SUPPLEMENTAL MATERIAL

Supplemental Tables

Table S1. Distribution of PI3K-C2γ expression in patients cohorts

	PI3K-C2γ expression			
	IHC score <1	IHC score ≥1	Row Totals	p-value*
Cohort 1	28 (38%)	45 (62%)	73	0.099
Cohort 2	17 (22.4%)	59 (77.6%)	76	
Cohort 3	15 (33.3%)	30 (66.7%)	45	
Column totals	60	134	194	

*chi-square test

Table S2. Biochemical analysis of Pik3c2g$^{+/+}$ (WT) and Pik3c2g$^{-/-}$ (KO) mice.

		WT		KO	
		mean	sd	mean	sd
liver function	ALT (U/L)	103	23	107	24,04163
	GGT (U/L)	0	0	0	0
	TP (g/dl)	4,9	0,7	4,6	0,565685
	ALB (g/dl)	2,05	0,15	2,05	0,212132
	TBIL (mg/dL)	0,1	1,7E-17	0,15	0,070711
	GLOB (g/dL)	2,85	0,55	2,55	0,353553
	ALB/GLOB	1,05	0,15	1,2	0,565685
	ALKP (U/L)	125	31	134	56,56854
renal function	CREA (mg/dL)	0,1	1,7E-17	0,1	0
	BUN (mg/dL)	24,5	6,5	22	8,485281
	PHOS (mg/dL)	7,5	0,9	7,6	0,777817
	Ca (mg/dL)	7,95	0,95	8,5	1,272792
pancreatic function	AMYL (U/L)	1912,5	137,5	1654	209,3036
	LIPA (U/L)	695	25	770	50,91169

ALT=alanine aminotransferase, GGT=g-glutamyl transferase, TP=total protein, ALB=albumin, TBIL=total bilirubin, GLOB=globulins, ALB/GLOB=albumin/globulin, ALKP=alkaline phosphatase, CREA=creatinine, BUN=blood urea nitrogen, PHOS=inorganic phosphate, Ca=calcium, AMYL=amylase, LIPA=lipase.

Statistical analysis did not show significant differences in all the parameters measured (P>0.05, n=3 mice, sd=standard deviation).

Table S3. Combination Index (C.I.) for CB 839 (CB) and Everolimus (EVR) treatment

C.I.	CB (uM)	EVR (uM)	CB (uM)	EVR (uM)	CB (uM)	EVR (uM)	CB (uM)	EVR (uM)	CB (uM)	EVR (uM)
	2.5	1.25	5	2.5	10	5	20	10	40	20
WT	0.95		0.83		0.71		0.82		0.94	
KO	0.88		0.28		0.36		0.49		0.75	

Supplemental Figure legends

Figure S1.

PI3K-C2γ loss decreases survival in patients and accelerates tumor development in KPC mice

(A) Gene expression analysis (scRNA-Seq) performed in PDAC datasets.

(B) Comparison of *PIK3C2G* expression in TCGA or ICGC PC cases stratified by Moffitt subtypes (basal-like and classical). Results are shown as mean ± SD (p=0.035 and p=0.013 by Wilcoxon rank-sum test).

(C) Correlation analysis of PI3K-C2γ and GATA6 expression in TCGA (n=148) or ICGC (n=95) PDAC datasets (p=0.014 and p<0.01 by Wilcoxon rank-sum test).

(D) PI3K-C2γ antibody validation by IHC on *Pik3c2g*$^{+/+}$ and *Pik3c2g*$^{-/-}$ mice (scale bar=100 μm).

(E) IHC assessment of the level of PI3K-C2γ expression in moderately and poorly differentiated PDACs from cohort #1 (scale bar=100 μm, n=73).

(F-G) Kaplan-Meier survival analysis showing the relationship between PI3K-C2γ expression (high: score ≥1 or low: score < 1) and overall survival (OS) (n= 76, p=0.035 by Mantel-Cox log-rank test) or progression free survival (PFS, n=76, p=0.318 by Mantel-Cox log-rank test) in cohort #2 of PDAC patients.

(H) Circulating levels of the pancreatic enzyme lipase in WT and KO mice fed with normal chow (CTRL) or metabolically challenging diet (HFD, n=4 mice).

(I-J) Assessment of number and grade of PanIN lesions in WT/KO KPC mice. Representative IHC staining for PI3K-C2γ (I) and quantification of the number of lesions per field (J). Arrows indicate PanIN lesions, asterisks (*) indicate normal acini, hashtags (#) indicates PDAC (scale bar=100 μm, n ≥ 6).

(K) Measurement of blood glucose levels of 1 month (1m) or 2 months (2m) old WT/KO KPC mice (n=3).

Data are shown as mean ± SEM, ANOVA followed by Bonferroni's post hoc test, otherwise indicated.

Figure S2.

PI3K-C2γ loss increases metastatization in KPC mice and accelerates PC cells growth

(A) Quantification of liver and lung macrometastasis in WT/KO KPC mice (n=6 mice, 3 sections per mice).

(B) IHC assessment of PI3K-C2γ expression levels of primary tumor cell lines derived from KPC WT/KO mice.

(C) Quantification of tumor growth arising from subcutaneously injected WT/KO KPC cell lines in syngeneic mice (n=6).

(D) Liver and lung macrometastasis count arising from subcutaneously injected WT/KO KPC cells in syngeneic mice (n=6 mice, 3 sections per mice).

(E) Relative invasive ability of WT/KO KPC cells (n=5).

(F-H) Real time qPCR analysis for *PIK3C2G* expression levels in Capan1 (F), MiaPaca2 (G) and Panc1 (H) after Crispr/Cas9 *PIK3C2G* editing and single cell selection (n=5).

