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Supplemental information

Combining the antianginal drug perhexiline with chemotherapy induces complete pancreatic cancer regression *in vivo*

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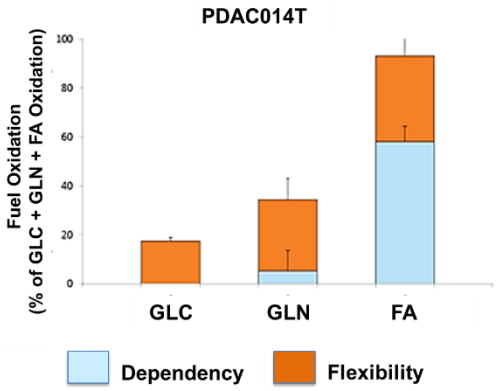
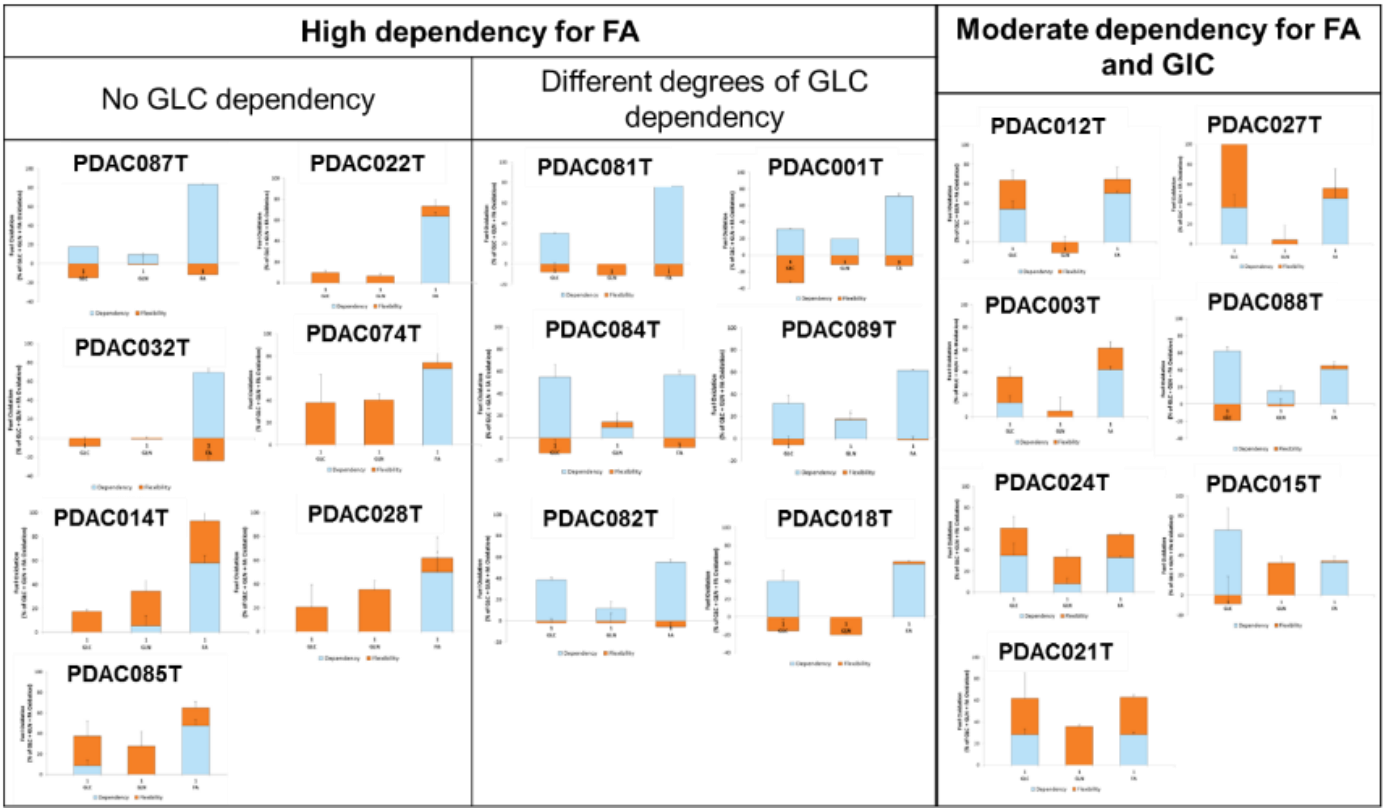
A


PDAC014T
Fuel Oxidation
(% of GLC + GLN + FA Oxidation)
100
80
60
40
20
0
GLC
GLN
FA
Dependency
Flexibility

