KRAS-regulated glutamine metabolism requires UCP2-mediated aspartate transport to support pancreatic cancer growth

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The oncogenic *KRAS* mutation has a critical role in the initiation of human pancreatic ductal adenocarcinoma (PDAC) since it rewires glutamine metabolism to increase NADPH production, balancing cellular redox homeostasis with macromolecular synthesis[1,2]. Mitochondrial glutamine-derived aspartate must be transported into the cytosol to generate metabolic precursors for NADPH production[2]. The mitochondrial transporter responsible for this aspartate efflux has remained elusive. Here, we show that mitochondrial uncoupling protein 2 (UCP2) catalyses this transport and promotes tumour growth. UCP2-silenced *KRAS*^{MUT} cell lines display decreased glutaminolysis, lower NADPH/NADP^{+} and GSH/GSSG ratios and higher ROS levels compared to wild-type counterparts. UCP2 silencing reduces glutaminolysis also in *KRAS*^{WT} PDAC cells but does not affect their redox homeostasis or proliferation rates. *In vitro* and *in vivo*, UCP2 silencing strongly suppresses *KRAS*^{MUT} PDAC cell growth. Collectively, these results demonstrate that UCP2 plays a vital role in PDAC as its aspartate transport activity connects mitochondrial and cytosolic reactions necessary for *KRAS*^{MUT} rewired glutamine metabolism[2] and thus it should be considered a key metabolic target for the treatment of this refractory tumour.

Energy production, redox homeostasis, and macromolecular synthesis in most cancer cells relies on glutamine consumption[3,4]. The main metabolites derived from the mitochondrial glutamine oxidation are aspartate and malate[4,5] which are transported to the cytosol where aspartate is used for nucleotide and protein synthesis and malate is used to generate NADPH for reductive biosynthesis and the cellular redox homeostasis[4]. In PDAC, oncogenic *KRAS* induces metabolic rewiring of the pentose phosphate pathway such that ribose-5-P biogenesis is decoupled from NADPH production[1]. This metabolic alteration encourages a glutamine addiction[2,6]. To fulfil the NADPH needs, *KRAS* triggers rewired glutamine metabolism in which mitochondrial glutamine-derived aspartate, once transported to the cytosol, is converted to oxaloacetate, malate and finally to pyruvate to produce NADPH[2] (Fig. 1a). Although this pathway has been known[2,7,8], the mitochondrial carrier responsible for aspartate efflux from mitochondria remains missing. One possible candidate is UCP2 (Fig. 1a).

[Figure1]

We recently showed that bacterially expressed and refolded UCP2 in artificial lipid vesicles can catalyse the transport of aspartate against phosphate plus a proton[9]. Furthermore, UCP2 is up-regulated at the translational level by glutamine[10,11], is required for macrophage and HepG2 glutamine oxidation[9,12], and it is overexpressed in PDAC compared with healthy tissue (Extended Data Fig. 1a). To assess the role of UCP2 in PDAC-rewired glutamine metabolism, we silenced UCP2 using RNA interference. Four PDAC cell lines, two $KRAS^{MUT}$, Patu8988T and Panc1, and two $KRAS^{WT}$, BxPC3 and KP2, were used. The efficiency of silencing was verified at both the transcript and protein levels (Extended Data Fig. 1b, c). Although all four cell lines showed significant differences in UCP2 transcript abundance, we did not find a proportional correspondence at the protein level (Extended Data Fig. 1c, d), this confirmed that UCP2 is regulated at both the transcriptional and translational levels[13]. UCP2 silencing did not alter the expression of other members of the mitochondrial UCP subfamily (UCP3-5)[14], which are expressed at levels much lower than those of UCP2 (Extended Data Fig. 1e, f), and UCP1 was absent in all cell lines. Detergent-solubilized mitochondrial extracts from UCP2-silenced cells reconstituted into artificial lipid vesicles[9] were unable to catalyse any significant aspartate/Pi transport activity (Extended Data Fig. 1g). We then assayed the effect of UCP2 silencing on cell proliferation and colony-forming capacity. All experiments were done in the presence of glutamine as UCP2 expression is glutamine-dependent[10,11] (Extended Data Fig. 1i). To identify the defect induced by the loss of mitochondrial efflux of aspartate by UCP2 silencing, all experiments were carried out in the presence of dialysed fetal bovine serum similar to that previously used to map the *KRAS*-rewired glutamine metabolism[2]. UCP2 silencing significantly decreased the proliferation rate and colony-forming capacity of $KRAS^{MUT}$ cells, Patu8988T and Panc1 cells, but not $KRAS^{WT}$ cells, BxPC3 and KP2 (Fig. 1b-f, and Extended Data Fig. 2a-d). The addition of aspartate to medium partially but significantly rescued the previously observed defects (Fig. 1g, and Extended Data Fig. 2a, b, e, f), confirming that UCP2 silencing depleted cytosolic aspartate.
Interestingly, the proliferation defect induced by UCP2 silencing was also partially rescued by the addition of glutamate to medium (Extended Data Fig. 2g). As glutamate cannot produce NADPH in the cytosol (Fig. 1a), we hypothesize that glutamate enters mitochondria

through the mitochondrial aspartate/glutamate carrier (AGC) where it produces aspartate by following the same reaction pathways as mitochondrial glutamate derived from glutamine. Importantly, AGC is expressed in the four PDAC cell lines, and UCP2 silencing does not alter its expression (Extended Data Fig. 2h). As AGC is a strict counter-exchanger, the entry of glutamate occurs only if aspartate exits[15]. The export of aspartate by AGC requires the entry of glutamate (Extended Data Fig. 2i) but this would result in the accumulation of C4 glutamine-derived metabolites in the matrix, inhibiting glutaminolysis[16]. As such, we hypothesize this pathway is only utilized when there are high concentrations of cytosolic glutamate. Furthermore, AGC transport activity is crucial for cell proliferation during glutamine starvation and its loss sensitizes tumours to CB-389, a glutaminase inhibitor[17]. Under glutamine-replete conditions in PDAC cells, glutamine is transported into the matrix through the mitochondrial glutamine transporter, SLC1A5_var, producing aspartate in the matrix[18] this requires a mitochondrial carrier able to catalyse a net efflux of aspartate. UCP2 is the only known mitochondrial carrier able to catalyse the exchange of aspartate against Pi plus a proton[9]; Interestingly, silencing of SLC1A5_var as well as that of UCP2 reduce gemcitabine resistance of PDAC cells[18,19].

The addition of reduced glutathione (GSH) or N-acetyl-cysteine (NAC) completely restored the growth defects seen in the absence of UCP2 (Fig. 2a and Extended Data Fig. 3a). This confirmed that the cytosolic depletion of aspartate in the $KRAS^{MUT}$ PDAC cells, induced by UCP2 silencing, decreased GSH availability[2] and altered cellular redox homeostasis. To dissect this point, we explored the effect of UCP2 silencing on the GSH/GSSG ratio. As expected UCP2 silencing decreased the GSH/GSSG ratios in the Patu8988T and Panc1 but not in $KRAS^{WT}$ cells (Fig. 2b and Extended Data Fig. 3b). These results were confirmed by the increase in ROS found in UCP2-silenced $KRAS^{MUT}$ cells (Fig. 2c and Extended Data Fig. 3c-i). Remarkably, UCP2 silencing slightly decreased ROS in the two $KRAS^{WT}$ cells. Because KRAS-dependent glutamine metabolism requires cytosolic aspartate derived from mitochondrial glutamine oxidation to reduce glutathione using cytosolic NADPH[2], we tested if the NADPH/$NADP^+$ ratio was decreased by UCP2 silencing. The results reported in Fig. 2d and Extended Data Fig. 3j confirmed that the lower GSH/GSSG ratio found in the Patu8988T and Panc1 cells was correlated with a lower NADPH/$NADP^+$ ratio, whereas the NADPH/$NADP^+$ ratio in $KRAS^{WT}$ cells was unaffected by UCP2 silencing. In support of these results, GSEA carried out on gene expression profile (GEP) of roughly 300 PDAC primary tumour samples and 43 PDAC cell lines, showed a significant association of UCP2 expression with genes involved in the “glutathione metabolism”, “oxidoreductase activity acting on NADH or NADPH”, “cellular response to oxidative stress” and “ROS metabolism” (Fig. 2e, Extended Data Fig. 3k, l and Supplementary Table 1). To gain more insight into the metabolic changes observed in PDAC cells upon UCP2 silencing, we carried out a targeted metabolomic analysis using uniformly ^{13}C-labelled Gln ([U-^{13}C]Gln) as a tracer.