(I) IHC assessment of PI3K-C2γ expression levels in WT/KO Panc1 cells (scale bar=100 μm)

(J-K) Cell growth assay of WT/KO Capan1 (J) and MiaPaca2 (K) cells (n=5).

(L-M) Western blot analysis of Erk phosphorylation in WT/KO KPC (L) and Panc1 (M) cells. Representative western blot images of whole-cell lysates probed with indicated antibodies.

Data are shown as mean ± SEM, Student t test (E-H) or ANOVA followed by Bonferroni's post hoc test (A,C,D,J,K).

Figure S3.

PI3K-C2γ loss induces mTORC1 hyperactivation upon glutamine deprivation

(A) IHC assessment of the level of expression of PCNA and pS6 in tumors arising from subcutaneously injected WT/KO Panc1 or KPC cells in syngeneic mice (scale bar=100 μm).

(B) Western blot analysis of mTORC1 activity in WT/KO KPC cells upon glutamine withdrawal (0 mM Gln) for 2 hours. Representative western blot images of whole-cell lysates probed with indicated antibodies.

(C-D) Western blot analysis (C) and quantifications (D) of mTORC1 activity in WT/KO Panc1 cells in normal culture condition (NC), upon FBS deprivation (-FBS) or glutamine withdrawal for 2 hours (-Gln, n=4).

(E) IHC assessment and quantification of the level of p4EBP1 expression in PDAC patients expressing high (score ≥ 1) or low (score <1) PI3K-C2γ levels from cohort #3 (n=45, Spearman's correlation analysis, scale bar=100 μm).

(F) IHC assessment of the level of pS6 expression in moderately-well/moderately and poorly differentiated PDACs from cohort #3 (scale bar=100 μm, n=45).

Data are shown as mean ± SEM, ANOVA followed by Bonferroni's post hoc test, otherwise indicated.

Figure S4.

AKT signaling is not affected by PI3K-C2γ loss

(A-B) Western blot analysis of Akt phosphorylation in WT/KO KPC cells transfected with tGFP, tGFP-labeled PI3K-C2γ WT or tGFP-labeled PI3K-C2γ KD in serum starved culture conditions (-INS, A) and stimulated with insulin where indicated (+INS, A) or upon glutamine deprivation (0 mM Gln, B) for 2 hours. Representative western blot images of whole-cell lysates probed with indicated antibodies.

(C-D) Western blot analysis of Akt phosphorylation in WT/KO Panc1 cells transfected with tGFP, tGFP-labeled PI3K-C2γ WT or tGFP-labeled PI3K-C2γ KD in serum starved culture conditions (-INS, C) and stimulated with insulin where indicated (+INS, C) or upon glutamine deprivation (0 mM Gln, D) for 2 hours. Representative western blot images of whole-cell lysates probed with indicated antibodies.

(E-F) mTOR kinase assay performed on WT/KO Panc1 cells upon 2 hrs glutamine starvation. Raptor or control IgG was immunoprecipitated (IP) from WT/KO Panc1 cells and analyzed for mTORC1 activity. Representative western blot images of IP and whole-cell lysates probed with indicated antibodies (E) and quantification of pS6K/mTOR ratio (F, n=5).

(G) ELISA quantification of whole-cell PI(3,4)P2 levels in WT/KO KPC cells upon glutamine withdrawal (0 mM Gln) for 2 hours (n>3).

(H-I) Localization of mTOR on Lamp1 positive vesicles in WT/KO Panc1 cells upon glutamine deprivation. Quantification (n>110 cells, H) and representative immunofluorescence images of mTOR (green), Lamp1 (magenta) and nuclei (blue) stainings (scale bar=5 μm, I).

Data are shown as mean ± SEM, by Student t test.

Figure S5.

PI3K-C2γ-derived PtdIns(3,4)P2 inhibits Arf1 activity

(A-B) Localization of PI3K-C2γ on Lamp1-positive lysosomes. Representative confocal images of tGFP-labeled PI3K-C2γ and miRFP-labeled Lamp1 in normal culture conditions (NC) and upon glutamine withdrawal (0 mM Gln) for 2 hours in Cos7 cells (A) (scale bar=10 μm). Quantification of PI3K-C2γ localization on Lamp1-positive endosomes (B). The ratio of PI3K-C2γ-Lamp1-positive lysosomes was adjusted by the mean number of Lamp1-positive spots in every experimental condition. Dashed white line defines cell limits. White arrows indicate co-localization of the indicated proteins (scale bar=10 μm, n=18).

(C-D) Localization of PI3K-C2γ and PI(3,4)P2 on Lamp1-positive lysosomes. Representative confocal images of tGFP-labeled PI3K-C2γ, mcherry-labeled PHX3 and miRFP-labeled Lamp1 in normal culture conditions (NC, C) or upon glutamine withdrawal (0 mM Gln, D) in Cos7 cells. Dashed white line defines cell limits. White arrows indicate co-localization of the indicated proteins (scale bar=10 μm).

(E) Quantification of the number of lysosomes positive for PI(3,4)P2 in Panc1 cells in normal culture conditions (NC) and upon glutamine withdrawal (0 mM Gln) for 2 hours (n=5).

(F) Quantification of the number of lysosomes positive for PI3K-C2γ and PI(3,4)P2 in Panc1 cells in normal culture conditions (NC) and upon glutamine withdrawal (0 mM Gln) for 2 hours. The ratio of PI3K-C2γ-Lamp1-positive lysosomes was adjusted by the mean number of Lamp1-positive spots in every experimental condition (n=5).

(G) Pull-down of endogenous active Arf1 in WT/KO KPC cell lines upon glutamine withdrawal for 2 hours. Representative western blot images of active Arf1 pull-down assay probed with indicated antibodies (PD: pull down, TL: total lysate, n=5).