B


High dependency for FA
No GLC dependency
Different degrees of GLC dependency
Moderate dependency for FA and GIC
PDAC087T
PDAC022T
PDAC081T
PDAC001T
PDAC012T
PDAC027T
PDAC032T
PDAC074T
PDAC084T
PDAC089T
PDAC003T
PDAC088T
PDAC014T
PDAC028T
PDAC082T
PDAC018T
PDAC024T
PDAC015T
PDAC085T
PDAC021T

C

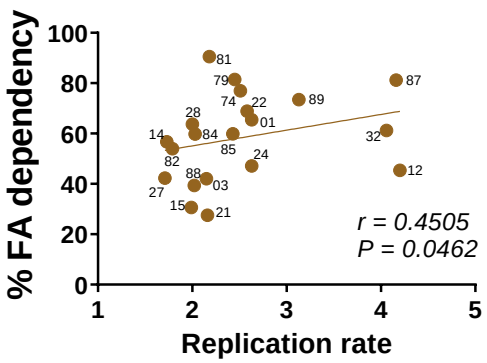

% FA dependency
100
80
60
40
20
0
1
2
3
4
5
Replication rate
81
79
74
22
89
87
28
01
14
84
85
24
32
82
88
03
12
27
15
21
r = 0.4505
P = 0.0462

Figure S1. Mitochondrial respiration of primary pancreatic cancer cells depends mainly on the Fatty Acid Oxidation (FAO) pathway (related to Figure 1). (A) Mitochondrial dependency and flexibility to oxidize three main energetic fuels: glucose (GLC), glutamine (GLN), and long-chain fatty acids (FA). Example of one primary PDAC cell. The rate of oxidation is expressed in percentage. **(B)** We addressed the mitochondrial dependency towards GLC, GLN, and FA, in 21 primary PDAC cells. Our results led us to classify them in two main groups. The first group consist in cells with high dependency on FA with no GLC dependence or different degrees of GLC dependency. The second group (one third of the total) consist in cells with moderate dependency for FA and GLC. The Perhexiline high responder xenograft PDAC084T (showing *in vivo* complete tumor regression induced by combination treatment in this study) is found in the high FA dependency group with moderate dependency for GLC. The Perhexiline low responder cells PDAC022T and PDAC032T are found in the high FA dependency group with no GLC reliance. Interestingly, the percentage of glutamine dependency is very low in all the tested cells. The graphs shown are representative of three independent experiments performed in triplicate, and data are the mean ± SD. **(C)** The FAO dependency of PDAC cells is related to their proliferation rate. The replication rate of each primary PDAC cell has been reported in our previous publication [S1]. The scatterplot displays the replication rate and the percentage of FA dependency from Figure 1B. Spearman's correlation coefficient and p value are shown, indicating statistically significant association between the two variables.

