell signaling), Anti-ATP5I (Rabbit, 1:500, #16483-1-AP, Proteintech, Rosemont, IL, USA), anti OXPHOS cocktail human (Mouse, 1:750, #ab110411, Abcam, Toronto, ON, Canada), anti-GAPDH (Goat, 1:3000, #NB300-320, Novus Biologicals, Centennial, CO, USA), anti-α-Tubuline (Mouse, #T6074, Sigma Aldrich) and anti-β-Actin (Mouse, 1:10000, #3700, Cell signaling). The following secondary antibodies were used: anti-rabbit IgG conjugated to HRP (Goat, 1:3000, #170-6515, Bio-Rad), anti-mouse IgG conjugated to HRP (Goat, 1:3000, #170-6516, Bio-Rad) and anti-goat IgG conjugated to HRP (donkey, #Sc-2020, Santa Cruz Biotechnology, Dallas, TX, USA).

Immunofluorescence

For immunofluorescence experiments, 50,000 - 100,000 cells were seeded on 1.5 mm thickness coverslips and were incubated for 48 h. As previously described[78], cells were washed twice with ice cold PBS, then were fixed with a 4% paraformaldehyde solution for 10 min at 4 °C and were washed 3x 10 min with a solution of PBS + 0.1 M glycine at rt with agitation. At this moment coverslips were sometime stored in PBS + 0.2 % Sodium Azide at 4°C until use. If stored, 3x 10 min washing steps with PBS were performed to remove the azide. Cells were then permeabilized with PBS + 0.2 % Triton X-100 and 3% Bovine Serum Albumin (BSA) for 5 min at rt. Then, cells were washed in a blocking solution (3 % BSA in PBS pH 7.4) 3x 10 min at rt with agitation and were then incubated with an appropriate diluted solution of primary antibody (in 3% BSA, PBS pH 7.4) o/n at 4°C in a humidified chamber. After primary antibody incubation, cells were washed in blocking solution 3x 10 min and were incubated with an appropriate diluted solution (3% BSA, PBS pH 7.4) of secondary AlexaFluor antibody for 1h at rt in the dark. Cells were then washed with PBS 3x 10 min and coverslips were mounted on glass coverslip in Vectashield mounting media containing DAPI (H-1200-10, Vector Laboratories, Newark, CA, USA) and were sealed off with nail polish and kept for a minimum of 24 hours at 4°C. Images were collected with the Zeiss Axio Imager Z2 upright microscope equipped with a CoolSNAP FX camera (Photometrics), Axiocam camera and ZEN 2 blue edition software and were analyzed using ImageJ software. Colocalization was assessed using the Job Plot profile functionality of ImageJ by determining fluorescence intensity for each pixel of each channel across a line drawn. For quantifications and colocalizations, raw data were exported to Prism (10.2.2, GraphPad) software to generate final figures. The following primary antibodies were used: Anti-ATP5I (Rabbit, 1:100, #16483-1-AP, Proteintech) and TOMM20 (Mouse, 1:100, #sc-104216, Santa Cruz Biotechnology). The following secondary antibodies were used: anti-mouse AF488 (Goat, 1:2000, #A-11029, Invitrogen), anti-rabbit AF568 (Goat, 1:2000, #A-11011, Invitrogen) and Streptavidin AF488(#S11223, Invitrogen).

Mitochondrial protein isolation

Mitochondrial crude extracts were obtained using Mitochondria Isolation Kit for Cultured cells (ab110170, Abcam) according to manufacturer's instructions. For each condition, 1x 15 cm cell culture dish of KP-4 cells was washed twice in ice-cold PBS, then the residual PBS was removed by aspiration. Cells were then scraped in ice-cold PBS and transferred in Eppendorf tubes. The following steps for mitochondrial isolation extraction were performed as described in the manufacturer's instructions. The pellet containing mitochondrial proteins was resuspended in lauryl maltoside buffer [1% lauryl maltoside, cOmplete-EDTA free Protease Inhibitor Cocktail (Roche, Laval, Qc, Canada), PhosSTOP (Roche), PBS pH 7.8]. The concentrations of mitochondrial proteins were determined using the bicinchoninic acid assay (BCA) (23225, Pierce). Of note, at the end of isolation, we obtained ~ 0.7-1 mg of mitochondrial proteins per 15 cm dish.

Pull-down assay

For pull-down assay, 1 mg of mitochondrial proteins were incubated with either 1 mM of biotin functionalized biguanide (BFB) or biotin functionalized amine (BFA) in Eppendorf tubes for 3h at rt with mild agitation. In parallel, Dynabeads MyOne streptavidin C1 (65001, Thermo Fisher Scientific) were washed and resuspended at least three times in lauryl maltoside (LM) buffer [1% lauryl maltoside, cOmplete-EDTA free Protease Inhibitor Cocktail (Roche), PhosSTOP (Roche), PBS pH 7.8]. After incubation with biotinylated molecules, beads (0.5 mg ~ 50 µL) were added to mitochondrial lysates and incubated for 30 min at 4°C with agitation. Beads were respectively recovered with a magnet, washed in LM buffer 3x 30 min at 4°C. Elution of bound proteins from beads in BFB condition was performed by adding 30 µL of 50 mM metformin in LM buffer. After an incubation time of 30 min at rt, beads were vortexed, pelleted with a magnet and the supernatant was collected in a new tube. Proteins were then denatured by adding 30 µL of 6x SDS-loading buffer (30% glycerol, 10% SDS, 1% bromophenol blue, 15% β-mercaptoethanol, 0.5 M Tris-