(H-I) Pull-down of endogenous active Arf1 in tumors taken from mice injected with WT/KO Panc1 (H) or KPC (I) cells. Representative western blot images of active Arf1 pull-down assay probed with indicated antibodies (PD: pull down, TL: total lysate, n=3).

Data are shown as mean ± SEM, by Student t test.

Figure S6.

PI3K-C2γ-derived PtdIns(3,4)P2 recruits Asap1 that inhibits Arf1 activity

(A) Quantification of Asap1 localization on Lamp1-positive lysosomes in WT/KO Panc1 cells. Results are shown as mean ± SEM (n = 10, *p < 0.05 by Student t test).

(B) Pull-down of WT and mutant Asap1 using PtdIns(3,4)P2-coated beads in Panc1 cells expressing mcherry, mcherry-Asap1 WT or mcherry-Asap1 R345L upon glutamine withdrawal for 2 hours. Representative western blot images of Asap1 pull-down assay probed with indicated antibodies (PD: pull down, TL: total lysate, n=5).

(C) Pull-down of WT and mutant Asap1 using PtdIns(4,5)P2-coated beads in Panc1 cells expressing mcherry, mcherry-Asap1 WT or mcherry-Asap1 R345L upon glutamine withdrawal for 2 hours. Representative western blot images of Asap1 pull-down assay probed with indicated antibodies (PD: pull down, TL: total lysate, n=5).

(D) Pull-down of endogenous active Arf1 in WT or Asap1 KO Panc1 cells expressing mcherry, mcherry-Asap1 WT or mcherry-Asap1 R345L upon glutamine withdrawal for 2 hours. Representative western blot images of active Arf1 pull-down assay and whole cell lysate probed with indicated antibodies (PD: pull down, TL: total lysate, n=5).

Figure S7.

PI3K-C2γ loss induces metabolic rewiring toward the anabolic use of glutamine

(A) KPC WT and KO cells were subjected to Seahorse XFe96 Mito Stress Test analysis and Oxygen Consumption Rate (OCR) was measured in real time in normal culture conditions or upon 1 hour of glutamine withdrawal (0 mM Gln 1 hr). Data are normalized on protein levels. Oligomycin is an adenosine triphosphate ATP synthase inhibitor (oligo), FCCP is the proton uncoupler carbonilcyanide p-triflouromethoxyphenylhydrazone, rotenone (Rot) is the respiratory complex I inhibitor, antimycin A (AntA) is with the respiratory complex III inhibitor (n=3).

(B-D) KPC WT/KO cells were subjected to Seahorse XFe96 Mito Stress Test analysis in normal culture conditions (2 mM Gln) or upon 1 hour of glutamine withdrawal (0 mM Gln 1 hr). Basal (B), maximal (C) and spare (D) OCR were measured and normalized on protein levels. Results are shown as mean ± SEM (n=3).

(E) Quantification of glucose consumption in normal culture conditions of KPC WT/KO cells. Results are shown as mean ± SEM (n=5).

(F) Quantification of lactate production in normal culture conditions of KPC WT/KO cell lines. Results are shown as mean ± SEM (n=5).

(G-H) Cell viability assay on KPC (G) and Panc1 (H) WT/KO cells at indicated glucose concentrations after 24hrs. Results are shown as mean ± SEM (n=5).

(I) Cell viability assay on KPC WT/KO cells at indicated glutamine concentrations after 24hrs. Results are shown as mean ± SEM (n=6).

(J) KPC WT/KO cell growth assay in normal culture condition (2 mM Gln) or upon glutamine depletion (0 mM Gln). Results are shown as mean ± SEM (#: KO 2 mM Gln vs KO 0 mM Gln; *: WT 0 mM Gln vs KO 0 mM Gln; n=6).

(K) Cell viability assay on KPC WT/KO cells in normal culture condition (2 mM Gln) or upon glutamine withdrawal (0 mM Gln) for 12 hrs and stimulated with BSA alone (BSA) or BSA-palmitate (BSA-Palm) for 24 hrs (n=6).

Data are shown as mean ± SEM, ANOVA followed by Bonferroni's post hoc test (B-D, G-K) or Student t test (E,F).

Figure S8.

PI3K-C2γ loss sensitizes to glutaminase inhibitors

(A) Cell viability assay on WT/KO Panc1 cells after 72 hrs treatment with BPTES at indicated concentrations (n=6).

(B) Cell viability assay on WT/KO KPC cells after 72 hrs treatment with BPTES at indicated concentrations (n=6).

(C) Cell viability assay on WT/KO KPC cells after 72 hrs treatment with CB-839 at indicated concentrations (n=6).

(D) Cell viability assay on WT/KO Panc1 cells after 48 hrs of treatment with CB 839, Everolimus or combination of the two drugs at indicated concentrations (n=6).

(E) Quantification of Caspase 3/7 activity in WT/KO Panc1 cells in normal culture condition (2 mM Gln) or upon glutamine withdrawal (0 mM Gln) and CB-839 or BPTES treatment for 24 hours (n=5).

(F-G) Western blot analysis of mTORC1 and Akt activation in WT/KO KPC (F) and Panc1 (G) cells upon Everolimus (EVR) or CB 839 treatment for 24 hours. Representative images of whole cell lysate probed with the indicated antibodies.

(H) Western blot analysis of phosphorylation status of AMPK in WT/KO Panc1 cells in normal culture condition (2 mM Gln, NT) or upon glutamine withdrawal (0 mM Gln) and CB-839 or BPTES treatment for 24 hours. Representative images of whole cell lysate probed with the indicated antibodies (top) and quantification of the pAMPK/AMPK ratio (bottom, n=5).