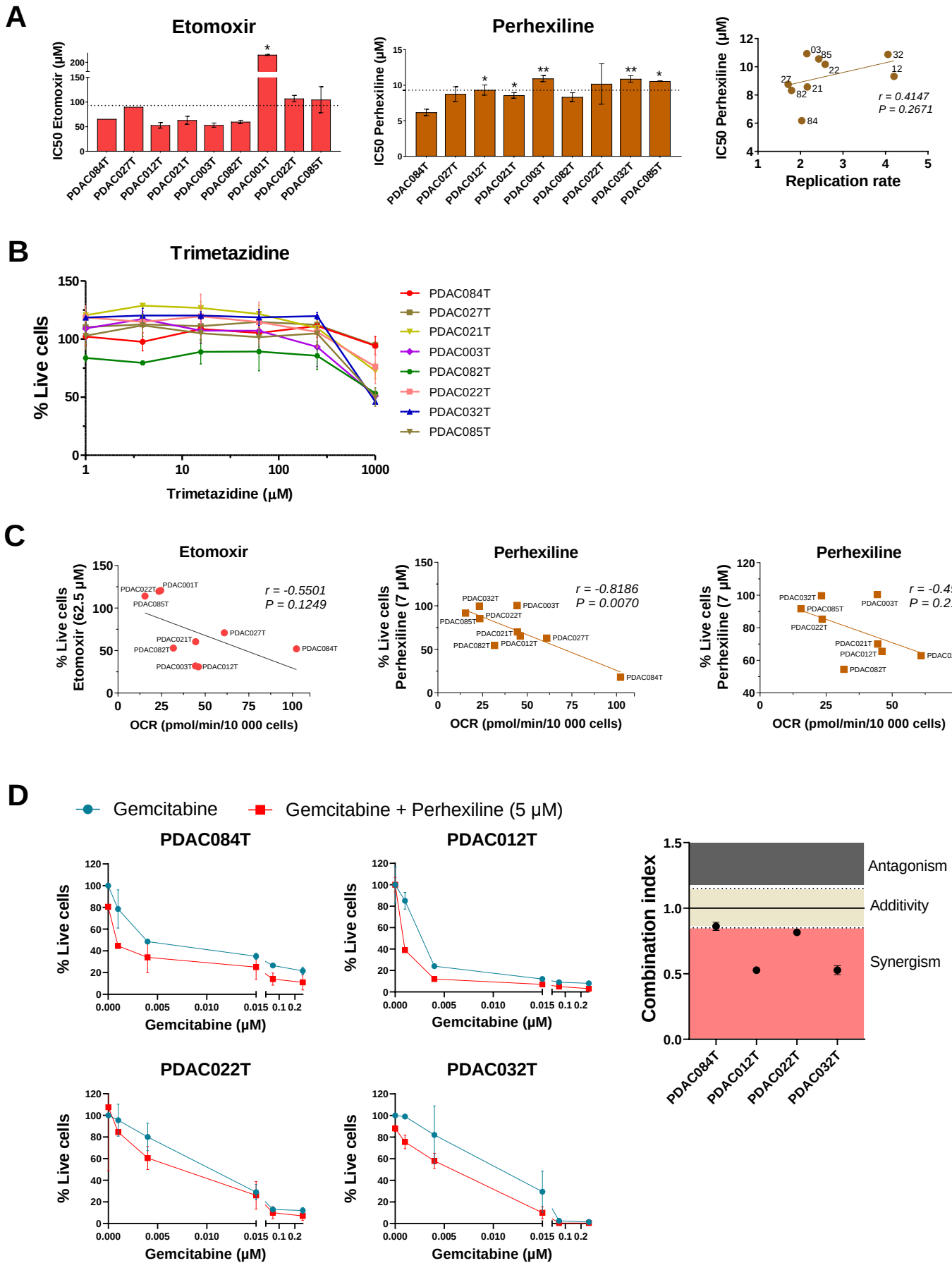

A
Etomoxir
IC50 Etomoxir (μM)
PDAC084T
PDAC027T
PDAC012T
PDAC021T
PDAC003T
PDAC082T
PDAC001T
PDAC022T
PDAC085T
Perhexiline
IC50 Perhexiline (μM)
PDAC032T
IC50 Perhexiline (μM)
Replication rate
r = 0.4147
P = 0.2671
B
Trimetazidine
% Live cells
Trimetazidine (μM)
C
Etomoxir
% Live cells
Etomoxir (62.5 μM)
r = -0.5501
P = 0.1249
OCR (pmol/min/10 000 cells)
Perhexiline
% Live cells
Perhexiline (7 μM)
r = -0.8186
P = 0.0070
r = -0.4922
P = 0.2154
D
Gemcitabine
Gemcitabine + Perhexiline (5 μM)
PDAC084T
PDAC012T
PDAC022T
PDAC032T
Gemcitabine (μM)
Combination index
Antagonism
Additivity
Synergism

Figure S2. Primary PDAC cells exhibit different sensitivities to FAO inhibitors, and the OXPHOS rates correlate with response to Perhexiline (related to Figure 2). (A) IC50 graphs of treatments with Etomoxir and Perhexiline. Data correspond to the mean (± SEM) of at least 2 independent experiments performed in triplicates, and the dot line is the average of the total of values. Unpaired t Test was used to calculate statistical significance * $p<0.05$, ** $p<0.01$. (Right) The Perhexiline sensitivity of PDAC cells is not related to their proliferation rate. The scatterplot displays the replication rate and the Perhexiline IC50. Pearson's correlation coefficient and p value are shown, indicating non-significant association between the two variables. **(B)** Dose-response curve of Trimetazidine treatment in primary PDAC cells. This curve is representative of two independent experiments performed in triplicate and data are the mean ± SD. **(C)** Scatterplots showing a negative correlation between the percentage of live cells treated with Perhexiline or Etomoxir and the basal mitochondrial respiration (Oxygen Consumption Rate, OCR) reported previously [S1]. The correlation between response to Perhexiline and OCR is statistically significant. The significance is lost when the high OXPHOS PDAC084T is excluded from the scatterplot (graph on the right). Pearson correlation and two tailed test were used. **(D)** (Left) Dose-response curves of PDAC cells treated with Gemcitabine with or without Perhexiline at low concentration (5 µM that alone has no impact on cell viability). (Right) Calculation of the combination index with the Chou and Talalay method for the 1 nM concentration of Gemcitabine, using two independent experiments.