[Figure 2]

UCP2 silencing did not produce a significant difference in the labelling of metabolites derived from glutamine utilisation, i.e. glutamate, aspartate, malate and 2-ketoglutarate. One explanation could be that the absence of UCP2 promotes a mitochondrial accumulation of labelled intermediates that would otherwise accumulate in the cytosol. To test this hypothesis, we fractionated cytosolic and organelle enriched metabolite pools[20] (detailed in Extended Data Methods). In both $KRAS^{WT}$ and $KRAS^{MUT}$ cells, UCP2 silencing led to an

accumulation of glutamine-derived metabolites in the organelle fraction and a decrease in the cytosolic fractions (Fig. 2f, g). Metabolomics data suggested that the loss of UCP2-dependent cataplerotic activity inhibited glutaminolysis in both *KRAS*^{WT} and *KRAS*^{MUT} cells. This was confirmed by the lower oxygen consumption rate of UCP2-silenced PDAC respiring on glutamine (Fig. 2h). These results establish the crucial role of UCP2 in glutamine metabolism[9,12] and the central nature of glutamine in redox homeostasis in *KRAS*^{MUT} cells, but not KRAS^{WT} cells. These initial results demonstrate: i) the vital role of UCP2 in the *KRAS*^{MUT} cells where rewired glutamine metabolism occurs[2]; ii) the higher levels of ROS found in the UCP2-silenced Patu8988T and Panc1 cells are due to impaired glutamine oxidation and not to UCP2-mediated uncoupling activity as no difference was found in the two *KRAS*^{WT} cell lines.

As mammalian cell lines present a very complex metabolic system, we confirmed the role of UCP2 as a mitochondrial aspartate exporter in yeast. Yeast cells require the malate-aspartate shuttle (MAS) for reduction of the cytosolic NADH derived from peroxisomal oleate oxidation[21] (Extended Data Fig. 4a). The deletion of the aspartate/glutamate carrier (AGC1), a mitochondrial transporter essential for the function of MAS in yeast, renders yeast cells unable to grow on oleate[21]. We hypothesised that if UCP2 catalysed the export of aspartate from mitochondria in exchange for inorganic phosphate plus a proton and operated together with the yeast mitochondrial glutamate/H^+ symporter (YMC2)[22], that aspartate/glutamate exchange activity would be restored (Extended Data Fig. 4b) along with the growth defect on oleate of the ΔAGC1 yeast strain. To exclude possible artefacts, we constructed an inactive UCP2 mutant to use as negative control where arginine at position 279 was mutated to glutamine. This highly conserved arginine is in the sixth transmembrane α-helix of UCP2[23,24] and is required for the transport activity of other mitochondrial carrier family (MCF) members[25,26]. As expected, the bacterial recombinant $UCP2_{R279Q}$ mutant was unable to catalyse any aspartate/Pi exchange activity once reconstituted into liposomes (Extended Data Fig. 4c). Confirming our hypothesis, the ΔAGC1 yeast strain expressing UCP2 was able to grow on oleate as sole carbon source (Fig. 2i). Importantly, the $UCP2_{R279Q}$ mutant, when expressed at equal levels to WT (Extended Data Fig. 4d), was unable to rescue the growth defect (Fig. 2i). To further prove that the artificial mitochondrial aspartate/glutamate exchange activity was due to the synergistic functioning of UCP2 and YMC2p, we created a double mutant ΔAGC1/ΔYMC2 where the expression of UCP2 was unable to restore the growth defect (Fig. 2j). Similar results were obtained when UCP2 was expressed in a yeast strain lacking the two isoforms of the oxoglutarate/malate carrier (ODC1 and ODC2), essential components of the MAS[21,27] (Extended Data Fig. 4e). To confirm that the complementation results observed in the ΔAGC1 strain were due to the aspartate transport catalysed by UCP2, we deleted aspartate transaminase (AAT1), the main mitochondrial aspartate-producing enzyme in the ΔAGC1 strain (Extended Data Fig. 4f). As expected UCP2 was unable to rescue the growth defect of this double mutant (Fig. 2k). Together our complementation studies suggest that *in vivo* UCP2 is unable to catalyse the aspartate/malate or malate/oxaloacetate exchange reactions, despite our previous finding that UCP2 transports malate, oxaloacetate, aspartate and Pi[9] *in vitro*, as this exchange activities would have rescued the growth defect of the ΔODC1/ΔODC2 and ΔAGC1/ΔAAT1 yeast strains (Extended Data Fig. 4g, h). Taken together our yeast complementation studies confirm that UCP2 is an aspartate/Pi + H^+ transporter *in vivo*.

We confirmed the effects of UCP2 silencing in two relevant physiological conditions, 3D organotypic cultures and xenograft models. 3D cultures were carried out on 100% Matrigel.

UCP2-silenced *KRAS*^{MUT} cells grew slower than the shRNA controls, formed smaller colonies and showed a significant increase in cell death (Fig. 3a and Extended Data Fig. 5a). The effects of UCP2 silencing in BXPC3 and KP2 (*KRAS*^{WT}) cells grown on Matrigel were quite similar to those previously obtained in 2D cultures (Fig. 3b and Extended Data Fig. 5b) as UCP2 silencing did not alter colony size nor growth rate. It should be noted that in the two *KRAS*^{WT} cell lines UCP2 silencing increased the cell death even though the effect was less substantial than that observed in *KRAS*^{MUT} cells.

[Figure 3]

To corroborate these data, we investigated the effect of UCP2 silencing in a tumour cell xenograft model. After cell implantation, the tumours were grown to a volume of 40-70 mm^3 thereafter mice were treated with doxycycline to silence UCP2. The loss of UCP2 severely arrested tumour growth in mice injected with the two *KRAS*^{MUT} cell lines whereas growth continued unabated in control shRNA cells (Fig. 3c and Extended Data Fig. 5c, d, g, i). As expected, UCP2 silencing did not exert a significant effect on BxPC3 and KP2 xenograft growth (Fig. 3d and Extended Data Fig. 5e, f, h, i). The increased expression of Ki67 confirmed the differences in tumour size (Fig. 3c-e and Extended Data, Fig. 5g, h, j). UCP2-silenced *KRAS*^{MUT} cell-derived tumours showed reduced GSH/GSSG and NADPH/$NADP^+$ ratios compared to those found in control tumours, whereas no significant differences were found in *KRAS*^{WT} cell-derived tumours (Fig. 3f, g and Extended Data Fig. 5k, l). These results confirmed the crucial role of UCP2 in the redox homeostasis of *KRAS*^{MUT} but not in *KRAS*^{WT} cells *in vivo*.

To further verify that the growth defect observed in the *KRAS*^{MUT} cells was due to UCP2 silencing, we carried out a rescue experiment by using constitutively active shRNA (sh#1) construct targeting the endogenous human mRNA at the 5'-UTR[9] and a doxycycline-inducible lentiviral vector expressing murine Ucp2 (mUcp2). In this system, endogenous human UCP2 was silenced and the addition of doxycycline induced the expression of mUcp2. The efficiency of UCP2 silencing was slightly lower than that obtained with the inducible system (Extended Data Fig. 1c, h), whereas the addition of doxycycline greatly induced the expression of mUcp2, bringing protein expression and the aspartate/Pi transport activity to a level comparable to that of Patu8988T cells transfected with the $control_{shRNA}$ (Extended Data Fig. 1c, h, g). mUcp2 expression restored proliferation and clonogenic growth defects in the hUCP2-silenced Patu8988T_{NI} cells (Fig. 4a and Extended Data Fig. 6a). mUcp2 expressing cells had significantly lower ROS levels than those found in the UCP2-silenced cells as well as in $control_{shRNA}$-transfected cells (Fig. 4b and Extended Data Fig. 6b). Higher ROS production in UCP2-silenced cells was associated with GSH/GSSG and NADPH/$NADP^+$ ratios lower than those of $control_{shRNA}$-transfected cells whereas mUcp2 expression rescued the ratios to those of the control cells (Fig. 4c, d). This suggested that mUcp2 was able to re-establish redox homeostasis in UCP2-silenced cells.