(I) Cell viability assay on WT/KO Panc1 cells after 48 hrs treatment with 1 μM Everolimus (EVR) in presence of 2 mM or 0.1 mM Glutamine (Gln, n=6).

Data are shown as mean ± SEM, ANOVA followed by Bonferroni's post hoc test.

Figure S9.

Glutaminase inhibitors reduce tumor growth in PI3K-C2γ-deficient xenograft models

(A) IHC assessment of the level of PCNA and pS6 in tumors taken from mice injected with WT/KO KPC cells treated with vehicle (NT), BPTES, Everolimus (EVR) or combination of the two drugs (COMBO) for 2.5 weeks.

(B) Coronal T_{2w} Magnetic Resonance images (B_0=7.1 T) of WT/KO KPC mice before the treatment or treated with vehicle (NT), BPTES, Everolimus (EVR) or combination of the two drugs (EVR) for 2.5 weeks. Light blu arrows indicate pancreatic tumor. Light blu arrow heads indicate tumor abdominal spread. Smallmlight blue small arrows indicate fuzzy edges.

(C) Kaplan-Meier curve for survival of KO KPC mice treated with either vehicle (NT) or combination (COMBO) of BPTES and Everolimus (n = 5 mice, p = 0.01 by Logrank test for trend).

(D) IHC assessment of the level of PCNA and pS6 in endogenous tumors taken from WT/KO KPC mice treated with vehicle (NT), BPTES, Everolimus (EVR) or combination of the two drugs (COMBO) for 2.5 weeks.

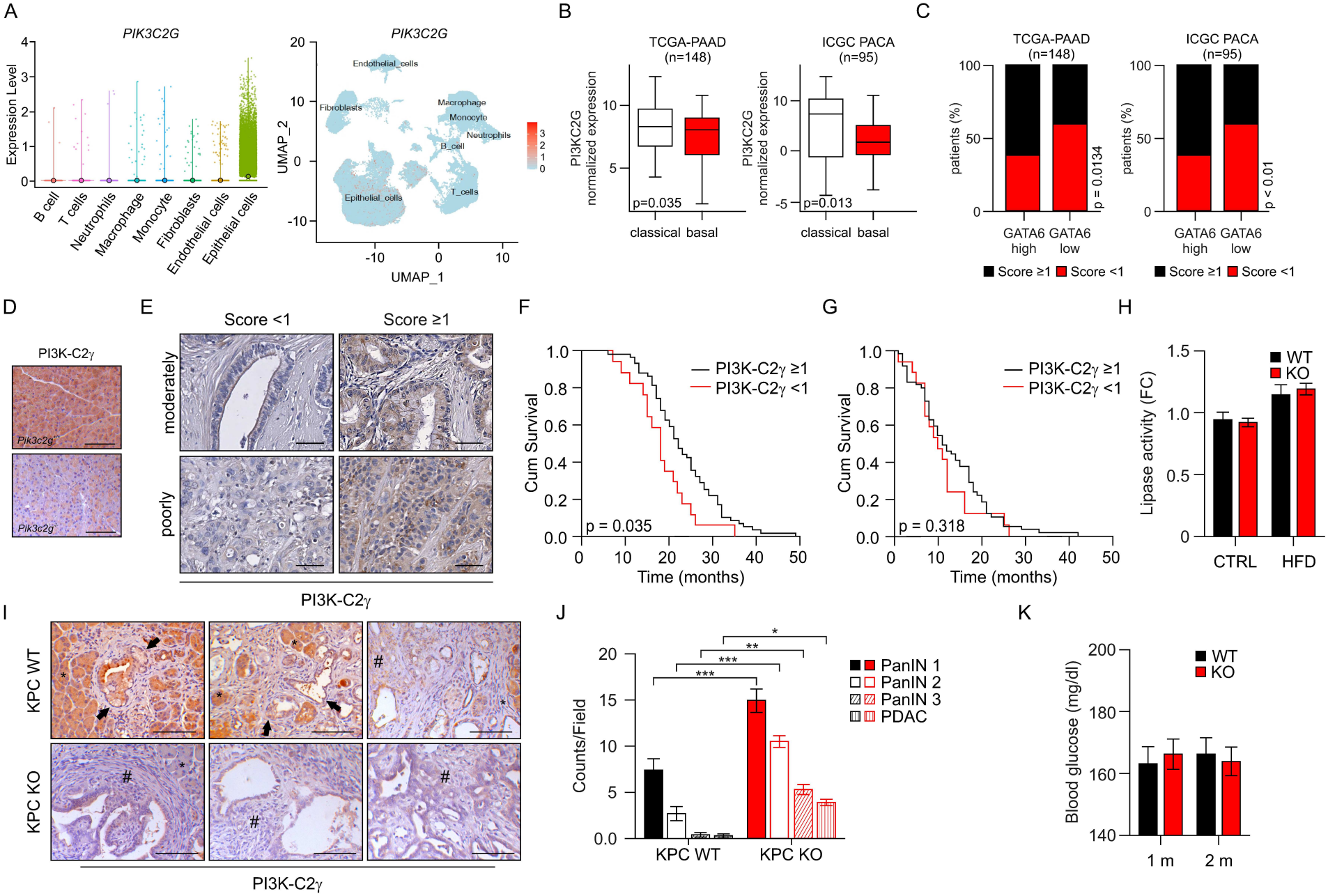
Figure S10

Graphical abstract

Schematic representation of the proposed working model and drug treatments.