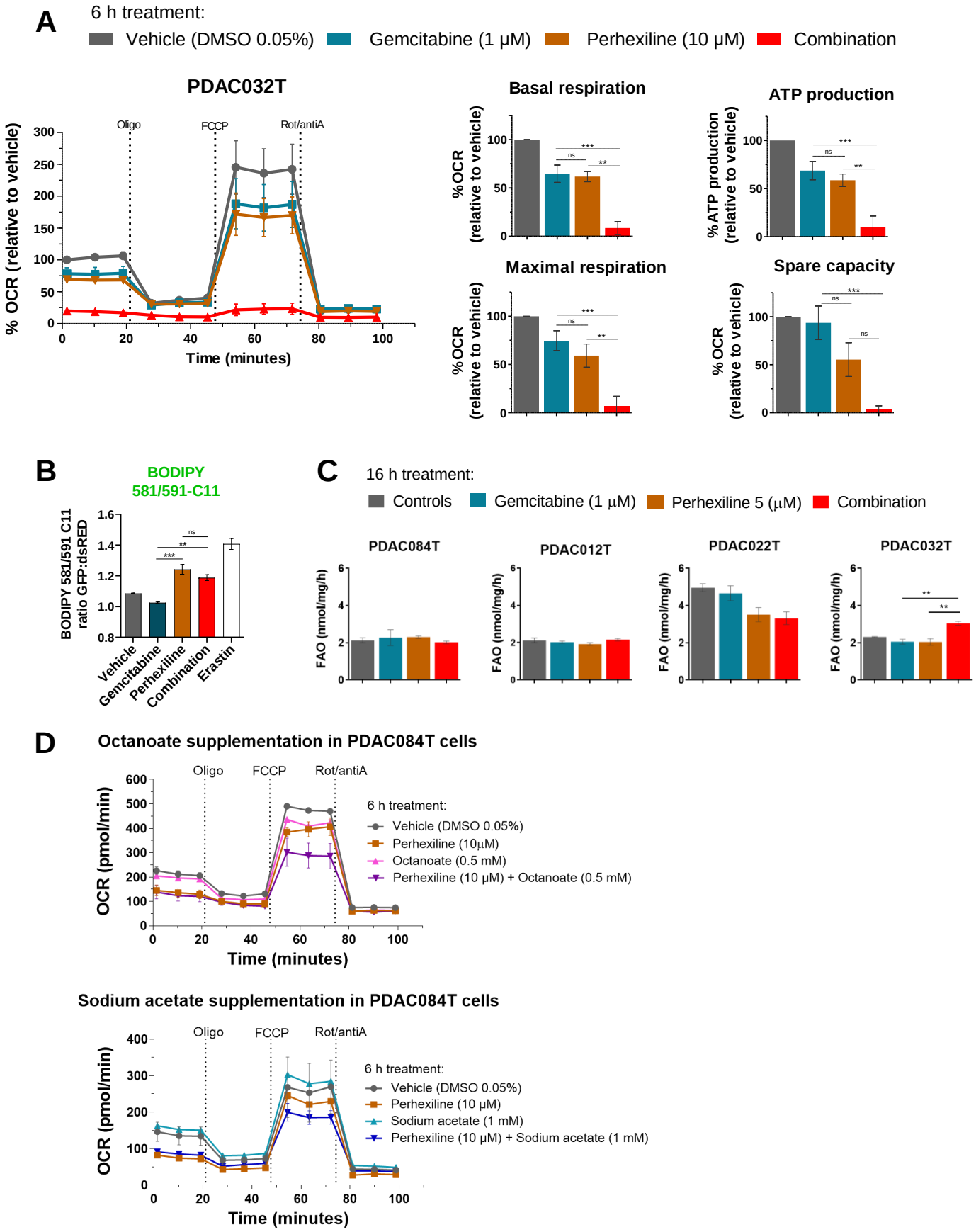
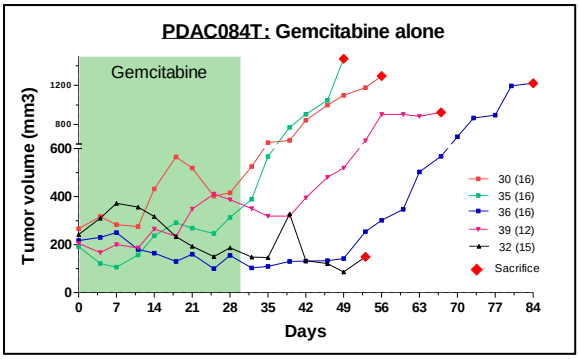
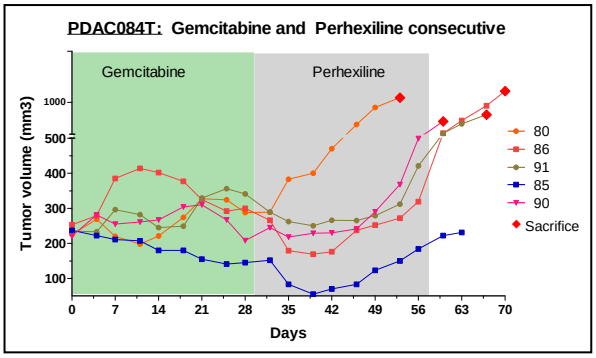
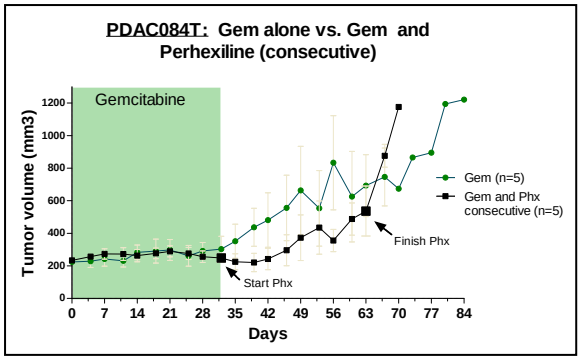