[Figure 4]

To confirm that doxycycline was not impacting respiration or altering redox homeostasis we tested constitutively UCP2-silenced Patu8988T_{NI} cells. Our results confirmed that the lower GSH/GSSG, NADPH/$NADP^+$ ratios and oxygen consumption rates in the presence of glutamine were independent of doxycycline (Extended Data Fig. 6c-e). In this system,

mUCP2 expression restored the cytosolic and organelle distribution of the [U-^{13}C] glutamine-derived metabolites of UCP2-silenced Patu8988T$_{NI}$ to control levels (Extended Data Fig. 6f). This suggested that control of redox homeostasis by mUcp2 expression occurred through the proper functioning of *KRAS* rewired glutamine metabolism.
These results were validated in the xenograft model. In this case, doxycycline was administrated to mice two days after cell implantation. mUcp2 expression restored the proliferation rate of the hUCP2-silenced Patu8988T$_{NI}$ cells, significantly increasing tumour mass (Fig. 4e and Extended Data Fig. 6g-i). UCP2 re-expression also restored GSH/GSSG and NADPH/NADP$^+$ ratios found in UCP2-silenced Patu8988T$_{NI}$-derived tumours to those found in the controls (Fig. 4f, g). Collectively these data prove that the observed differences in the two *KRAS*^{MUT} PDAC cell lines were dependent on the expression of UCP2.
To verify the existence of any direct causal link between the acquisition of *KRAS* mutation and dependence on UCP2, we expressed the *KRAS*$_{G12V}$ mutant, carried by Patu8988T cells, in BxPC3 cells. *KRAS*$_{G12V}$ expression (Extended Data Fig. 7a) did not alter the expression of UCP2 at the transcript or protein level (Extended Data Fig. 7b, c). Interestingly, *KRAS*$_{G12V}$ increased the proliferation rate and the clonogenic capacity of BxPC3 cells in a UCP2-dependent manner (Fig. 4h, i). These findings indicate that *KRAS* mutations do not regulate UCP2 expression, however its biochemical function is crucial to support the increase of cell proliferation induced by mutated *KRAS*.

Together, these results demonstrate that the unknown mitochondrial transporter, which catalyses mitochondrial aspartate efflux in *KRAS*-rewired glutamine metabolism[2], is UCP2 (Fig. 1a). We demonstrate in an *in vivo* model that UCP2 acts as a metabolite transporter like all other members of the MCF[28]. Additionally, we show that the role UCP2 plays in ROS handling is linked to its substrate transport function rather than an uncoupling activity. Moreover, increased mitochondrial phosphate concentration produced by UCP2-dependent exchange reactions[9] would activate phosphate-dependent glutaminase[29] (Fig. 1a). It should be noted that malate, a reaction product of mitochondrial glutamine oxidation and used by the cytosolic malic enzyme to generate NADPH[4,5], is efficiently exported by UCP2 in exchange for phosphate plus a proton[9]. In brief, the above reported results together with the glutamine addiction found in many cancer cells, the increased expression of UCP2 in many tumours[30] and UCP2's tight regulation by glutamine[10] make UCP2 an important metabolic target in PDAC and other glutamine-dependent cancers.

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Contributions
S.R., C.P., A.V. D.F. and L.C performed most of the experiments *in vitro;* F.D. performed the *in vitro* reconstitution experiments; I.P. performed the cytofluorimetric analyses; S.N.B. and F.M.L. performed the respirometry experiments, R.G., G.E.D. and G.A. performed the GS-MS metabolomics experiments; P.S. carried out the RT-PCR experiments; Y.L., C.M.T.M. and F.P. performed the yeast experiments; V.R., R.M., V.D. performed the xenograft experiments; M.R.G., R.C. and S.J.R. performed the organotypic cell culture experiments; M.C.V. performed GSEA and analysis on GEP; L.P., V.D. and G.F. conceived and designed the

study. W.S., L.P., V.D., E.M., C.L.R. and G.F. interpreted results and wrote the manuscript. G.F. acquired funding.

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References

1 Ying, H. *et al.* Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. *Cell* **149**, 656-670, doi:10.1016/j.cell.2012.01.058 (2012).

2 Son, J. *et al.* Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature* **496**, 101-105, doi:10.1038/nature12040 (2013).

3 Hensley, C. T., Wasti, A. T. & DeBerardinis, R. J. Glutamine and cancer: cell biology, physiology, and clinical opportunities. *J Clin Invest* **123**, 3678-3684, doi:10.1172/JCI69600 (2013).

4 Altman, B. J., Stine, Z. E. & Dang, C. V. From Krebs to clinic: glutamine metabolism to cancer therapy. *Nat Rev Cancer* **16**, 619-634, doi:10.1038/nrc.2016.71 (2016).

5 DeBerardinis, R. J. *et al.* Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc Natl Acad Sci U S A* **104**, 19345-19350, doi:10.1073/pnas.0709747104 (2007).

6 Abrego, J. *et al.* GOT1-mediated anaplerotic glutamine metabolism regulates chronic acidosis stress in pancreatic cancer cells. *Cancer Lett* **400**, 37-46, doi:10.1016/j.canlet.2017.04.029 (2017).

7 Chakrabarti, G. *et al.* Targeting glutamine metabolism sensitizes pancreatic cancer to PARP-driven metabolic catastrophe induced by ss-lapachone. *Cancer Metab* **3**, 12, doi:10.1186/s40170-015-0137-1 (2015).

8 Wang, Y. P. *et al.* Arginine Methylation of MDH1 by CARM1 Inhibits Glutamine Metabolism and Suppresses Pancreatic Cancer. *Mol Cell* **64**, 673-687, doi:10.1016/j.molcel.2016.09.028 (2016).

9 Vozza, A. *et al.* UCP2 transports C4 metabolites out of mitochondria, regulating glucose and glutamine oxidation. *Proc Natl Acad Sci U S A* **111**, 960-965, doi:10.1073/pnas.1317400111 (2014).

10 Pecqueur, C. *et al.* Uncoupling protein 2, in vivo distribution, induction upon oxidative stress, and evidence for translational regulation. *J Biol Chem* **276**, 8705-8712, doi:10.1074/jbc.M006938200 (2001).

11 Rupprecht, A., Moldzio, R., Modl, B. & Pohl, E. E. Glutamine regulates mitochondrial uncoupling protein 2 to promote glutaminolysis in neuroblastoma cells. *Biochim Biophys Acta Bioenerg* **1860**, 391-401, doi:10.1016/j.bbabio.2019.03.006 (2019).

12 Nubel, T. *et al.* Modified glutamine catabolism in macrophages of Ucp2 knock-out mice. *Biochim Biophys Acta* **1777**, 48-54, doi:10.1016/j.bbabio.2007.11.002 (2008).

13 Donadelli, M., Dando, I., Fiorini, C. & Palmieri, M. UCP2, a mitochondrial protein regulated at multiple levels. *Cell. Mol. Life Sci.* **71**, 1171-1190, doi:10.1007/s00018-013-1407-0 (2014).

14 Bouillaud, F., Couplan, E., Pecqueur, C. & Ricquier, D. Homologues of the uncoupling protein from brown adipose tissue (UCP1): UCP2, UCP3, BMCP1 and UCP4. *Biochim Biophys Acta* **1504**, 107-119, doi:10.1016/s0005-2728(00)00241-3 (2001).

15 Palmieri, L. *et al.* Citrin and aralar1 are Ca2+-stimulated aspartate/glutamate transporters in mitochondria. *EMBO J.* **20**, 5060-5069, doi:papers3://publication/doi/10.1093/emboj/20.18.5060 (2001).