Supplemental Figures

Figure S1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Neutrophils
Macrophage
Monocyte
Fibroblasts
Endothelial cells
Epithelial cells
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UMAP_2
B
TCGA-PAAD (n=148)
ICGC PACA (n=95)
PI3KC2G normalized expression
p=0.035
p=0.013
classical basal
C
patients (%)
p = 0.0134
p < 0.01
GATA6 high
GATA6 low
Score ≥1
Score <1
D
PI3K-C2γ
Pik3c2g-/-
Pik3c2g
E
Score <1
Score ≥1
moderately
poorly
PI3K-C2γ
F
Cum Survival
PI3K-C2γ ≥1
PI3K-C2γ <1
p = 0.035
Time (months)
G
p = 0.318
H
Lipase activity (FC)
WT
KO
CTRL
HFD
I
KPC WT
KPC KO
PI3K-C2γ
J
Counts/Field
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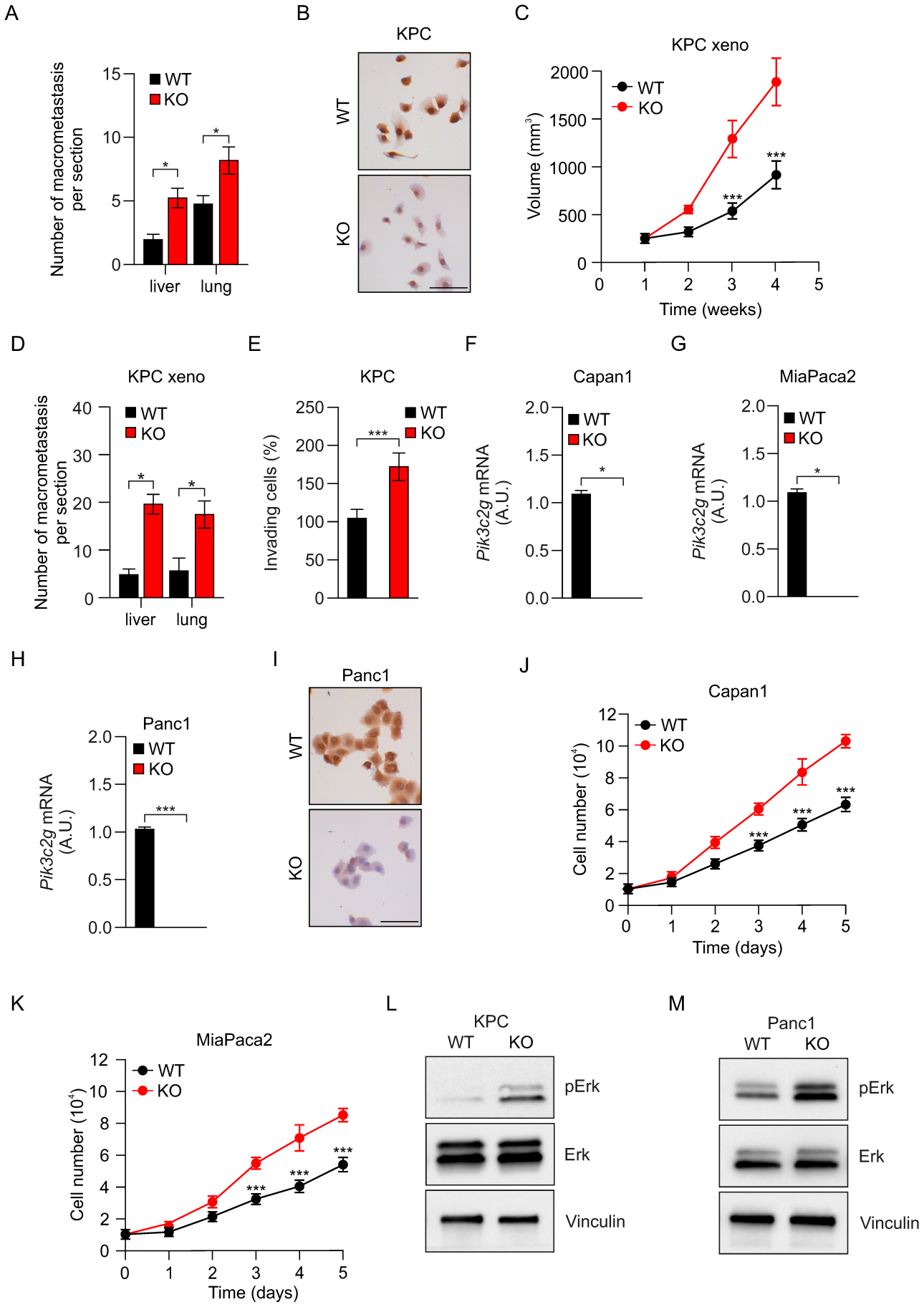
PanIN 2
PanIN 3
PDAC
K
Blood glucose (mg/dl)
1 m
2 m