A
6 h treatment:
Vehicle (DMSO 0.05%)
Gemcitabine (1 μM)
Perhexiline (10 μM)
Combination
PDAC032T
% OCR (relative to vehicle)
Oligo
FCCP
Rot/antiA
Time (minutes)
Basal respiration
ATP production
Maximal respiration
Spare capacity
%OCR (relative to vehicle)
%ATP production (relative to vehicle)
B
BODIPY 581/591-C11
BODIPY 581/591 C11 ratio GFP:dsRED
Vehicle
Gemcitabine
Perhexiline
Combination
Erastin
C
16 h treatment:
Controls
Gemcitabine (1 μM)
Perhexiline 5 (μM)
Combination
PDAC084T
PDAC012T
PDAC022T
PDAC032T
FAO (nmol/mg/h)
D
Octanoate supplementation in PDAC084T cells
OCR (pmol/min)
6 h treatment:
Vehicle (DMSO 0.05%)
Perhexiline (10μM)
Octanoate (0.5 mM)
Perhexiline (10 μM) + Octanoate (0.5 mM)
Sodium acetate supplementation in PDAC084T cells
Perhexiline (10 μM)
Sodium acetate (1 mM)
Perhexiline (10 μM) + Sodium acetate (1 mM)

Figure S3. The combination of Gemcitabine with Perhexiline enhances the energetic and oxidative stress induced by Perhexiline in primary pancreatic cancer cells, independently of FAO inhibition (related to Figure 3). (A) Mitochondrial respiration (Oxygen Consumption Rate, OCR) was measured on a Seahorse oxygraph in PDAC032T cells after 6 hours of Gemcitabine (1 µM), Perhexiline (10 µM), or combination treatments at same dose that drugs alone. DMSO (0.05%) was used as vehicle for the controls. The basal and maximal respiration, ATP production by mitochondria, and spare respiratory capacity were calculated. Values are presented as percentage of the vehicle-treated. Data are mean ± SEM of three independent experiments performed in triplicates. p values calculated from One-Way ANOVA test; ** p<0.01, *** p<0.001. **(B)** PDAC084T cells were treated with Gemcitabine (1 µM), Perhexiline (7 µM) or the combination for 24 hours, and processed for flow cytometry to quantify lipid peroxidation using BODIPY 581/591 C11 staining. Cells treated with Erastin (2 µM) were used as positive controls. We measured the two forms of the probe, the reduced (dsRED) and oxidized form (GFP), then the ratio oxidized/reduced was calculated as shown. Data are mean of three independent experiments ± SEM performed in duplicates. p values calculated from One-Way ANOVA test; ** p<0.01, *** p<0.001. **(C)** FAO measurement by labeling with ^{3}H-palmitate and quantification of $^{3}H_2O$. Perhexiline treatment is not decreasing FAO level except in PDAC022T cells. Data are mean of two independent experiments ± SEM performed in duplicates. p values calculated from Two-Way ANOVA test; ** p<0.01 **(D)** OCR was measured on a Seahorse oxygraph 6 h after treatment with Perhexiline (10 µM) or DMSO control, with or without supplementation with either octanoate 0.5 mM (Top) or sodium acetate 1 mM (Bottom). Same experiments were done with other concentrations of octanoate (0.1, 1, and 5 mM) and sodium acetate (2 and 5 mM) with similar outcome. No rescue of Perhexiline-induced OCR decrease was observed indicating that it could not be due to FAO inhibition.