16 Owen, O. E. The key role of anaplerosis and cataplerosis for citric acid cycle function. *J. Biol. Chem.* **277**, 30409-30412, doi:papers3://publication/doi/10.1074/jbc.R200006200 (2002).
17 Alkan, H. F. *et al.* Cytosolic Aspartate Availability Determines Cell Survival When Glutamine Is Limiting. *Cell Metab* **28**, 706-720 e706, doi:10.1016/j.cmet.2018.07.021 (2018).
18 Yoo, H. C. *et al.* A Variant of SLC1A5 Is a Mitochondrial Glutamine Transporter for Metabolic Reprogramming in Cancer Cells. *Cell Metab* **31**, 267-283 e212, doi:10.1016/j.cmet.2019.11.020 (2020).
19 Dalla Pozza, E. *et al.* Role of mitochondrial uncoupling protein 2 in cancer cell resistance to gemcitabine. *Biochim. Biophys. Acta* **1823**, 1856-1863, doi:papers3://publication/doi/10.1016/j.bbamcr.2012.06.007 (2012).
20 Lee, W. D., Mukha, D., Aizenshtein, E. & Shlomi, T. Spatial-fluxomics provides a subcellular-compartmentalized view of reductive glutamine metabolism in cancer cells. *Nat Commun* **10**, 1351, doi:10.1038/s41467-019-09352-1 (2019).
21 Cavero, S. *et al.* Identification and metabolic role of the mitochondrial aspartate-glutamate transporter in Saccharomyces cerevisiae. *Mol Microbiol* **50**, 1257-1269, doi:10.1046/j.1365-2958.2003.03742.x (2003).
22 Porcelli, V. *et al.* Molecular identification and functional characterization of a novel glutamate transporter in yeast and plant mitochondria. *Biochim Biophys Acta Bioenerg* **1859**, 1249-1258, doi:10.1016/j.bbabio.2018.08.001 (2018).
23 Palmieri, F. Mitochondrial transporters of the SLC25 family and associated diseases: a review. *J Inherit Metab Dis* **37**, 565-575, doi:10.1007/s10545-014-9708-5 (2014).
24 Monne, M., Miniero, D. V., Iacobazzi, V., Bisaccia, F. & Fiermonte, G. The mitochondrial oxoglutarate carrier: from identification to mechanism. *J Bioenerg Biomembr* **45**, 1-13, doi:10.1007/s10863-012-9475-7 (2013).
25 Tessa, A. *et al.* Identification of novel mutations in the SLC25A15 gene in hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome: a clinical, molecular, and functional study. *Hum Mutat* **30**, 741-748, doi:10.1002/humu.20930 (2009).
26 Fiermonte, G. *et al.* An adult with type 2 citrullinemia presenting in Europe. *N Engl J Med* **358**, 1408-1409, doi:10.1056/NEJMc0707353 (2008).
27 Palmieri, L. *et al.* Identification in Saccharomyces cerevisiae of two isoforms of a novel mitochondrial transporter for 2-oxoadipate and 2-oxoglutarate. *J Biol Chem* **276**, 1916-1922, doi:10.1074/jbc.M004332200 (2001).
28 Palmieri, F. The mitochondrial transporter family (SLC25): physiological and pathological implications. *Pflugers Arch* **447**, 689-709, doi:10.1007/s00424-003-1099-7 (2004).
29 Cassago, A. *et al.* Mitochondrial localization and structure-based phosphate activation mechanism of Glutaminase C with implications for cancer metabolism. *Proc Natl Acad Sci U S A* **109**, 1092-1097, doi:10.1073/pnas.1112495109 (2012).
30 Baffy, G. Uncoupling protein-2 and cancer. *Mitochondrion* **10**, 243-252, doi:10.1016/j.mito.2009.12.143 (2010).

Figure legends

Fig.1 | UCP2 silencing reduces the availability of cytosolic aspartate in PDAC cell lines. a, The rewired glutamine metabolism induced by $KRAS^{MUT}$ in PDAC[2]. In red, the enzymes involved in the *KRAS*-dependent glutamine metabolism[2]. **b-f,** UCP2 silencing reduces the proliferation rate and the clonogenic capacity of

KRAS^{MUT} PDAC cells. The histogram data were referred to control shRNA transfected cells – dox (f). **g,** Aspartate in the medium partially rescues the proliferation growth defect of UCP2-silenced Patu8988T and Panc1 cells. Doxycycline was always present in b-e and g. Asp, aspartate; Glu, glutamate; Gln, glutamine; Mal, malate; 2-KG, 2-ketoglutarate; Pyr, pyruvate; OA, oxaloacetate, GR, glutathione reductase; ME1, malic enzyme; GPT1, glutamate-pyruvate transaminase; GOT1 and GOT2, glutamate-oxaloacetate transaminase; GDH, glutamate dehydrogenase; GLS, glutaminase; MDH1 malate dehydrogenase; SLC1A5_var, mitochondrial glutamine carrier. Values represent means ± SD of two biologically independent triplicates. Statistical significance was calculated by unpaired two-tailed Student's *t* test. Welch correction was applied to panels **b**, **f** and sh#2 (-Asp) *vs* sh#2 (+Asp) (**g**).

Fig. 2 | The UCP2 aspartate/Pi activity connects mitochondrial and cytosolic reactions necessary for rewired glutamine metabolism and regulates the redox homeostasis of PDAC cells. a, GSH or NAC in the medium fully rescues the growth defect of UCP2-silenced Patu8988T cells. **b,** UCP2 silencing reduces the GSH/GSSG ratio only in *KRAS*^{MUT} cells. **c,** UCP2 silencing increases ROS levels only in *KRAS*^{MUT} cells. **d,** UCP2 silencing reduces the NADPH/NADP^{+} ratio only in *KRAS*^{MUT} cells. The histogram data were referred to control$_{shRNA}$ transfected cells (**b**-**d**). **e,** In PDAC, UCP2 expression levels are associated with those of genes involved in redox homeostasis. The GSEA panel shows the enrichment of gene sets related to UCP2 expression in roughly 300 PDAC primary tumour samples from E-MTAB-6134 dataset. NES, normalized enrichment score. **f, g,** UCP2 silencing alters the cytosol/matrix distribution of the glutamine-derived metabolites in PDAC cells. **h,** UCP2-silenced PDAC cells show a reduced oxygen consumption rates (OCR) in the presence of glutamine, values represent means ± SD of three biologically independent experiments. **i-k,** UCP2 functions as an aspartate/Pi exchanger in yeast cells. Serial dilutions of different yeast cell models expressing UCP2 or UCP2$_{R279Q}$ were spotted on YP plate in the presence of oleate. Values represent means ± SD of two biologically independent triplicates (**a**-**d**, **f** and **g)**. Statistical significance was calculated by unpaired two-tailed Student's *t* test. Welch correction was applied to sh#1 (-NAC) *vs* sh#1 (+NAC), sh#1 (-GSH) *vs* sh#1 (+GSH) (**a**), Ctr *vs* sh#2 (**b**), Mito_Ctr$_{NI}$ *vs* Mito_sh#1$_{NI}$ (Glu) (**f**), Cito_Ctr$_{NI}$ *vs* Cito_sh#1$_{NI}$, Mito_Ctr$_{NI}$ *vs* Mito_sh#1$_{NI}$ (Glu), Cito_Ctr$_{NI}$ *vs* Cito_sh#1$_{NI}$ (Mal) (**g**). Asp, aspartate; Glu, glutamate; Mal, malate; 2-KG, 2-ketoglutarate.

Fig. 3 | UCP2 silencing inhibits *KRAS*^{MUT} PDAC cell growth in organotypic 3D and xenograft models. a, b, UCP2 silencing reduces growth of organotypic cultures of *KRAS*^{MUT} PDAC cells. Representative microscopic images of growth and death (red signal) of PDAC cells grown on 100% Matrigel ECM in the presence of doxycycline. Scale bar = 500 μm. Histograms show the growth and the death of cells cultured for seven days. The growth data were normalized to those measured at the beginning of the experiment (T_0), whereas the death data were referred to the control$_{shRNA}$ transfected cells. **c, d,** UCP2 silencing inhibits the proliferation of *KRAS*^{MUT} PDAC cells *in vivo*. Representative images of tumour sizes and Ki-67 immunostaining, taken at the end point, generated by PDAC cells injected in female SCID mice (n = 5). Inset shows localization at higher magnification (40X), scale bar=25 μm. **e,** Percentage of Ki67 positive PDAC cells. **f, g,** UCP2 silencing reduces the GSH/GSSG and NADPH/NADP^{+} ratios only in *KRAS*^{MUT} cell xenografts, the histogram data were referred to control$_{shRNA}$ xenografts. Values represent means ± SD of two biologically independent triplicates (**a** and **b**) and five mice (**e**-**g**). Statistical significance was calculated by unpaired two-tailed Student's *t* test. Welch correction was applied to **a**, (death) and Ctr *vs* sh#2 (**f**).

Fig. 4 | UCP2 is essential for the proliferation of PDAC cells expressing mutated *KRAS*. a-d, The expression of murine Ucp2 rescues the growth defect of UCP2-silenced Patu8988T$_{NI}$ cells and re-establishes the redox homeostasis. Histogram data were referred to control$_{shRNA}$ transfected cells carrying the empty expression vector (EV) (**b**-**d**). **e,** mUcp2 expression increases the proliferation of UCP2-silenced Patu8988T$_{NI}$ cells *in vivo*. Representative images of tumour sizes and Ki-67 immunostaining, taken at the end point, generated by PDAC cells injected in female SCID mice (n = 5). Inset shows localization at higher magnification (40X), scale bar=25 μm. **f, g,** mUcp2 expression re-establishes the redox homeostasis in xenografts. Histogram data were referred to control$_{shRNA}$ xenografts carrying the empty expression vector (EV) (**f**, **g**). **h, i,** UCP2 silencing reduces the proliferation rate and the clonogenic capacity of BxPC3 cells expressing mutated *KRAS.* The histogram data were referred to control shRNA transfected BxPC3 cells expressing *KRAS*^{WT} (**i**). Values represent means ± SD of two biologically independent triplicates (**a**-**d**, **h** and **i**) and five mice (**f** and **g**). Statistical significance was calculated by unpaired two-tailed Student's *t* test (**a**, **c**, **d** and **f**-**i**) and unpaired two-tailed Mann–Whitney test (**b**). Welch correction was applied to Ctr_*KRAS*$_{G12V}$ *vs* sh#1_*KRAS*$_{G12V}$ (**h**). Patu8988T$_{NI}$ and BxPC3$_{NI}$, cells transfected with non-inducible lentiviral plasmids.