HCl pH 6.8) to the mixture and boiled 3x 5 min at 95°C. Elution of bound proteins for BFA condition and remaining proteins on beads for BFB condition were achieved by boiling and vortexing the corresponding beads in 60 µL of 6x SDS loading buffer 3x 5 min at 95°C. After denaturation of proteins, all samples were loaded into SDS–PAGE. The resulting gel was stained with Coomassie brilliant blue R-250 (#33445225GM, Thermo Fisher Scientific) then washed with a destaining solution (40% water, 50% methanol, 10% acetic acid). Gel segments were then excised, digested with trypsin and dried overnight in a cold trap (Labconco, Kansas City, MO, USA). Three samples for each elution condition were prepared in two technical replicates and were then analyzed by the Mass spectrometry platform at IRIC (Institute for Research on Immunology and Cancer). It's worth mentioning that for pull-down validation, same experiments were performed using western-blot analysis with anti ATP5I antibody after protein denaturation steps (see immunoblotting section).

Constructs

For recombinant protein expression in bacteria, full-length of human ATP5I sequence was PCR-amplified and cloned using BamH1/EcoRI restriction sites into a pET-TEV vector (Addgene, Watertown, MA, USA) for the expression of a N-terminal 6x-His-tagged protein. This plasmid was obtained as a kind gift from Dr. J.G. Omichinski (University of Montreal, Montreal). For lentiviral mediated transduction in KP-4 cells, annealed and phosphorylated oligos for two RNA guides (sgRNAs) targeting two regions of ATP5I's cDNA (sgATP51 #1 and sgATP5I #2) and one RNA guide targeting GFP (sgGFP) sequence were subcloned into BsmBI restriction site of lentiCRISPRv2 plasmid (#52961, Addgene). For re-expression of ATP5I in control (sgGFP) and ATP5I knockout (KO) (sgATP5I#1 and #2) cells, full-length human ATP5I sequence with modified codons (same amino acid but different nucleic acid sequence) was generated by PCR site-directed mutagenesis and cloned using BamH1/EcoRI restriction sites into MSCV retroviral vector. All constructs were confirmed by DNA sequencing.

Constructs primers used:

Target	5' forward primer	3' reverse primer
For recombinant protein expression:		
ATP5I (ATP5ME)	GAATGGATCCGCCGCCATGGTGCC ACCGGTGCA	ATCGGAATTCTCACTTTAATATGCTGTCATC TTCTGCC
For lentiviral mediated transduction		
sgGFP		GGGCGAGGAGCTGTTCACCG
sgATP5I #1		CGTAGGCCACACCGAGGAAC
sgATP5I #2		TGGCTCCGTAGGCCACACCG
For PCR site-directed mutagenesis		
	GAATGGATCCGCCGCCATGGTGCC ACCGGTGCA	CGCACCATATGCGACCCCTAGAAAGAGCGC GGAGTAGCGGCCGAGCTTGAT
	GCGCTCTTTCTAGGGGTCGCATAT GGTGCGACGCGCTACAATTACCTA A	ATCGGAATTCTCACTTTAATATGCTGTCATC TTCTGCC

Protein expression and purification

N-terminal 6x-His-tagged ATP5I was expressed in Rosetta E. coli BL21 competent cells (#70954, Addgene) for the expression of eukaryotic proteins that contain rare codons. To overexpress recombinant protein as suggested[79], cells were grown at 37°C in terrific broth (TB) medium supplemented with 1% of ethanol (v/v), 100 mg/mL ampicillin and 50 mg/mL chloramphenicol to an OD_{600nm} of 0.8. Expression was induced for 4h at 30°C with 1 mM isopropyl β-D-1-thiogalactopyranoside then cells were harvested by centrifugation (15 min, 5000 rpm at 4°C). Bacterial pellets were then solubilized in buffer A (6 M guanidine, 500 mM NaCl, 20 mM NaH_2PO_4 pH 8) for 30 min at 4°C and lysed by sonication. Cell lysate was then harvested by centrifugation (30min, 13 000 rpm at 4°C) and after filtration (0.45µm) the supernatant was loaded in an

immobilized metal ion affinity chromatography column (HisTrap FF, Cytiva) with buffer A. After removing non-specifically bound molecules (flow through), the column was washed with buffer B (8M urea, 500 mM NaCl, 20 mM NaH_2PO_4 pH 8) then with buffer C (20 mM imidazole, 500 mM NaCl, 20 mM NaH_2PO_4 pH 8) until UV absorbance returned to baseline. After washing, the column was eluted with buffer D (500 mM imidazole, 500 mM NaCl, 20 mM NaH_2PO_4 pH 8) and the resulting eluate was loaded in a desalting column (HiPrep 26/10, Cytiva) with a PBS containing 125 mM NaCl, 5 mM DTT, PBS pH 7.4) and concentrated in a 3 kDa centrifugal filter (Amicon) to 1.5 mg/mL. Of note, all purification steps were done with AKTA pure chromatography system.