Figure S2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lung
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KPC
WT
KO
C
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KPC xeno
Number of macrometastasis per section
E
KPC
Invading cells (%)
F
Capan1
Pik3c2g mRNA (A.U.)
G
MiaPaca2
H
Panc1
I
J
Capan1
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KPC
pErk
Erk
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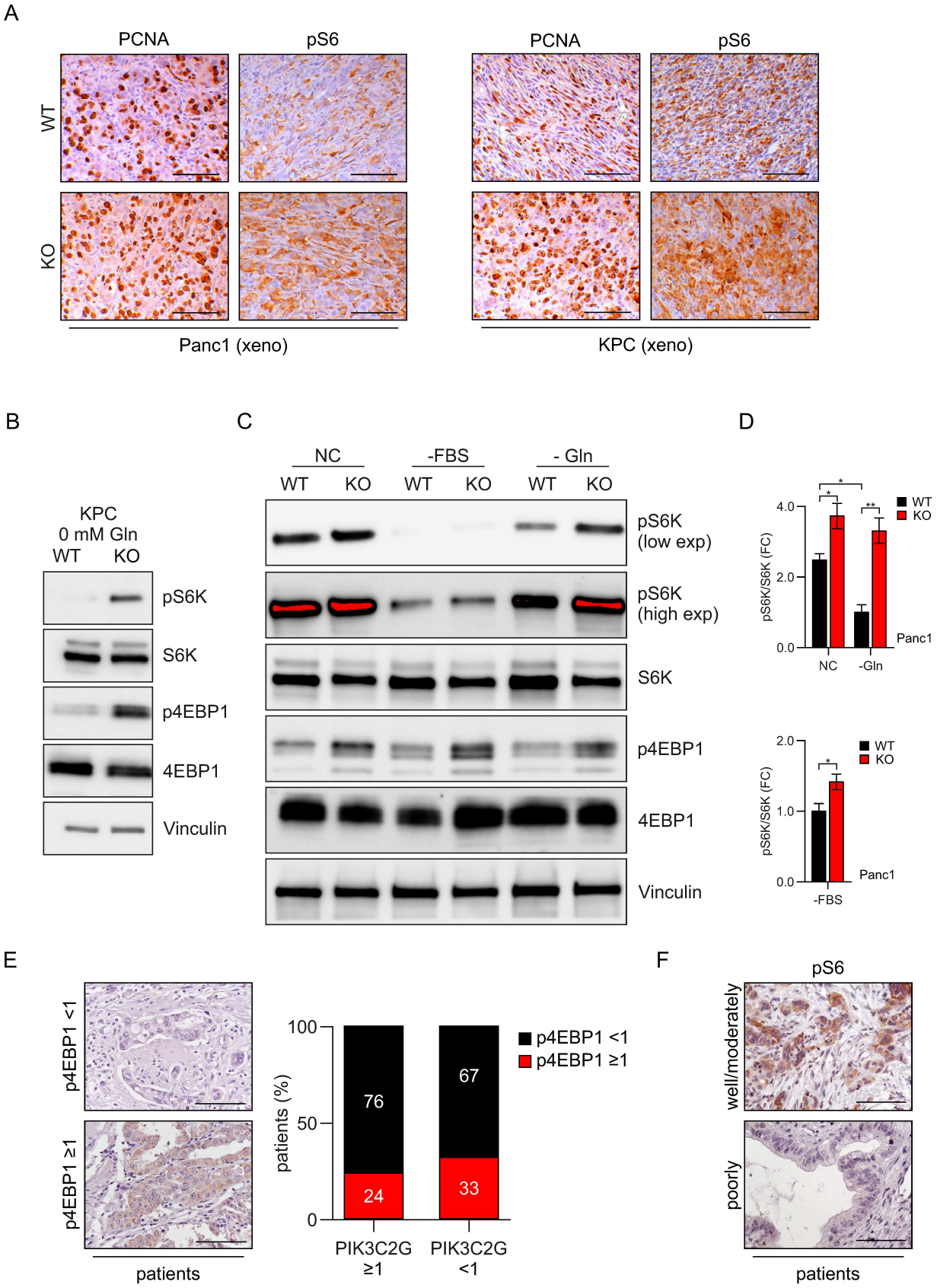
M
Panc1

Figure S3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PCNA
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S6K
p4EBP1
4EBP1
Vinculin
C
NC
-FBS
- Gln
WT KO WT KO WT KO
pS6K (low exp)
pS6K (high exp)
S6K
p4EBP1
4EBP1
Vinculin
D
pS6K/S6K (FC)
WT
KO
Panc1
NC
-Gln
-FBS
E
p4EBP1 <1
p4EBP1 ≥1
patients
patients (%)
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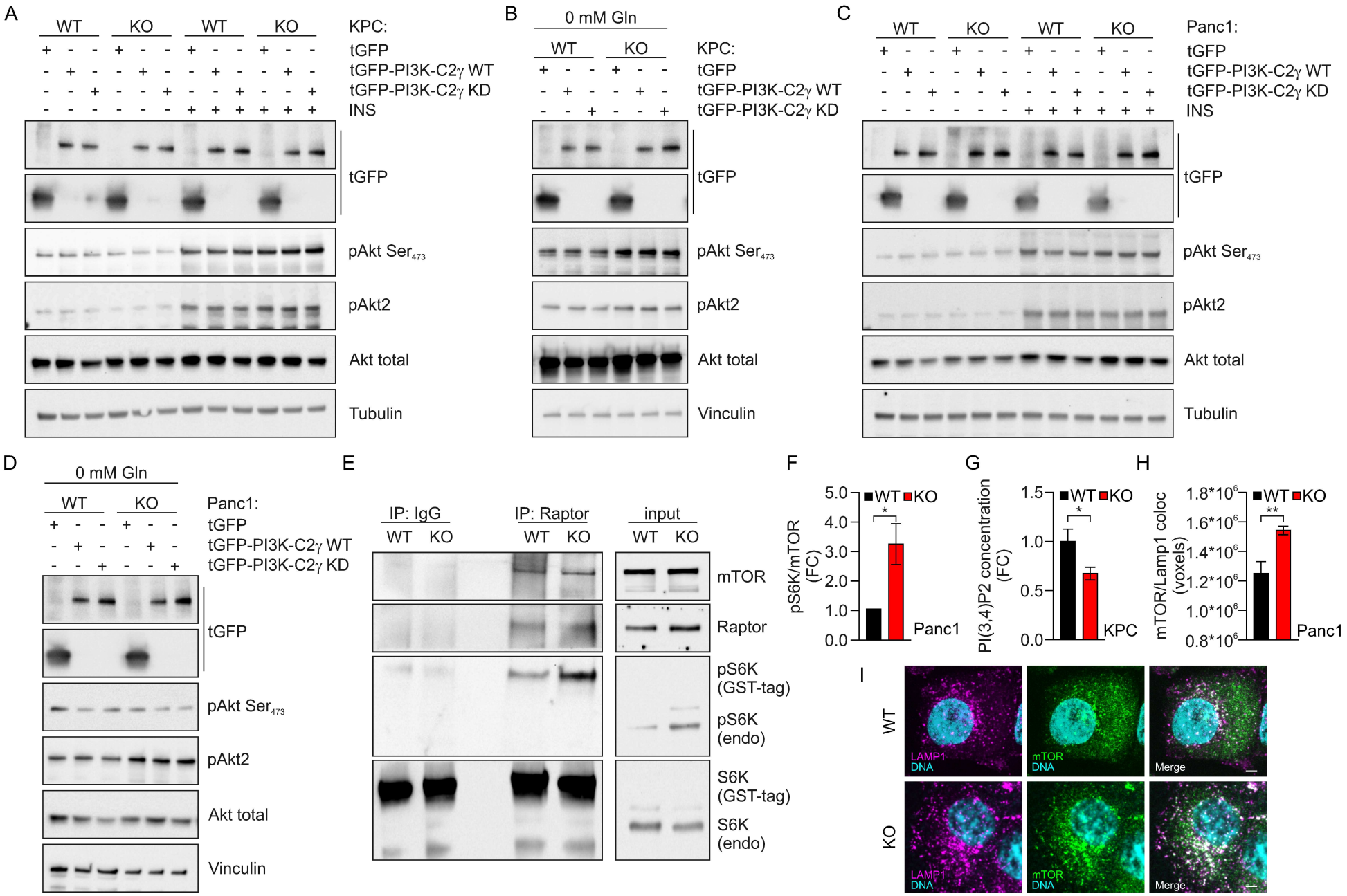
PIK3C2G <1
F
pS6
well/moderately
poorly
patients