Methods

Cell culture. Cell lines Patu8988T (ACC-162), Panc1 (CRL-1469) and BxPC3 (CRL-1687) were from ATCC and KP2 (JCRB0181) from JCRB Cell Bank. Although in the COSMIC database (https://cancer.sanger.ac.uk/cell_lines/sample/overview?id=1298218) an unverified heterozygous mutation (c.34G>C, p.G12R) is reported for KP2 cells, we used this cell line as the second $KRAS^{WT}$ control since, at transcript levels, in these cells we found an allelic imbalance in favour of wild-type allele as it is shown in two examples of forward and reverse sequences of PCR fragments (amplified from a first-strand cDNA) carried out along the study (Extended Data Fig. 8). Furthermore, based on previously published results[31], we estimate that less than 20% of the pancreatic KP2 cell line population contain the mutated *KRAS* variant.

Cells were continuously cultured in 100 U/ml penicillin/streptomycin (Euroclone, ECB3001D), routinely checked for mycoplasma contamination and used before passage 20-25. RPMI-1640 (ECB9006L) and DMEM (ECB7501L) media, tetracycline Negative Fetal Bovin Serum (FBS) (ECS0182L), dialysed Fetal Bovine Serum (dFBS) (Hyclone, SH30079.03), glutamine (ECB3000D) and glucose (SE2270001) were purchased from Euroclone. GSH reduced ethyl ester (G1404), N-Acetyl-L-cysteine (NAC) (A7250), doxycycline hyclate (D9891) and D-5030 medium (D5030) without glucose, glutamine, pyruvate and non-essential amino acids were obtained from Sigma. Cells were cultured in the following media (specific growth media): Patu8988T and Panc1 in DMEM and BxPC3 and KP2 in RPMI, the media of all cell lines were supplemented with 10% FBS, 10 mM glucose and 2 mM glutamine. To check effects of UCP2 silencing on PDAC cells, specific growth media and FBS were replaced with D-5030 medium and dialyzed FBS (dFBS).

Lentiviral plasmids. The Tet-pLKO-puro-Scrambled plasmid, used as non-targeting shRNAs (shCtr) in the inducible silencing experiments, was obtained from Addgene (plasmid#47541)[32]. The pLKO-puro-Scrambled plasmid, used in the non-inducible silencing experiments, was obtained from Addgene (plasmid#1864)[33]. The sequences of the two UCP2 silencing shRNAs are the following: GTTCCTCTATCTCGTCTTG[9] (sh#1) and CGGCCTGTATGATTCTGTCAA (TRCN0000060145) (sh#2). sh#1 and sh#2 were cloned AgeI/XbaI in the Tet-pLKO-puro (Addgene, plasmid#21915)[34] (inducible silencing) and, only sh#1 in the pLKO-puro-Scrambled by replacing the shCtr sequence (non-inducible silencing). For the murine UCP2 (mUcp2) expression, the coding sequence was cloned NheI/MluI in the inducible expression plasmid pCW57-MCS1-P2A-MCS2 (Blast) (Addgene, plasmid#80921)[35]. Recombinant lentiviral particles were produced by transient transfection of 293T cells following a standard protocol. For the rescue experiments, Patu8988T cells were first transfected with the control_pLKO.1 and sh#1_pLKO.1 lentiviral particles and selected on puromycin (Invivogen, ant-pr-1). Once checked the efficiency of silencing, the control cells were transfected with the pCW57 empty vector ($pCW57_{EV}$) and the sh#1 cells were transfected with $pCW57_{EV}$ or $pCW57_{mUcp2}$ and selected on blasticidin (Invivogen, ant-bl-05). The lentiviral plasmids used to express $KRAS_{G12V}$ in BxPC3 cells were the two pLKO.1 vectors used in the rescue experiments. Briefly, in the BamHI and KpnI digested control_pLKO.1 and sh#1_pLKO.1 plasmids, we replaced the puromycin with the blasticidin resistance gene (BSD) amplified by PCR using the pCW57-MCS1-P2A-MCS2 (Blast) as template. The forward primer carried a BglII restriction site, compatible with BamHI site, whereas the reverse

primer carried a BamHI and KpnI restriction sites downstream the stop codon of the blasticidin resistance gene (Extended Data Fig. 9a, b). *KRAS*$_{G12V}$ coding sequence was amplified by PCR using a first-strand cDNA, derived from Patu8988T cells mRNA, as template. The forward primer carried an HA-Tag upstream the starting methionine of *KRAS*. The PCR fragment, carrying an XbaI and SalI restriction sites at the 5' and 3' ends, respectively, was cloned in the pULTRA plasmid (Addgene, plasmid #24129)[36], in frame with EGFP and a "self-cleaving" P2A peptide sequences. Once verified the sequences of all plasmids, we digested the pULTRA plasmid with BglII and KpnI, obtaining the KRAS expression cassette made up of the human ubiquitin C promoter (UbC) which controlled the EGFP-P2A-*KRAS*$_{G12V}$ module and the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) sequence at the 3' end. The expression cassette was cloned in the control_pLKO.1 (Blast) and sh#1_pLKO.1 (Blast) plasmids digested BamHI and KpnI, downstream the blasticidin resistance gene (Extended Data Fig. 9c, d). In this way, we constructed two all-in-one lentiviral plasmids expressing *KRAS*$_{G12V}$ as well as the scramble or the sh#1 shRNAs. Lentiviral particles production and cells transfection was carried out as reported above.

Yeast studies. The yeast strain used in all experiments was W303 (*MATa {leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15}*). The yeast gene deletions were constructed using the PCR-mediated gene disruption technique[37]. The hUCP2 and hUCP2R279Q ORFs were cloned HindIII/XhoI in the yeast expression vector pYES2 in which the inducible GAL1 promoter had been replaced with the constitutive TDH3 promoter. Both proteins carried a V5-tag at their C-termini. The endogenous yeast deleted genes AGC1, AAT1, YMC2 and ODC1 were cloned in the centromeric vector pRS416 whereas ODC2 in the pRS415 and used to verify the growth defect phenotype in oleate. For the initial propagation, yeast cells were grown in rich (YP) medium, containing 2% Bacto-peptone and 1% yeast extract, supplemented with 2% glucose. The final pH was adjusted to 4.5. The growth in oleate was carried out by replacing the glucose with 0.5 mM of oleate (Sigma-Aldrich #O1008) dissolved in tergitol (Sigma-Aldrich #15S9)[21].

qRT-PCR. Total RNA was extracted using the Blood/Tissue Total RNA extraction Mini kit (Fisher Molecular Biology) according to the manufacturer's protocol. The first strand cDNA was synthesised using the Prime Script RT Master Mix KIT (Takara, RR036B). The qPCR reactions were performed using an ABI Prism 7900 HT Real Time PCR system (Applied Biosystems). The TaqMan-MGB assays for UCP1 (ID: Hs00222453_m1), UCP2 (ID: Hs01075227_m1), UCP3 (ID: Hs01106052_m1), UCP4 (ID: Hs00188687_m1), UCP5 (ID: Hs01073976_m1) and PPIA (ID: Hs04194521_s1) were purchased from Life Technologies. To quantify murine Ucp2, the qPCR was performed using the SYBR Select Master Mix (Applied Biosystems). The primers based on the cDNA sequences of the investigated genes were designed with Primer Express 3.0 (Applied Biosystems, Life Technologies) and purchased from Invitrogen (Life Technologies). Gene primer sequences used in the qPCR analyses were: PPIA forward 5'-CATACGGGTCCTGGCATCTT-3' and PPIA reverse 5'-TCCATGGCCTCCACAATATTC-3'; Human UCP2 forward 5'-CACCTTTCCTCTGGATACTGCTAAA-3' and Human UCP2 reverse 5'-CACTGGCCCCTGACTTTCTC-3'; Mouse Ucp2 forward 5'-TCATCACTTTCCCTCTGGATACC-3'and Mouse Ucp2 reverse 5'-GCGCACTAGCCCTTGACTCT-3'.