Surface plasmon resonance (SPR)

Experiments were performed at 25°C with a portable SPR (P4SPR) device from Masson's laboratory (WO/2010/130045, Affinité instruments) using a compatible prism coated with a 200 nm streptavidin (SA) derivatized carboxymethyldextran hydrogel (Xantec bioanalytics) for immobilisation of biotinylated ligands. For each solution, a volume of 300 µL was injected in the flow cells comprising a multifluidic channel cell and a reference cell. After having stabilized the baseline (~30 min) with running buffer (125 mM NaCl, 5 mM DTT, PBS pH 7.4), biotin functionalized biguanide chloride salt was immobilized on SA sensor chip at 125 µM (concentration experimentally determined to saturate streptavidin sites). Then, the chip was washed with running buffer until stabilising the baseline. After that, several concentrations of purified recombinant ATP5I were injected until saturation of the signal. Of note, between each concentration added, washing steps were performed with running buffer to remove non-specifically bound molecules. Finally, the surface was regenerated with urea (2M) prepared in milli-Q-water. It's worth nothing that due to the low dissociation constant of biotin/streptavidin interaction, biotinylated ligands are almost irreversibly bound that make it difficult to regenerate the chip. The sensorgrams were analysed with P4SPR Control v2.022 software and final figures were exported to Prism (10.2.2, GraphPad) using a pseudo-first order association kinetics equation for each concentration. For binding affinity curve, each steady state for each concentration were exported to Prism (10.2.2, GraphPad) software to generate the figure using a one site-specific binding equation. Of note, compounds were dissolved in the running buffer.

Generation of stable ATP5I knockout cells

Knocking out ATP5I was realized in KP-4 cell line using lentiviral CRISPR/Cas9 technology with sgRNAs against ATP5I or a control sgRNA (see construct section). First, 5×10^6 293T cells were seeded in 10 cm plates and grown for 16 hours. They were then transfected using the calcium-phosphate precipitation method as previously described[80] with 3 µg of lentiCRISPRv2 plasmid vector, 2 µg of the *pCMV-dR8.2* plasmid and 1 µg of the *VSV-G* envelope protein expression plasmid (both king gift of Dr. N. Bardeesy). After 16 h, 10 mM sodium butyrate (Sigma Aldrich) was added for a minimum of 6 h, and then the medium was changed. In parallel, target cells were seeded to obtain 60% confluence the following day. Media from the transfected cells were collected the following day, filtered through a 0.45 µm filter. This viral soup was supplemented with 4 µg/mL polybrene (Sigma Aldrich) and 10% fresh media before being placed on KP-4 receiving cells. Viral soup was removed 24 hours later for fresh DMEM cell culture media (see cell culture section) supplemented with 100 $\mu g.mL^{-1}$ sodium pyruvate (#600-110-EL, Wisent) and 50 $\mu g.mL^{-1}$ uridine (#URD222.10, Bioshop) to help them growth in case mitochondrial functions were affected[81]. KP-4 infected cells were selected 6 hours after media change with 2ug/mL puromycin (#400-160-EM, Wisent). After two weeks in culture to ensure time alterations in the targeted regions with CRISP/CAS9, cells were replated highly diluted to isolate clones of cells. Many clones were grown and characterized by ATP5I immunoblots to select proper ATP5I knock-out.

Retroviral infections

ATP5I re-expression was performed by retroviral-mediated expression of wild-type ATP5I in control (sgGFP) and ATP5I KO cells (sgATP5I #1 and #2) with a MSCV-ATP5I vector (see constructs).

For retroviral infections, 5×10^6 Phoenix-Ampho packaging cells were seeded in 10 cm cell culture dishes and grown for 24 hours. Then, cells were transfected with 20 µg of MSCV-ATP5I vector and 10 µg of Helper envelope protein expression plasmid using calcium phosphate method as previously described[80]. The next day, sodium butyrate was added at 10mM to the dishes for 6 hours and then fresh medium was added. In parallel, target cells were seeded to obtain 60-70% on confluence the day of infection. Two days following transfection, the viral soup from one 10 cm dish was filtered through a 0.45µm filter, supplemented with 4 $\mu g.ml^{-1}$ polybrene (#H-9268, Sigma Aldrich) and 1/10 (v/v) of fresh media and then used to infect one 10 cm dish of target cells. After at least 12 h post infection, infected cells were seeded into a new dish for passage. After at least 4 hours of adherence, infected cells were selected using 50 µg/mL hygromycin (#400-141-UG, Wisent). Of note, the selection was maintained for the length of the experiments.

Quantitative PCR (qPCR)

RNA isolation and qPCR quantifications were performed exactly as previously described[77] using the following primers (see below, BioCorp, Pierrefonds, Qc, Canada). Mitochondrial to nuclear DNA ratio were performed similarly by qPCR but with primer targeting DNA and on 30 ng of genomic DNA purified from cultures KP-4 cells. For the isolation, one 6 cm plate of sub confluent KP-4 cells were rinsed twice in PBS, collected by scraping in 500µL of SNET lysis buffer (5 mM EDTA, 400 mM NaCl, 1% SDS, 400 ug/mL Proteinase K, 20mM Tris-HCL pH8.0 [Sigma Aldrich]) and incubated at 55°C overnight. Equal volume of phenol:chloroform (Sigma Aldrich) was added and left to agitate for 30 min at rt. After a 5-minute centrifugation (16,000g), the aqueous phase was transferred to a new tube and precipitated by an equal volume of isopropanol, mixed well and DNA was collected by centrifugation at maximum speed (16,000g) for 15 minutes at 4°C. Pellet was washed with 70% ethanol, air dried and resuspended in TE.