Figure S4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Tubulin
B
0 mM Gln
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KPC:
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pAkt Ser473
pAkt2
Akt total
Vinculin
C
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Akt total
Vinculin
E
IP: IgG
IP: Raptor
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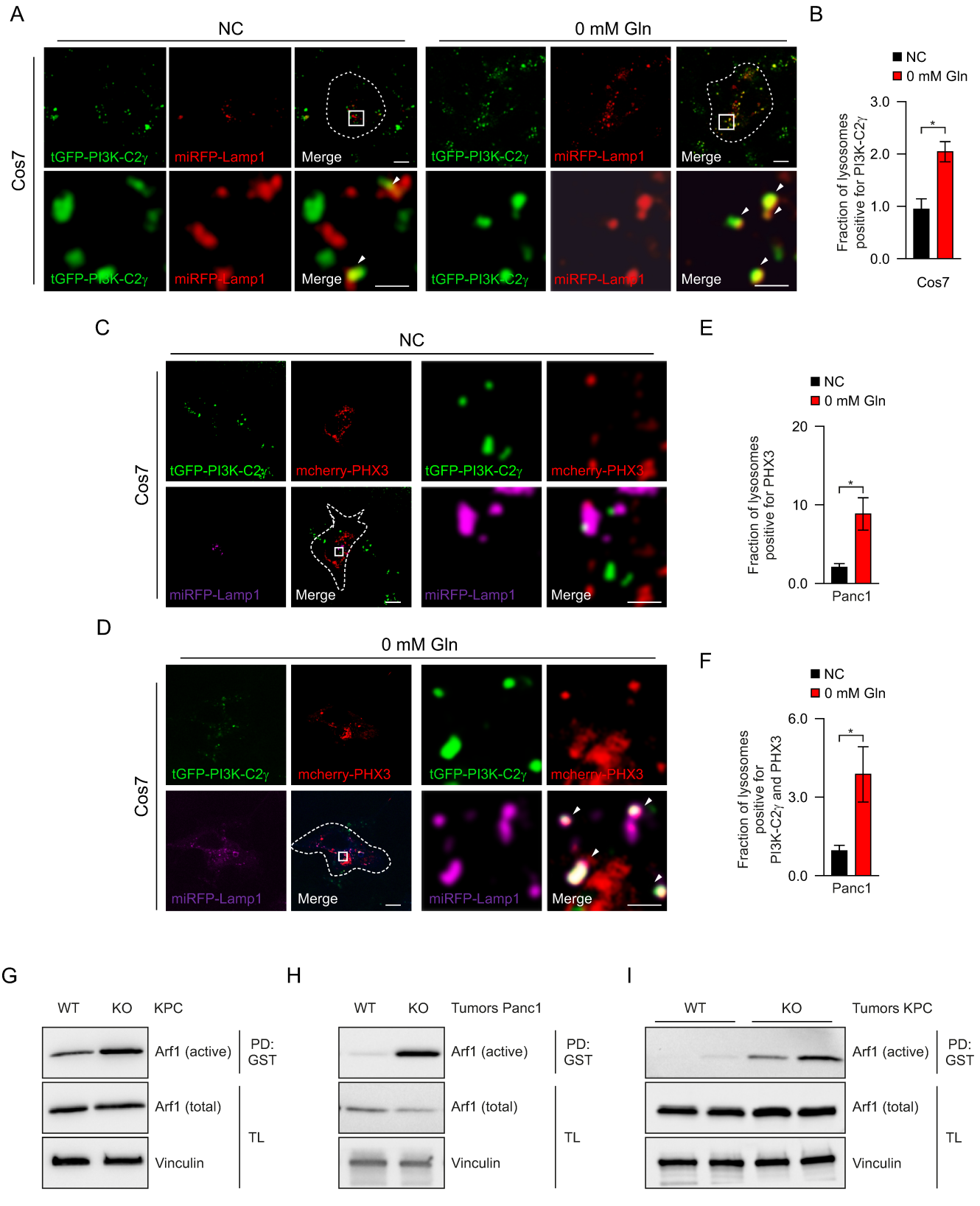
mTOR
Raptor
pS6K (GST-tag)
pS6K (endo)
S6K (GST-tag)
S6K (endo)
F
pS6K/mTOR (FC)
WT KO
Panc1
G
PI(3,4)P2 concentration (FC)
WT KO
KPC
H
mTOR/Lamp1 coloc (voxels)
WT KO
Panc1
I
WT
KO
LAMP1 DNA
mTOR DNA
Merge