The specificity of the PCR amplification was checked with the heat dissociation protocol following the final cycle of PCR. To correct for differences in the amount of starting first-

strand cDNAs, the human peptidylprolyl isomerase A (PPIA) gene was amplified in parallel as a reference gene[38]. Fluorescence data were calculated using the ABI SDS software 2.4 (Applied Biosystems). The relative quantification of mRNA for UCPs genes was performed according to the comparative method ($2^{-\Delta\Delta Ct}$), where ΔCt sample is Ct sample - Ct reference gene, and Ct is the threshold cycle[39]. For each silenced cell lines, the value of $2^{-\Delta\Delta Ct}$ indicates the fold change in mRNA values relative to the ΔCt of control cell line (calibrator). For analyses, reverse transcribed cDNA from three biological replicates was used and three technical replicates were analysed for each biological replicate. The following doxycycline concentrations were used for UCP2 silencing: 500 ng/ml (Patu8988T and Panc1), 250 ng/ml (BxPC3) and 100 ng/ml (KP2); 500 ng/ml for the induction of mUcp2 in Patu8988T_{NI}.

Western blot analysis. PDAC cell lysates (about 100 µg of proteins) were separated by SDS-PAGE and transferred on nitrocellulose membrane by a Trans-Blot Turbo Transfer System - Protein Blotting System (Bio-Rad). Samples were not heated before loading. Membranes were blocked in Tris-buffered saline (TBS) containing 1% BSA and 0.1% Tween 20 (TBS-T), before incubation with the primary antibody overnight at 4 °C. The membranes were washed with TBS-T and incubated with the appropriate dilution of the HRP-conjugated secondary antibody for 1 hour at room temperature and washed again. Immunoblotting on yeast cells was carried out as above, cell lysates were prepared as previously described[40]. Briefly, yeast cells were resuspended in 100 µl distilled water and 100 µl 0.2 M NaOH, incubated for 5 min at room temperature and centrifuged at 12000 rpm. The pellet was resuspended in 50 µl SDS sample buffer and boiled for 3 min before loading. V5-tagged UCP2 and $UCP2_{R279Q}$ were detected with an anti-V5 monoclonal antiserum. A rabbit antiserum against yeast porin[41], a kind gift of Dr. Pelosi, was used for protein normalization. The SuperSignal™ West Pico PLUS Chemiluminescent Substrate Kit (ThermoFisher #34577) was used to immunodecorate UCP2 and a standard ECL kit (Pierce™) was used for COX4, HA-tagged $KRAS_{G12V,}$ V5-tagged UCP2, yeast porin and AGC1/2. Protein specific signals were detected densitometrically, 5 min of acquisition for UCP2 and few seconds for COX4, HA-tagged $KRAS_{G12V}$, V5-tagged UCP2, yeast porin and AGC1/2. The following antibodies were used: anti-UCP2 (murine homemade antibody, 1:20000), anti-AGC1/2 (rabbit homemade antibody, 1:5000), anti-HA (BioLegend, anti-HA.11 epitope, clone 16B12, 1:5000), anti-V5 (Cell Signaling, #13202, 1:5000), anti-COX4 (Santa Cruz Biotechnology, Antibody (F-8) sc-376731, 1:5000) and anti-yeast porin (rabbit homemade antibody, 1:5000); the following secondary antibodies were used: a Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP (ThermoFisher Scientific Pierce™ #31430) 1:50000 (UCP2), 1:10000 (HA-tagged $KRAS_{G12V}$), 1:20000 (COX4) and a Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP (ThermoFisher Scientific Pierce™ #31460) 1:10000 (V5-tagged UCP2), 1:10000 (AGC1/AGC2) and 1:10000 (yeast porin).

Transport assays. Mitochondria were isolated from PDAC cell lines by using a mitochondrial isolation kit (Thermo Scientific, no. 89874) with the Halt Protease Inhibitor Mixture (Thermo Scientific, no. 78415) according to the manufacturer's instructions. 100 µg of isolated mitochondria were solubilized for 45 min on ice in a buffer containing 2% TX-114 (Sigma-Aldrich, X114-500ML), 1 mM EDTA, 10 mM PIPES, pH 7.4 and cardiolipin (4 mg/ml, final concentration) (Sigma-Aldrich, C0563) and centrifuged at 13.800 x g for 15 min at 4 °C. Triton-solubilized proteins were immediately incorporated into phospholipid vesicles by cyclic removal of the detergent using a hydrophobic column (Amberlite, BioRad, 152-3920)[42]. The composition of the initial mixture used for reconstitution was: 90 µl of Triton-

solubilized extract, 70 µl of Triton X-114 (10% w/v), 110 µl of L-α-phosphatidylcholine from egg yolk (10% w/v) (Sigma-Aldrich, 61771) in the form of sonicated liposomes, 20 mM phosphate (Pi) or 10 mM ATP (Sigma-Aldrich, A2383), 10 mM Pipes (pH 7.0), 0,8 mg/ml cardiolipin and water to a final volume of 700 µl.

Transport was started by adding [^{14}C]aspartate (1 mM) (Moravek, MC-139) or [^{14}C]ADP (50 µM) (PerkinElmer, NEC559050UC) (outside the proteoliposomes and stopped after 30 minutes with a mixture of pyridoxal-5′-phosphate (10 mM) (Sigma-Aldrich, 82870) and bathophenanthroline (10 mM) (Sigma-Aldrich, 146617). All reconstitution and transport assay steps were carried out at 25 °C at the same internal and external pH value (pH 7.0). Finally, the external radioactivity was removed from each sample of proteoliposomes by a Sephadex G-75 column (GE Healfthcare, 17-0051-01); the proteoliposomes were eluted with 50 mM NaCl, and their radioactivity was measured[43]. The reconstitution and transport assays of the bacterially expressed UCP2 and UCP2$_{R279Q}$ were carried as described above and reported previously[9].

Cell proliferation and clonogenic assays. An appropriate number of cells was seeded in 6X multi-well plates in the specific growth media with doxycycline (500 ng/ml (Patu8988T and Panc1), 250 ng/ml (BxPC3) and 100 ng/ml (KP2)). The next day, media were changed with D-5030 supplemented with 2 mM glutamine, 10 mM glucose, 10% dFBS and doxycycline. In some experiments, 2 mM aspartate, 1 mM glutamate, 4 mM glutathione reduced ethyl ester or 4 mM NAC were added. Medium was not substituted during the experiment even though similar results were obtained by changing the medium every two days. The proliferation rate was regularly determined from day 1 to day 9. Cells were counted by an automated Scepter TM Counter Cells (proliferation assay). After 2-3 weeks, colonies were gently washed with PBS (Lonza BE17-516F), fixed in 80% methanol and stained with 0.5% crystal violet. After images acquisition, colonies were counted (clonogenic assay).

GSH/GSSG and NADP$^+$/NADPH assays. 2-4 x 10^6 PDAC cells grown in D-5030 and 30-60 mg of frozen tumours were assayed. NADPH /NADP$^+$ ratios were determined using the NADPH/NADP$^+$ assay kit (Biovision, K347) according to the manufacturer's instructions. After cell lysis, the NADPH/NADP$^+$ inhibiting enzymes were removed using a 10 kD spin column (Vivaspin® 500, Sigma, GE28-9322-25). The GSH/GSSG ratios were determined using the Glutathione Fluorescent Detection Kit (ThermoFisher, EIAGSHF) according to the manufacturer's instructions. In both assays the concentration was obtained by comparison to standard curves.

Measurement of total ROS. The four human PDAC cell lines were plated in specific growth media with doxycycline. The next day, the medium was changed to D-5030 medium supplemented with 2 mM glutamine, 10 mM glucose, 10% dFBS and doxycycline. After four days 2 x 10^5 cells per sample were stained with 10 µM 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Molecular Probes, Eugene) for 30 min at 37°C. After staining, the cells were washed and measured using an Attune Nxt Acoustic Focusing Cytometer (Life Technologies Corporation). Analysis was performed and data visualized using the Attune™ NxT v2.6 Software.