qPCR primers used:

Target	5' forward primer	3' reverse primer
For mRNA expression:		
HMBS	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC
TBP	GCTGGCCCATAGTGATCTTTGC	CTTCACACGCCAAGAAACAGTGA
NDUFB8	CCGCCAAGAAGTATAATATGCGT	GTATCCACACGGTTCCTGTTGT
SDHB	CACAGCTCCCCGTATCAAGAAA	TGCATGATCTTCGGAAGGTCAAA
UQCRC2	TTCAGCAATTTAGGAACCACCCA	GGTCACACTTAATTTGCCACCAA
COXII (MT-CO2)	CCGCCATCATCCTAGTCCTCAT	GGTGGCCAATTGATTTGATGGT
ATP5A(ATP5F1A)	TATGACGACTTATCCAAACAGGC	CGGGAGTGTAGGTAGAACACAT
For mitochondrial/nuclear ratio:		
MT-DNA	GATTTGGGTACCACCCAAGTATTG	GTACAATATTCATGGTGGCTGGCA
Nuclear-DNA	TTCACTTCCCCTTGGCCACAACAT	TGTTCCATGCAGGGGAAAACAAGC

NAD^+/NADH quantification

For NAD^+/NADH quantifications, 5×10^5 cells were seeded in 10 cm cell culture dishes for technical duplicates and incubated for 48h. NAD^+/NADH ratio were quantified using a fluorometric kit assay (#MAK460-1KT, Sigma Aldrich) following manufacturer's instructions. For sample preparation, 9×10^5 cells were pelleted and homogenized with corresponding extraction buffers to be in the linear range of the kit. Fluorescence intensities were measured at λ_{Ex} = 530 nm/λ_{Em} = 585 nm on SPARK 10 M (TECAN) and data were exported to Prism (10.2.2, GraphPad) software to generate final figures.

Seahorse experiments

Seahorse XFe96 cell culture microplates (Agilent) were coated with 50 µg/mL of Poly-D-Lysine (#A3890401, Thermo Fisher Scientific) in D-PBS (#311-425-CL, Wisent) for 1 hour at room temperature and extensively washed with sterile water. Seahorse XFe96 sensor cartridges (Agilent) were calibrated according to the manufacturer's protocol. Assay media was prepared as

follows: 828 g DMEM without L-Glutamine, phenol red, sodium pyruvate, and sodium bicarbonate (#219-060-XK, Wisent), 29.2 mg L-glutamine (#AC386032500, Fisher Chemical), 450.4 mg D-glucose (#D-16-500, Fisher Chemical), 100 µL HEPES 1M (#330-050, Wisent), completed at 100 mL with Milli-Q water and adjusted pH at 7.2. Cells were plated in Poly-D-Lysine-coated microplates at a density of 40,000 cells/well in 100 µL of assay media. Following a 30-minute incubation at room temperature, 75 µL of assay media was added to each well, and the plate was transferred to an XFe96 analyzer. Typical runs were composed of the following steps: 1) baseline measurements for 18 minutes (3 cycles, each of 3-minute mixing and 3-minute measurement), 2) single drug injection (5 mM metformin, 100 µM phenformin, or sterile water as vehicle control), and 3) measurements over a period of 10.5 hours (63 cycles, each of 6-minute mixing, 1-minute pause, and 3-minute measurement). Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were extracted from the Wave software (Agilent) and all time points were normalized to the first measurement. Data were then exported to Prism (10.2.2, GraphPad) software to generate final figures.

Crystal violet cell number assay

The crystal violet staining retention assay[82] was used to estimate cell growth and viability by measuring absorbance at 590 nm in a microplate reader (SpectraMax 190 microplates, Molecular Devices).

For the growth assay 5,000 cells were seeded in 4 different 6-well cell culture dishes in technical triplicates and incubated for 4 different time points up to 6 days. Of note, media was replaced every 48 hours. At indicated times, cells were washed twice in PBS and fixed in 1% glutaraldehyde solution (PBS) for 10 min at rt. After fixation, cells were washed twice in PBS. Once all time point were collected, plates were stained in a 0.05 % crystal violet solution (PBS) for 30 min with agitation. After staining, plates were washed five times in water and were dried for 24 hours. The next day, crystal violet retained in cells were solubilized in 10 % acetic acid solution for 15 min with agitation and transferred into a 96 well plates for absorbance measurement. Data were then exported to Prism (10.2.2, GraphPad) software to generate final figures.

For the cell viability assay, 1,000 cells were seeded in 96 well plates in technical triplicates. After 24 hours, cells were treated at different concentrations with the corresponding drugs and were grown for 72 hours. Crystal violet staining was performed as described for the growth assay. The half minimal effective concentrations were obtained using a fit a curve with non-linear regression log(inhibitor) vs. response -variable slope (four parameters) from the corresponding dose response curves with Prism (10.2.2, GraphPad) software. Importantly, because growth rate was affected in ATP5I KO cells generating variabilities, cell viability assays comparing ATP5I KO with control cells were done in media supplemented with pyruvate and uridine (see generation of stable ATP5I KO cells section). Of note, drugs were dissolved in the corresponding media.