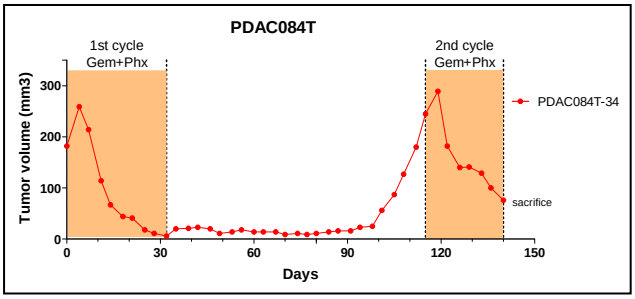
A

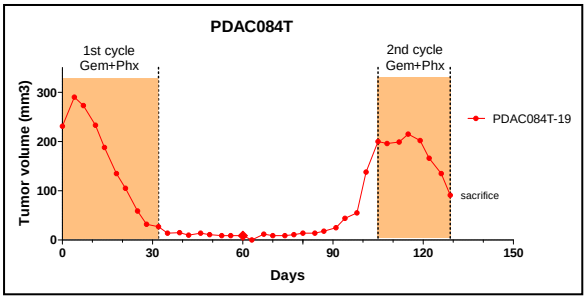

PDAC084T: Gemcitabine alone
Gemcitabine
Tumor volume (mm3)
Days
30 (16)
35 (16)
36 (16)
39 (12)
32 (15)
Sacrifice


PDAC084T: Gemcitabine and Perhexiline consecutive
Gemcitabine
Perhexiline
Tumor volume (mm3)
Days
80
86
91
85
90
Sacrifice


PDAC084T: Gem alone vs. Gem and Perhexiline (consecutive)
Gemcitabine
Tumor volume (mm3)
Days
Gem (n=5)
Gem and Phx consecutive (n=5)
Finish Phx
Start Phx

B


PDAC084T
1st cycle Gem+Phx
2nd cycle Gem+Phx
Tumor volume (mm3)
Days
PDAC084T-34
sacrifice


PDAC084T
1st cycle Gem+Phx
2nd cycle Gem+Phx
Tumor volume (mm3)
Days
PDAC084T-19
sacrifice

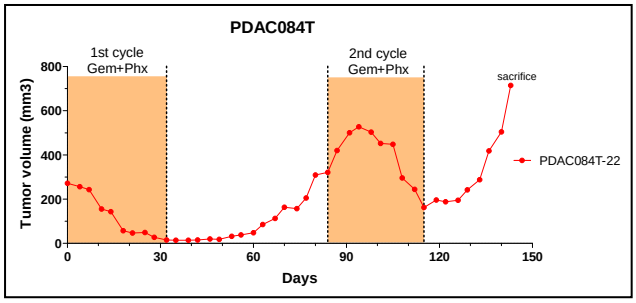

PDAC084T
1st cycle Gem+Phx
2nd cycle Gem+Phx
Tumor volume (mm3)
Days
sacrifice
PDAC084T-22

Figure S4. Efficacy of Perhexiline treatment in combination with Gemcitabine in a sequential therapeutic scheme, and in a relapse context (related to Figure 4A). (A) We aimed to compare the efficacy of the combination treatment of Gemcitabine with Perhexiline in a simultaneous or sequential administration in the PDAC084T xenograft model. For the sequential administration, we treated a cohort of mice with Gemcitabine alone during one month, followed by Perhexiline treatment alone also for one month. Interestingly, we observed that this sequential therapeutic strategy does not induce complete tumor regression contrary to a simultaneous treatment; however, it seems to delay the tumor growth in comparison with the sole Gemcitabine therapy. **(B)** The xenograft model PDAC084T show complete tumor regression upon Gemcitabine plus Perhexiline treatment for one month. Then, we monitored the mice after the tumor regression, and we found out that tumors relapsed after a certain period of time. To address the potential of our combination therapeutic strategy in this relapse context, we treated the tumor-bearing mice with a second cycle of Perhexiline and Gemcitabine. Remarkably, we observed that the tumors remain sensitive to the combination strategy (these mice were sacrificed before one-month treatment for *ex vivo* analysis).