Oxygen consumption rate (OCR). PDAC cells (Patu8988T and BxPC3) were plated into XF Cell Culture Microplates (Seahorse BioScience) in specific growth media with doxycycline and incubated overnight at 37°C and 5% CO_2. The next day, the medium was replaced with D-5030 supplemented with 2 mM glutamine, 10 mM glucose, 10% dFBS and doxycycline, pH 7.4. On the third day, to measure OCR, the medium was replaced with the Seahorse XF base medium (Agilent, 103193-100), supplemented with 2 mM of glutamine and the plate was

incubated at 37°C without CO_2 for 1 h. During the experiment, 2 µM oligomycin (Sigma, 04876), 0.5 µM FCCP (Sigma, C2759), and 1 µM antimycin A (Sigma, A8674) + 1 µM rotenone (Sigma, R8875) were sequentially injected. The experiments were run using a Seahorse XFe96 Analyzer and data analysis was carried out with Wave Desktop 2.2 software. The raw data were normalized to the total protein amount per well measured at the end of the experiment.

UCP2 expression analysis in PDAC and gene search enrichments analysis (GSEA). Transcript abundance of UCP2 from PDAC primary tumour samples, normal pancreatic tissues and PDAC cell lines was determined by analysing gene expression profiling (GEP) of different publicly available datasets[44-49]. All the GEP (HG-U133_Plus 2.0 and HG_U219 arrays from Affimetrix platform) were processed by using RMA (Robust Multi-array Average) normalization method by means of RMAExpress v1.2.0. Probes were then collapsed to unique genes by selecting the ones with the maximal average expression for each gene and the expression values were log2 transformed. The difference of UCP2 expression between PDAC samples and normal pancreas was determined by Mann-Whitney test with the R statistical programming environment (v3.4.3). The sources data used to perform UCP2 expression analysis in PDAC primary tumour samples and cell lines are reported in the Supplementary Table 2. GSEA[50] was performed on publicly available PDAC datasets[46,51] by using continuous UCP2 expression level to label tumour samples. Genes were ranked by Pearson metric method. We then tested roughly 200 gene sets related to different metabolic pathways gathered from MSigDB collection (https://www.gsea-msigdb.org/gsea/msigdb/collections.jsp).

Stable isotope analysis using GC-MS.

Rapid subcellular fractionation and metabolite extraction. Doxycycline-induced PDAC cells were cultured in 6-well in D-5030 medium supplemented with 10 mM glucose, 10% dFBS plates and 2 mM of [U-^{13}C] glutamine for 24 h. The cytosolic and mitochondrial-enriched pools separation was carried out as previously described[20] with some modifications, i.e. the digitonin (Sigma, D5628) cell permeabilization was carried out on adherent cells and the amount of digitonin used was much lower. Briefly, after tracer incubation, the 6-well plate was placed on ice and cells were washed twice with ice-cold phosphate buffer saline, then they were incubated for 2 minutes with 300 µl of a buffer containing NaCl (150 mM), $MgCl_2$ (5 mM), $KHCO_3$ (20 mM), pH7.4, and digitonin (Patu8988T, 100 µg and BxPC3, 200 µg per million of cells). The supernatant (cytosolic fraction) was removed and immediately quenched in 1 ml of methanol/chloroform (1:1, -20°C). Permeabilized cells were washed once with the permeabilization buffer without digitonin, quenched with 500 µl of methanol (-60 °C), scraped from the well and collected in a tube containing 800 µl of chloroform/water (1,67:1) (mitochondrial enriched fraction). The two fractions were vortexed for 5 min at 4 °C, centrifuged at 14000 rpm for 10 min at 4° C, the upper phase recovered, dried using a Speedvac and stored at -80 °C until the derivatization. During metabolites extraction, tartaric acid was used as internal standards to normalize the efficiency of derivatization. The integrity of mitochondria in the permeabilization buffer was checked: i) by respirometry, using the correct n. of cells/amount of digitonin ratio, PDAC cells efficiently respired succinate and the respiration was inhibited by oligomycin and uncoupled by FCCP; ii) PDAC cells transfected with a mitochondria-targeted GFP (mGFP)[52] and treated with the permeabilization buffer did not release any fluorescence from mitochondria upon four minutes of treatment (Extended Data Fig. 3m). Similar results were obtained if digitonin was replaced by 10 nM of rFPO (XF PMP)[53]. Images were acquired with

63x plan apo objective every 20 secs with an exposure time of 100 msecs (MetaMorph software 6.1).

Derivatisation. Dried sample were dissolved in 50 µL methoxamine reagent (SIGMA) and sonicated for 10 min. Samples were then kept at 40 °C for 90 min, then at 70 °C for 30 min following the addition of 70 µl MTBSTFA +1 % TBDMCS (ThermoFisher Scientific)[54]. Samples were next transferred to vials equipped with inserts.

GC-MS measurements. A Waters® Micromass® Quattro micro™ GC Triple Quadrupole mass spectrometer, directly interfaced to an Agilent 6890N GC was used to analyse the samples. Samples (2 µL) were injected in splitless mode into the GC and then separated with a fused silica DB-5MS+DG capillary column (30 m, 0.25 mm inside diameter, 0.25 µm thickness of the inner liquid in the column).The injector temperature was set to 240°C. High purity helium was used as a carrier gas at a constant flow rate of 1 mL/min. The column temperature was initially kept at 70°C for 2 min, ramped up to 140 °C at 3 °C/min, ramped up to 150 °C at 1 °C/min and ramped up to 280 °C at 3 °C/min. The interphase and ion source temperatures were 280°C and 240°C, respectively. Data were acquired in Selected Ion Monitoring (SIM)[54] mode and the integrated peak area of the targeted labelled ions was normalised by the peak area of tartrate. All the metabolites were quantified using five points external calibration curves over the range of 1-1000 ng/metabolite. The quantity of metabolite fraction analysed was adjusted to the corresponding protein concentration calculated upon processing a parallel 6-well plate. Data were acquired and processed using MassLynx 4.0 software.

Organotypic 3D culture. PDAC cells were seeded at an appropriate density in 96-well plates on top of an extracellular matrix gel prepared by Matrigel (Corning Matrigel Growth Factor Reduced Basement Membrane Matrix, Phenol Red-Free) with serum-free specific growth media, to a final concentration of 7 mg/ml. One hundred microliters/well was plated into 96-well plates and incubated for 60 min in a 37°C incubator allowing the gels to solidify. The next day D-5030 medium was added and growth was maintained for a total of 7 days, with a change of medium every two days. After 5 or 7 days in these growth conditions, colonies were photographed using the TE200 microscope (Nikon USA, Garden City, NY, USA). Cell viability was measured using the Resazurin cell viability assay (Immunological Sciences, Rome, Italy). Resazurin (10 µl) was added to each 100 µl of medium according to the manufacturer's instructions and fluorescence was measured after ~3 h. Cell death was assayed by incubating the cells overnight at 37°C with Ethidium Homodimer-1 (16 nM). The images with ethidium homodimer-1 were acquired with a 4X air objective using a Nikon EclipseTi-S epifluorescence microscope and analysed for integrated density using ImageJ (http://rsb.info.nih.gov/ij/).

Xenograft studies. For subcutaneous xenografts, 5 weeks old female SCID mice C.B-17/IcrHanHsd-Prkdc^{scid} congenic strain of Balb/cAnIcr were purchased from ENVIGO RMS srl, (Udine, Italy). The mice were housed with 12-hour light/dark cycle at 23±1°C and 50±5 % humidity, and ad libitum diet and water were provided. Control, UCP2-silenced and rescued PDAC cells (Patu8988T (7 x 10^5), Panc1, BxPC3 and KP2 (1.5-2 x 10^6)) suspended in 200 µl Hanks Buffered Saline Solution (HBSS), were injected subcutaneously into the interscapular region of mice (5 animals for each group). Tumour length and width were measured with a calliper and the volume was calculated according to the formula (length x width^2)/2. No littermate controls were used. When the tumour sizes reached the volume of 40-70 mm^3, animals were fed with doxycycline water (doxycycline 2 g l^{-1}, sucrose 20 g l^{-1}). For the rescue experiment doxycycline water was administrated two days after the injection. Tumour

volume was measured once per week. We state that for 3 out of 60 mice used in the xenograft experiments the final tumour volume exceeded of about 10% the maximal tumour volume (2 cm^3) established by our animal care committee for implanted tumours in the interscapular region (Extended Data Fig. 5c and 6g). We also state that all tumour bearing animals, for all the duration of the experiment, were observed daily to screen for behavioural changes resulting from tumour burden and were respected all endpoints established by our animal care committee as: i) absence of tumour ulceration; ii) parental care; iii) food and water intake; iv) no loss body weight; v) no locomotion limitation; vi) no cachexia; we also state that no animal presented any of these endpoints for all the duration of the experiment. At the end of each experiment, animals were euthanized, and the xenografts were harvested and subjected to downstream end-point analysis. All xenograft experiments with human PDAC lines were approved by Italian Ministry of Health under protocol number 418/2015-PR. All animals were maintained and handled in accordance with the recommendation of the Guidelines for the Care and Use of Laboratory Animals and experiments were approved by the Animal Care Committee of University of Calabria (OPBA), Italy.