Chemogenomic CRISPR/Cas9 KO screen in NALM-6 cells

The Genome-wide pooled CRISPR/Cas9 KO screens made in the presence of chemicals inhibiting growth were performed by the ChemoGenix platform (IRIC, Université de Montréal; *https://chemogenix.iric.ca/*) as previously described[83]. Briefly, a NALM-6 clone bearing an integrated doxycycline-inducible Cas9 expression cassette generated by lentiviruses made from pCW-Cas9 (Addgene #50661) was transduced with the genome-wide KO EKO sgRNA library[83] (278,754 different sgRNAs). After thawing the library from liquid N_2 and letting it recover in 10% FBS RPMI for 1 day, KOs were induced for 7 days of culture with 2 ug/mL doxycycline. The pooled library was then split in different T-75 flasks (28×10^6 cells per flask; a representation of 100 cells/sgRNA) in 70 mL at 4×10^5 cells/mL. Cells were treated with 16 mM Metformin (using 1M stock solution prepared in media) or 70 nM rotenone (Sigma, R8875) or 2µM oligomycin A (Tocris Bioscience, 4110) (both from 1000X stock solutions prepared in DMSO) for 8 days with monitoring of growth every 2 days, diluting back to 4×10^5 cells/mL and adding more compounds to maintain same final

concentration whenever cells reached 8×10^5 cells/mL. Over that period, treated cells had respectively 1.47, 2.93 & 4.14 population doublings whereas no solvent or DMSO controls had about 7.5. Cells were collected, genomic DNA extracted using the Gentra Puregene kit according to manufacturer's instructions (QIAGEN), and sgRNA sequences PCR-amplified as described[83]. SgRNA frequencies were obtained by next-generation sequencing (Illumina NextSeq 500). Reads were aligned using Bowtie2.2.5 in the forward direction only (norc option) with otherwise default parameters and total read counts per sgRNA tabulated. Context-dependent chemogenomic interaction scores were calculated from comparing sgRNA frequency changes against negative controls from using a modified version of the RANKS algorithm[83] which uses guides targeting similarly essential genes as controls to distinguish condition-specific chemogenomic interactions from non-specific fitness/essentiality phenotypes. Raw read counts are available upon request directly from the ChemoGenix platform.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (10.2.2) software. Unpaired two-tailed Student's *t*-test or Ordinary one-way ANOVA with compensation of multiple comparison with Sida'k were used to determine significance except for seahorse analysis where paired two-tailed Student's *t*-test and RM one-way ANOVA were preferred. A value of $p < 0.05$ was considered significant. All specific statistical details can be found in the corresponding legends.

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Reviewer #1 (Public review):

Summary:

In the manuscript entitled 'The Role of ATP Synthase Subunit e (ATP5I) in 1 Mediating the Metabolic and Antiproliferative 2 Effects of Biguanides', Lefrancois G et al. identifies ATP5I, a subunit of F1Fo-ATP synthase, as a key target of medicinal biguanides. ATP5I stabilizes F1Fo-ATP synthase dimers, essential for cristae morphology, but its role in cancer metabolism is understudied. The research shows ATP5I interacts with a biguanide analogue, and its knockout in pancreatic cancer cells mimics biguanide treatment effects, including altered mitochondria, reduced OXPHOS, and increased glycolysis. ATP5I knockout cells resist biguanide-induced antiproliferative effects, but reintroducing ATP5I restores the effects of metformin and phenformin. These findings highlight ATP5I as a promising mitochondrial target for cancer therapies. The manuscript is well written.

Strengths:

Demonstrated the experiments in systematic and well-accepted methods.

Weaknesses:

The significance of the target molecule and mechanisms may help in understanding the molecular mechanisms of metformin.

https://doi.org/10.7554/eLife.102680.1.sa3

Reviewer #2 (Public review):

Summary:

The mechanism(s) by which the therapeutic drug metformin lowers blood glucose in type 2 diabetes and inhibits cell proliferation at higher concentrations remain contentious. Inhibition of complex 1 of the mitochondrial respiratory chain with consequent changes in cellular metabolites which favour allosteric activation of phosphofructokinase-1, allosteric inhibition of fructose bisphosphatase-1 and cAMP signalling and activation of AMPK which phosphorylates transcription factors are candidate mechanisms. The current manuscript proposes the e-subunit of ATP-synthase as a putative binding protein of biguanides and demonstrates that it regulates the expressivity of the Complex 1 protein NDUFB8.

Strengths:

(1) The metformin conjugate and metformin show comparable efficacy on inhibition of cell proliferation in the millimolar range.

(2) Demonstration of compromised expression of the Complex I protein NDUFB8 by the ATP5I knockout and its reversal by ATP5I expression is an important strength of the study. This shows that the decreased "sensitivity" to metformin in the ATP5I knock-out cells could be due to various proteins.

(3) Demonstration of converse effects of ATP5I KO and re-expression ATP5I on the NAD/NADH ratio.

Weaknesses:

(1) The interpretation of the cellular co-localization of the biotin-biguanide conjugate with TOMM20 (Figure 1-D) as mitochondrial "accumulation" of the conjugate is overstated because it cannot exclude binding of the conjugate to the mitochondrial membrane. It would have been more convincing if additional incubations with the biotin-biguanide conjugate in combination with metformin had shown that metformin is competitive with the biotin-conjugate.

(2) The manuscript reports the identification of 69 proteins by mass spectrometry of the pull-down assay of which 31 proteins were eluted by metformin. However, no Mass Spectrometry data is presented of the peptides identified. The methodology does not state the minimum number of peptides (1, 2?) that were used for the identification of the 31/69 proteins.