Enriched Pathways | KEGG
activated
suppressed
Ribosome
Nucleotide excision repair
Maturity onset diabetes of the young
Base excision repair
DNA replication
Retinol metabolism
African trypanosomiasis
Fanconi anemia pathway
Olfactory transduction
Taste transduction
Autoimmune thyroid disease
Oxidative phosphorylation
2−Oxocarboxylic acid metabolism
Chemical carcinogenesis
Bile secretion
Complement and coagulation cascades
Hypertrophic cardiomyopathy
Metabolism of xenobiotics by cytochrome P450
Drug metabolism − cytochrome P450
Neuroactive ligand−receptor interaction
Dilated cardiomyopathy
Viral protein interaction with cytokine and cytokine receptor
Herpes simplex virus 1 infection
Vitamin digestion and absorption
Linoleic acid metabolism
Staphylococcus aureus infection
Calcium signaling pathway
Fat digestion and absorption
RNA polymerase
Nicotine addiction
GnRH secretion
Vascular smooth muscle contraction
Cell adhesion molecules
Pancreatic secretion
alpha−Linolenic acid metabolism
GABAergic synapse
Oxytocin signaling pathway
Vasopressin−regulated water reabsorption
Thermogenesis
Circadian entrainment
Glutamatergic synapse
Serotonergic synapse
Morphine addiction
Gap junction
Cardiac muscle contraction
Rap1 signaling pathway
Ras signaling pathway
Ether lipid metabolism
MAPK signaling pathway
Retrograde endocannabinoid signaling
Tyrosine metabolism
Phenylalanine metabolism
Fatty acid degradation
0.1
0.2
0.3
0.4
0.5
0.6
GeneRatio
Count
50
100
150
200
p.adjust
0.01
0.02
0.03
0.04

Figure S5. Significantly enriched pathways in PDAC cells comparing high responders to Perhexiline versus low responders (related to Figure 5A). Transcriptomic analysis of significantly enriched activated and suppressed KEGG pathways. The vertical items are the names of KEGG terms. The depth of the color represents the adjusted p-value and the area of circle in the graph means gene counts.

Total Fatty Acids

Perhexiline (High vs. Low responders)