Immunohistochemical analysis. Tumours were fixed in 4% formalin, embedded in paraffin and sectioned (5 µm). The sections were mounted on slides precoated with poly-lysine, and then deparaffinised and dehydrated (seven to eight serial sections). Immunohistochemical experiments were performed after heat-mediated antigen retrieval, using human Ki67 (1:100 Clone MIB-1 DAKO, Denmark) primary antibodies at 4 °C overnight. Then, a biotinylated specific IgG was applied for 1 h at room temperature, followed by the avidin biotin horseradish peroxidase complex (VECTASTAIN® ABC KIT, UNIVERSAL Vector Laboratories, CA). Immunoreactivity was visualized by using DAB Peroxidase (HRP) Substrate Kit, 3,3'-diaminobenzidine (VECTOR® Vector Laboratories, CA). The immunostained slides of tumour samples were evaluated by light microscopy using 20X magnification (Olympus BX51 microscope).

Scoring system. Six to seven serial sections were scored in a blinded manner for each sample by two independent observers. To quantify the proliferation index, the percentage of Ki-67-positive cells was counted in 10 random fields at 20X magnification (Olympus BX51 microscope).

Statistical analysis. Graphpad Prism software V7 was used to conduct the statistical analysis of all data, except for qPCR and Seahorse data where Microsoft Excel was used. Data are presented as mean ± SD. All quantitative results were assessed by unpaired two-tailed Student's *t*-test or Mann–Whitney test after confirming that the data met appropriate assumptions. The Student's t-test assumed two-tailed distributions to calculate statistical significance between groups. Unless otherwise indicated, for all *in vitro* experiments, 2 independent experiments with three replicates were analysed. For mouse studies, tumour development and staining intensity of tissue sections were scored in a 'blinded' manner. GC-MS studies were also carried out in a 'blinded' manner. $P < 0.05$ was considered statistically significant and the P value is indicated in the figures.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data Availability

Source data are provided with this paper.

All data used for expression analysis and GSEA were publicly available.
The datasets using HG133plus2 from Affymetrix included Array Express accession code E-MEXP-2780; GEO accession codes GSE15471, GSE22780, GSE16515, GSE32676, GSE42952 and GSE36133.
The dataset using HG_U219 from Affymetrix included Array Express accession code E-MTAB-6134.
The unverified heterozygous mutation (c.34G>C, p.G12R) for KP2 cells is reported in the Catalogue of Somatic Mutations in Cancer (COSMIC), sample ID COSS1298218.
There no restriction on data availability, any additional information supporting the data within this paper and the findings of this study are available from the corresponding authors upon request.

References

31 Gao, J. *et al.* Validation of targeted next-generation sequencing for RAS mutation detection in FFPE colorectal cancer tissues: comparison with Sanger sequencing and ARMS-Scorpion real-time PCR. *BMJ Open* **6**, e009532, doi:10.1136/bmjopen-2015-009532 (2016).
32 Rudin, C. M. *et al.* Comprehensive genomic analysis identifies SOX2 as a frequently amplified gene in small-cell lung cancer. *Nat Genet* **44**, 1111-1116, doi:10.1038/ng.2405 (2012).
33 Sarbassov, D. D., Guertin, D. A., Ali, S. M. & Sabatini, D. M. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**, 1098-1101, doi:10.1126/science.1106148 (2005).
34 Wiederschain, D. *et al.* Single-vector inducible lentiviral RNAi system for oncology target validation. *Cell Cycle* **8**, 498-504, doi:10.4161/cc.8.3.7701 (2009).
35 Barger, C. J., Branick, C., Chee, L. & Karpf, A. R. Pan-Cancer Analyses Reveal Genomic Features of FOXM1 Overexpression in Cancer. *Cancers (Basel)* **11**, doi:10.3390/cancers11020251 (2019).
36 Lou, E. *et al.* Tunneling nanotubes provide a unique conduit for intercellular transfer of cellular contents in human malignant pleural mesothelioma. *PLoS ONE* **7**, e33093, doi:10.1371/journal.pone.0033093 (2012).
37 Wach, A. PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in S. cerevisiae. *Yeast* **12**, 259-265, doi:10.1002/(SICI)1097-0061(19960315)12:3%3C259::AID-YEA901%3E3.0.CO;2-C (1996).
38 Agrimi, G., Russo, A., Scarcia, P. & Palmieri, F. The human gene SLC25A17 encodes a peroxisomal transporter of coenzyme A, FAD and NAD+. *Biochem J* **443**, 241-247, doi:10.1042/BJ20111420 (2012).
39 Bustin, S. A. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* **25**, 169-193 (2000).
40 Kushnirov, V. V. Rapid and reliable protein extraction from yeast. *Yeast* **16**, 857-860, doi:10.1002/1097-0061(20000630)16:9<857::AID-YEA561>3.0.CO;2-B (2000).
41 De Marcos Lousa, C., Trezeguet, V., Dianoux, A. C., Brandolin, G. & Lauquin, G. J. The human mitochondrial ADP/ATP carriers: kinetic properties and biogenesis of wild-type and mutant proteins in the yeast S. cerevisiae. *Biochemistry* **41**, 14412-14420, doi:10.1021/bi0261490 (2002).

42 Li, Y. *et al.* Functional characterization of the partially purified Sac1p independent adenine nucleotide transport system (ANTS) from yeast endoplasmic reticulum. *J Biochem* **164**, 313-322, doi:10.1093/jb/mvy054 (2018).
43 Vozza, A. *et al.* Biochemical characterization of a new mitochondrial transporter of dephosphocoenzyme A in Drosophila melanogaster. *Biochim Biophys Acta* **1858**, 137-146, doi:10.1016/j.bbabio.2016.11.006 (2017).
44 Badea, L., Herlea, V., Dima, S. O., Dumitrascu, T. & Popescu, I. Combined gene expression analysis of whole-tissue and microdissected pancreatic ductal adenocarcinoma identifies genes specifically overexpressed in tumor epithelia. *Hepatogastroenterology* **55**, 2016-2027, doi:papers3://publication/uuid/CC44A547-7F3A-4827-924A-66971F37C930 (2008).
45 Pei, H. *et al.* FKBP51 affects cancer cell response to chemotherapy by negatively regulating Akt. *Cancer Cell* **16**, 259-266, doi:10.1016/j.ccr.2009.07.016 (2009).
46 Barretina, J. *et al.* The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* **483**, 603-607, doi:10.1038/nature11003 (2012).
47 Donahue, T. R. *et al.* Integrative survival-based molecular profiling of human pancreatic cancer. *Clin Cancer Res* **18**, 1352-1363, doi:10.1158/1078-0432.CCR-11-1539 (2012).
48 Van den Broeck, A., Vankelecom, H., Van Eijsden, R., Govaere, O. & Topal, B. Molecular markers associated with outcome and metastasis in human pancreatic cancer. *J Exp Clin Cancer Res* **31**, 68, doi:10.1186/1756-9966-31-68 (2012).
49 Winter, C. *et al.* Google goes cancer: improving outcome prediction for cancer patients by network-based ranking of marker genes. *PLoS Comput Biol* **8**, e1002511, doi:10.1371/journal.pcbi.1002511 (2012).
50 Subramanian, A. *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* **102**, 15545-15550, doi:10.1073/pnas.0506580102 (2005).
51 Puleo, F. *et al.* Stratification of Pancreatic Ductal Adenocarcinomas Based on Tumor and Microenvironment Features. *Gastroenterology* **155**, 1999-2013 e1993, doi:10.1053/j.gastro.2018.08.033 (2018).
52 Marchi, S., Bonora, M., Patergnani, S., Giorgi, C. & Pinton, P. Methods to Assess Mitochondrial Morphology in Mammalian Cells Mounting Autophagic or Mitophagic Responses. *Methods Enzymol* **588**, 171-186, doi:10.1016/bs.mie.2016.09.080 (2017).
53 Divakaruni, A. S. *et al.* Thiazolidinediones are acute, specific inhibitors of the mitochondrial pyruvate carrier. *Proc Natl Acad Sci U S A* **110**, 5422-5427, doi:10.1073/pnas.1303360110 (2013).
54 Young, J. D., Allen, D. K. & Morgan, J. A. Isotopomer measurement techniques in metabolic flux analysis II: mass spectrometry. *Methods Mol Biol* **1083**, 85-108, doi:10.1007/978-1-62703-661-0_7 (2014).