(3) The validation of ATP5I was based on the use of recombinant protein (which was 90% pure) for the SPR and the use of a single antibody to ATP5I. The validity of the immunoblotting rests on the assumption that there is no "non-specific" immunoactivity in the relevant mol wt range. Information on the validation of the antibody would be helpful.

(4) Knock-out of ATP5I markedly compromised the NAD/NADH ratio (Fig.3A) and cell proliferation (Figure 3D). These effects may be associated with decreased mitochondrial membrane potential which could explain the low efficacy of metformin (and most of the data in Figures 3-5). This possibility should be discussed. Effects of [metformin] on the NAD/NADH ratio in control cells and ATP5I-KO would have been helpful because the metformin data on cell growth is normalized as fold change relative to control, whereas the NAD/NADH ratio would represent a direct absolute measurement enabling comparison of the absolute effect in control cells with ATP5I KO.

(5) Figure-6 CRISPR/Cas9 KO at 16mM metformin in comparison with 70nM rotenone and 2 micromolar oligomycin (in serum-containing medium). The rationale for the use of such a high concentration of metformin has not been explained. In liver cells metformin

concentrations above 1mM cause severe ATP depletion, whereas therapeutic (micromolar) concentrations have minimal effects on cellular ATP status. The 16mM concentration is ~2 orders of magnitude higher than therapeutic concentrations and likely linked to compromised energy status. The stronger inhibition of cell proliferation by 16mM metformin compared with rotenone or oligomycin raises the issue of whether the changes in gene expression may be linked to the greater inhibition of mitochondrial metabolism. Validation of the cellular ATP status and NAD/NADH with metformin as compared with the two inhibitors could help the interpretation of this data.

https://doi.org/10.7554/eLife.102680.1.sa2

Reviewer #3 (Public review):

Most of the data are based on measurements of the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measured by the Seahorse analyser in control and ATP5l KO cells. However, these measurements are conducted by a single injection of a biguanide, followed over time and presented as fold change. By doing so, the individual information on the effect of metformin and derivate on control and KO cells are lost. In addition, the usual measurement of OCR is coupled with certain inhibitors and uncouplers, such as oligomycin, FCCP, and Antimycin A/rotenone, to understand the contribution of individual complexes to respiration. Since biguanides and ATP5l KO affect protein levels of components of complex I and IV, it would be informative to measure their individual contributions/effects in the Seahorse. To further strengthen the data, it would be helpful to obtain measurements of actual ATP levels in these cells, as this would explain the activation of AMPK.

The authors report on alterations in mitochondrial morphology upon ATP5l KO, which is measured by subjective quantifications of filamentous versus puncta structures. Fiji offers great tools to quantify the mitochondrial network unbiasedly and with more accuracy using deconvolution and skeletonization of the mitochondria, providing the opportunity to measure length, shape, and number quantitatively. This will help to understand better, whether mitochondria are really fragmented upon ATP5l KO and rescued by its re-introduction.

Finally, the authors report in the last part of the paper a genetic CRISPR/Cas9 KO screen in NALM-6 cells cultured with high amounts of metformin to identify potential new mediators of metformin action. It is difficult to connect that to the rest of the paper because a) different concentrations of metformin are used and b) the metabolic effects on energy consumption are not defined. They argue about the molecular function of the obtained hits based on literature and on a comparison of the pattern of genetic alterations based on treatments with known inhibitors such as oligomycin and rotenone. However, a direct connection is not provided, thus the interpretation at the end of the results that "the OMA1-DEL1-HRI pathway mediates the antiproliferative activity of both biguanides and the F1ATPase inhibitor oligomycin" while increasing glycolysis, needs to be toned down. This is an interesting observation, but no causality is provided. In general, this part stands alone and needs to be better connected to the rest of the paper.

https://doi.org/10.7554/eLife.102680.1.sa1

Author response:

> ***Reviewer #1 (Public review):***
>
> *The significance of the target molecule and mechanisms may help in understanding the molecular mechanisms of metformin.*

We greatly appreciate the reviewer's insightful comment regarding the significance of the target molecule and its mechanisms in understanding the molecular actions of metformin. ATP5I is responsible for the dimerization of the F_1F_0-ATPase(1-3). Hence, we propose conducting BN-PAGE followed by a western blot using the β-subunit of the F1 domain of F1F0-ATP synthase to investigate whether metformin affects its dimerization. This will provide a more direct evidence of the on target action of metformin on ATP5I. Due to the high abundance of F_1F_0-ATP synthase in cells and the slow ability of metformin to enter mitochondria, we plan to perform long-term treatments (3 and 6 days) with high concentrations of metformin (10 mM) to enhance the likelihood of detecting subtle yet biologically relevant shifts in the monomer and dimer populations. Prolonged exposure is expected to reveal the cumulative effects of metformin on F_1F_0-ATP synthase dimers/monomers ratio. We do not expect that metformin will totally mimic the cumulative effect of the dimerization as in ATP5I KO cells but we think it will be important to report to what extent this ratio is affected.