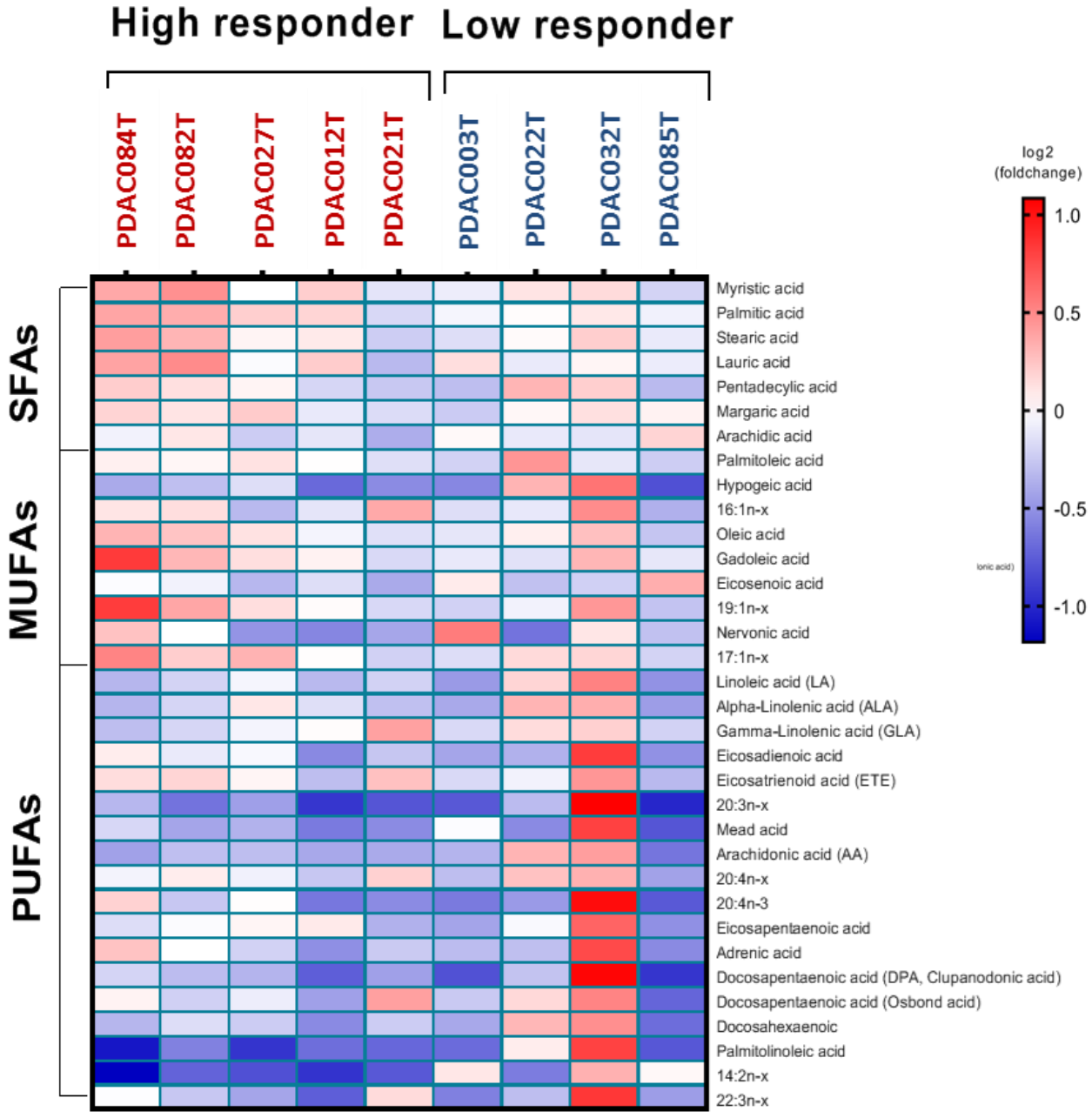


Figure S6. Fatty acid metabolomic analysis in PDAC cells comparing high responders to Perhexiline versus low responders (related to Figure 5A and S5). Heatmap representing fatty acids abundance comparing the high versus low responder groups to Perhexiline treatment. Red and blue colors indicate positive and negative correlation, whereas the white color indicates the absence of correlation. SFAs = Saturated fatty acids, MUFAs = Monounsaturated fatty acids, PUFAs = Polyunsaturated fatty acids.

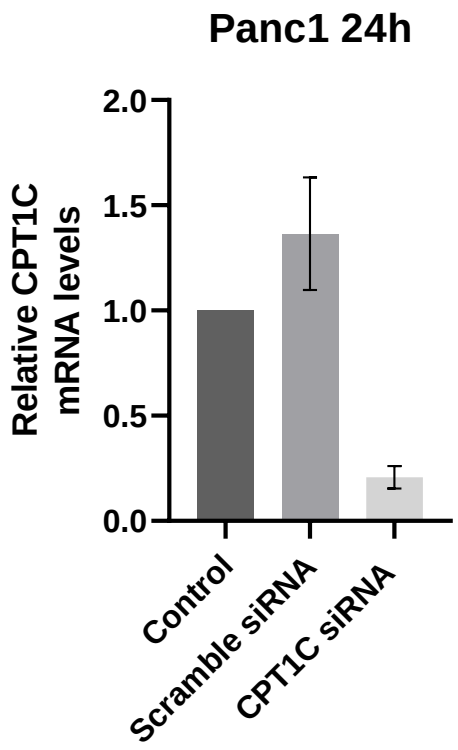


Figure S7. CPT1C downregulation in siRNA-transfected Panc-1 cells (related to Figure 5F). Cells were seeded and transfected in triplicates for 24 h, then pooled before performing RT-qPCR in triplicates to determine CPT1C mRNA levels. Data are mean (± SEM) of triplicates and representative of three independent assays.

Table S1. Cell density of primary PDAC cells used for the Seahorse Mito Fuel Flex Test experiments (related to Figure 1 and S1A-B).

Cell line	Cellular density
PDAC001T	30 000
PDAC003T	30 000
PDAC012T	50 000
PDAC014T	40 000
PDAC015T	20 000
PDAC018T	15 000
PDAC021T	30 000
PDAC022T	40 000
PDAC024T	30 000
PDAC027T	20 000
PDAC028T	40 000
PDAC032T	40 000
PDAC074T	20 000
PDAC079T	30 000
PDAC081T	40 000
PDAC082T	30 000
PDAC084T	20 000
PDAC085T	40 000
PDAC087T	30 000
PDAC088T	40 000
PDAC089T	50 000

Supplemental reference

S1. Masoud, R., Reyes-Castellanos, G., Lac, S., Garcia, J., Dou, S., Shintu, L., Abdel Hadi, N., Gicquel, T., El Kaoutari, A., Diémé, B., et al. (2020). Targeting Mitochondrial Complex I Overcomes Chemoresistance in High OXPHOS Pancreatic Cancer. Cell Reports Medicine *1*, 100143. 10.1016/j.xcrm.2020.100143.