> ***Reviewer #2 (Public review):***
>
> *(1) The interpretation of the cellular co-localization of the biotin-biguanide conjugate with TOMM20 (Figure 1-D) as mitochondrial "accumulation" of the conjugate is overstated because it cannot exclude binding of the conjugate to the mitochondrial membrane. It would have been more convincing if additional incubations with the biotin-biguanide conjugate in combination with metformin had shown that metformin is competitive with the biotin-conjugate.*

We appreciate the reviewer's insightful comment and agree that the resolution provided by fluorescence microscopy makes it challenging to pinpoint the specific mitochondrial compartment where the biotin-biguanide conjugate localizes, even with additional markers such as TOMM20 antibodies for the inner mitochondrial membrane. While it remains a possibility that the conjugate binds to the mitochondrial surface, another plausible explanation is that the biotin moiety may facilitate entry into mitochondria through a biotin-specific transporter, adding further mechanistic intricacies. Furthermore, while a competition assay with metformin might help investigate interactions with mitochondrial targets and transporters (OCT family), it would not compete for biotin-mediated transport. Thus, while we acknowledge the reviewer's suggestion, we believe such an experiment may not provide conclusive evidence regarding the conjugate's mitochondrial localization or mechanism of entry. Instead, we will revise the manuscript to more accurately describe the findings as "mitochondrial association" rather than "mitochondrial accumulation," ensuring that our interpretation remains consistent with the resolution and limitations of the data presented.

> *(2) The manuscript reports the identification of 69 proteins by mass spectrometry of the pull-down assay of which 31 proteins were eluted by metformin. However, no Mass Spectrometry data is presented of the peptides identified. The methodology does not state the minimum number of peptides (1, 2?) that were used for the identification of the 31/69 proteins.*

Concerning the mass spectrometry results, our intention was to provide a comprehensive table summarizing these findings in a separate data sheet, as part of the data availability section. To address the reviewer's comment and ensure full transparency, we will include this

table as supplementary material in the revised manuscript. Additionally, we will update the methodology section to explicitly state these criteria and ensure clarity regarding the identification process.

> *(3) The validation of ATP5I was based on the use of recombinant protein (which was 90% pure) for the SPR and the use of a single antibody to ATP5I. The validity of the immunoblotting rests on the assumption that there is no "non-specific" immunoactivity in the relevant mol wt range. Information on the validation of the antibody would be helpful.*

Regarding the recombinant protein used for SPR, its purity was evaluated using a Coomassie-stained gel. For the antibody used in immunoblotting, its specificity was validated through knockout cell lines, ensuring minimal concerns about non-specific immunoactivity within the relevant molecular weight range. Unfortunately, the KO data comes in the paper after the first immunoblots are presented. In the revised manuscript, we will clearly outline these validation steps in the methods section and additional manufacturer documentation for the antibody we used.

> *(4) Knock-out of ATP5I markedly compromised the NAD/NADH ratio (Fig.3A) and cell proliferation (Figure 3D). These effects may be associated with decreased mitochondrial membrane potential which could explain the low efficacy of metformin (and most of the data in Figures 3-5). This possibility should be discussed. Effects of [metformin] on the NAD/NADH ratio in control cells and ATP5I-KO would have been helpful because the metformin data on cell growth is normalized as fold change relative to control, whereas the NAD/NADH ratio would represent a direct absolute measurement enabling comparison of the absolute effect in control cells with ATP5I KO.*

The mitochondrial membrane potential depends on a functional electron transport chain which drives proton pumping from the matrix to the intermembrane space. Metformin can decrease the mitochondrial membrane potential and this usually explained as a consequence of complex I inhibition(4). It has been published the metformin requires this membrane potential to accumulate in mitochondria so the actions of metformin are self-limiting due to this requirement. The reviewer is right that ATP5I KO cells could be resistant to metformin because they may have a lower membrane potential. We do not believe this to be the case because the response to phenformin, another biguanide that can enter mitochondria through the membrane without the need of the OCT transporters(5), is also affected in ATP5IKO cells. Of note, compensatory mechanisms such as enhanced glycolysis, as observed in ATP5I-KO cells (elevated ECAR and increased sensitivity to 2-D-deoxyglucose), and the ATPase activity of F_1F_0-ATP synthase could potentially help maintain membrane potential suggesting that this might not be an issue in the ATP5I KO cells. We will discuss these possibilities in the revised manuscript.

Nevertheless, to experimentally address this point, we propose measuring mitochondrial membrane potential using tetramethylrhodamine methyl ester (TMRE) and ATP levels using luciferase-based assays (CellTiter-Glo) in ATP5I-KO cells.

Regarding the NAD+/NADH in both control and KO cells may not be very helpful because this ratio can be corrected by LDH which is induced as part of the glycolytic adaptation that occurs after inhibition of respiration. Since our KO cells have been propagated already for several passages, the extent of this adaptation is likely different from metformin-treated cells. As we mentioned in answering Reviewer 1, we will provide a more direct measurement of metformin acting on ATP5I: the levels of F1F0-ATPase dimers and monomers.

> *(5) Figure-6 CRISPR/Cas9 KO at 16mM metformin in comparison with 70nM rotenone and 2 micromolar oligomycin (in serum-containing medium). The rationale for the use of*

> *such a high concentration of metformin has not been explained. In liver cells metformin concentrations above 1mM cause severe ATP depletion, whereas therapeutic (micromolar) concentrations have minimal effects on cellular ATP status. The 16mM concentration is ~2 orders of magnitude higher than therapeutic concentrations and likely linked to compromised energy status. The stronger inhibition of cell proliferation by 16mM metformin compared with rotenone or oligomycin raises the issue of whether the changes in gene expression may be linked to the greater inhibition of mitochondrial metabolism. Validation of the cellular ATP status and NAD/NADH with metformin as compared with the two inhibitors could help the interpretation of this data.*

To address the reviewer's final comment, we would like to clarify the rationale behind our experimental approach. NALM-6 cells are very glycolytic, have low respiration rates, and weak dependence on ATP5I (DepMap score: -0.47)(6). The concentration of 16 mM metformin was chosen based on the IC50 for this cell line. This approach aligns with our focus on the anticancer mechanism of action rather than the antidiabetic effects of metformin. Both ATP status and NAD^+/NADH ratios will depend on the extent of the compensatory glycolysis. On the other hand, our genetic screening evaluates cell proliferation as an integration of all metabolic activities required for the process. This unbiased screening revealed a common pathway affected by metformin and oligomycin different that the pathway affected by rotenone, which is consistent with the finding that metformin acts of the F_1F_0ATPase.

> ***Reviewer #3 (Public review):***
>
> *(1) Most of the data are based on measurements of the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measured by the Seahorse analyser in control and ATP5I KO cells. However, these measurements are conducted by a single injection of a biguanide, followed over time and presented as fold change. By doing so, the individual information on the effect of metformin and derivate on control and KO cells are lost. In addition, the usual measurement of OCR is coupled with certain inhibitors and uncouplers, such as oligomycin, FCCP, and Antimycin A/rotenone, to understand the contribution of individual complexes to respiration. Since biguanides and ATP5I KO affect protein levels of components of complex I and IV, it would be informative to measure their individual contributions/effects in the Seahorse. To further strengthen the data, it would be helpful to obtain measurements of actual ATP levels in these cells, as this would explain the activation of AMPK.*

We appreciate the reviewer's observations regarding the Seahorse measurements and acknowledge the potential limitations of presenting the data as fold change. Due to experimental challenges in maintaining KP-4 and ATP5I-KO cells with sufficient nutrients, caused by their rapid glucose uptake and subsequent lactate production, it was more practical to present the Seahorse results in this format. Using inhibitors at each time point during the Seahorse experiment was not feasible, as the delay between inhibitor injections and the corresponding changes in oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) would introduce variability and complicate the interpretation of dynamic responses. Nevertheless, we recognize the importance of understanding the contributions of specific respiratory complexes to OCR and ECAR. To address this, we will include a representative figure showcasing a typical Seahorse analysis, highlighting ATP turnover and proton leak after oligomycin addition, maximal respiration with FCCP, and disruption with rotenone and antimycin A. While these experiments are inherently complex due to the metabolic demands of ATP5I-KO cells, this approach will provide a clearer breakdown of mitochondrial activity. Furthermore, as mentioned in our response to Reviewer 2, we will measure ATP levels using a luciferase-based assay (CellTiter-Glo) in both control and ATP5I-KO cells to better explain AMPK activation. This will provide additional

context to strengthen the interpretation of mitochondrial function and metabolic compensation mechanisms in these cells.

> *(2) The authors report on alterations in mitochondrial morphology upon ATP5I KO, which is measured by subjective quantifications of filamentous versus puncta structures. Fiji offers great tools to quantify the mitochondrial network unbiasedly and with more accuracy using deconvolution and skeletonization of the mitochondria, providing the opportunity to measure length, shape, and number quantitatively. This will help to understand better, whether mitochondria are really fragmented upon ATP5I KO and rescued by its re-introduction.*

Concerning the analysis of mitochondrial morphology, we acknowledge the potential benefits of using Fiji and additional plugins such as MiNA for more accurate and unbiased quantification. Indeed, this approach could provide stronger evidence for mitochondrial fragmentation upon ATP5I-KO and its potential rescue by ATP5I reintroduction. We will consider integrating this methodology into our analysis to enhance the precision and robustness of our findings.

> *(3) Finally, the authors report in the last part of the paper a genetic CRISPR/Cas9 KO screen in NALM-6 cells cultured with high amounts of metformin to identify potential new mediators of metformin action. It is difficult to connect that to the rest of the paper because a) different concentrations of metformin are used and b) the metabolic effects on energy consumption are not defined. They argue about the molecular function of the obtained hits based on literature and on a comparison of the pattern of genetic alterations based on treatments with known inhibitors such as oligomycin and rotenone. However, a direct connection is not provided, thus the interpretation at the end of the results that "the OMA1-DEL1-HRI pathway mediates the antiproliferative activity of both biguanides and the F1ATPase inhibitor oligomycin" while increasing glycolysis, needs to be toned down. This is an interesting observation, but no causality is provided. In general, this part stands alone and needs to be better connected to the rest of the paper.*

NALM-6 are very glycolytic, have low respiration rates, and weak dependence on ATP5I(6), forcing us to use higher concentrations of metformin to inhibit their growth. Recent results show that metformin targets PEN2 in the cytosol to increase AMPK activity, controlling both the glucose lowering and the life span extension abilities of metformin 7. This work raises the question whether the antiproliferative and anticancer effects of metformin are due to a mitochondrial activity or are controlled by this new pathway of AMPK activation. Hence, the genetic screening was performed to unbiasedly find how metformin works. The results provide compelling evidence for mitochondria and in particular the ATP synthase as potential targets of metformin and a foundation for future studies. We will revise the text and abstract to better reflect the exploratory nature of this finding and ensure clarity.

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