Figure S5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Merge
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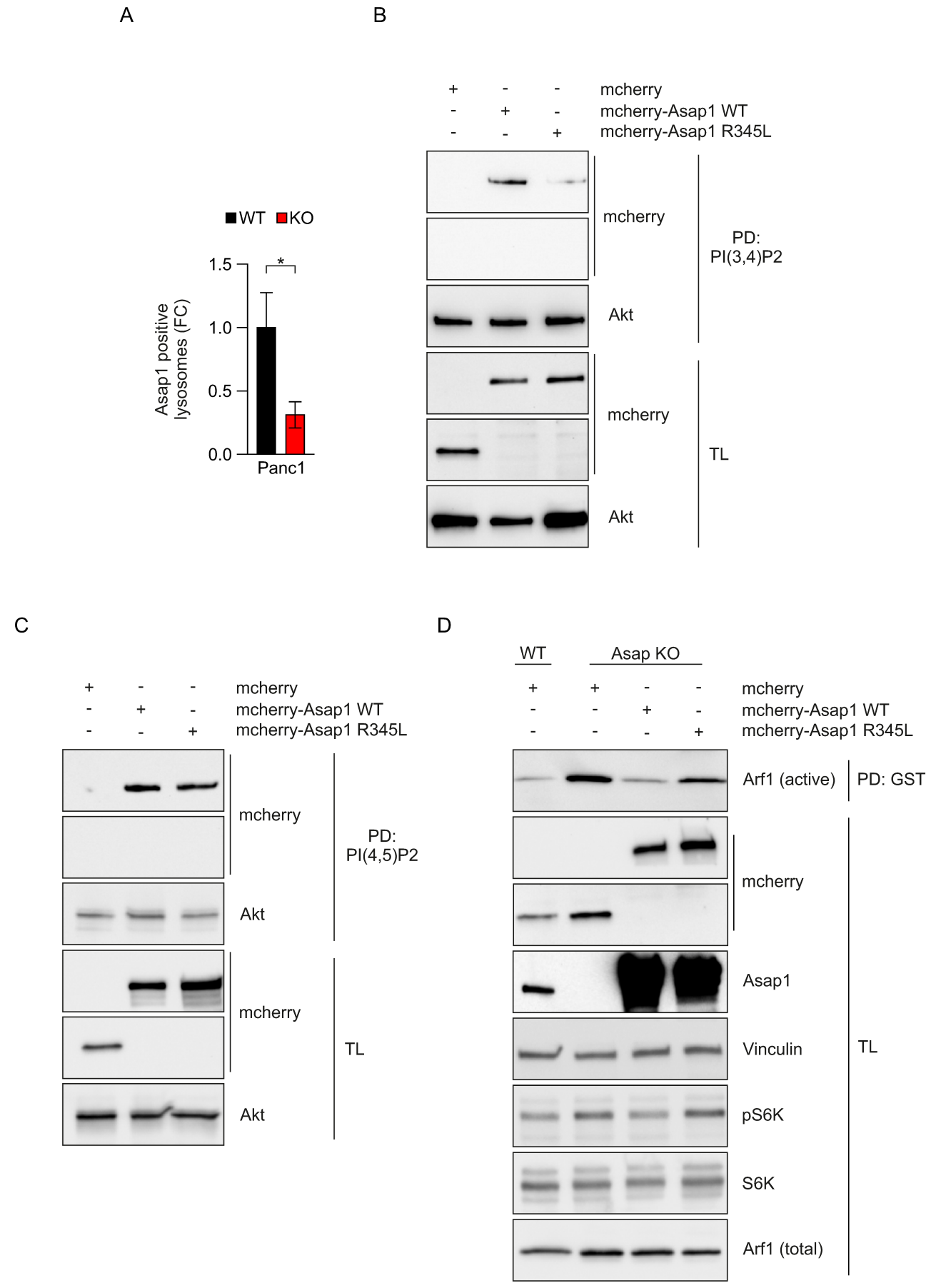
3.0
2.0
1.0
0.0
*
Cos7
C
NC
mcherry-PHX3
D
0 mM Gln
E
Fraction of lysosomes positive for PHX3
20
10
0.0
Panc1
F
Fraction of lysosomes positive for PI3K-C2γ and PHX3
6.0
3.0
G
WT
KO
KPC
Arf1 (active)
PD: GST
Arf1 (total)
TL
Vinculin
H
Tumors Panc1
I
Tumors KPC

Figure S6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B
WT
KO
Asap1 positive lysosomes (FC)
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1.0
0.5
0.0
*
Panc1
+ - - mcherry
- + - mcherry-Asap1 WT
- - + mcherry-Asap1 R345L
mcherry
Akt
PD: PI(3,4)P2
mcherry
Akt
TL
C
D
+ - - mcherry
- + - mcherry-Asap1 WT
- - + mcherry-Asap1 R345L
mcherry
Akt
PD: PI(4,5)P2
mcherry
Akt
TL
WT
Asap KO
+ + - - mcherry
- - + - mcherry-Asap1 WT
- - - + mcherry-Asap1 R345L
Arf1 (active)
PD: GST
mcherry
Asap1
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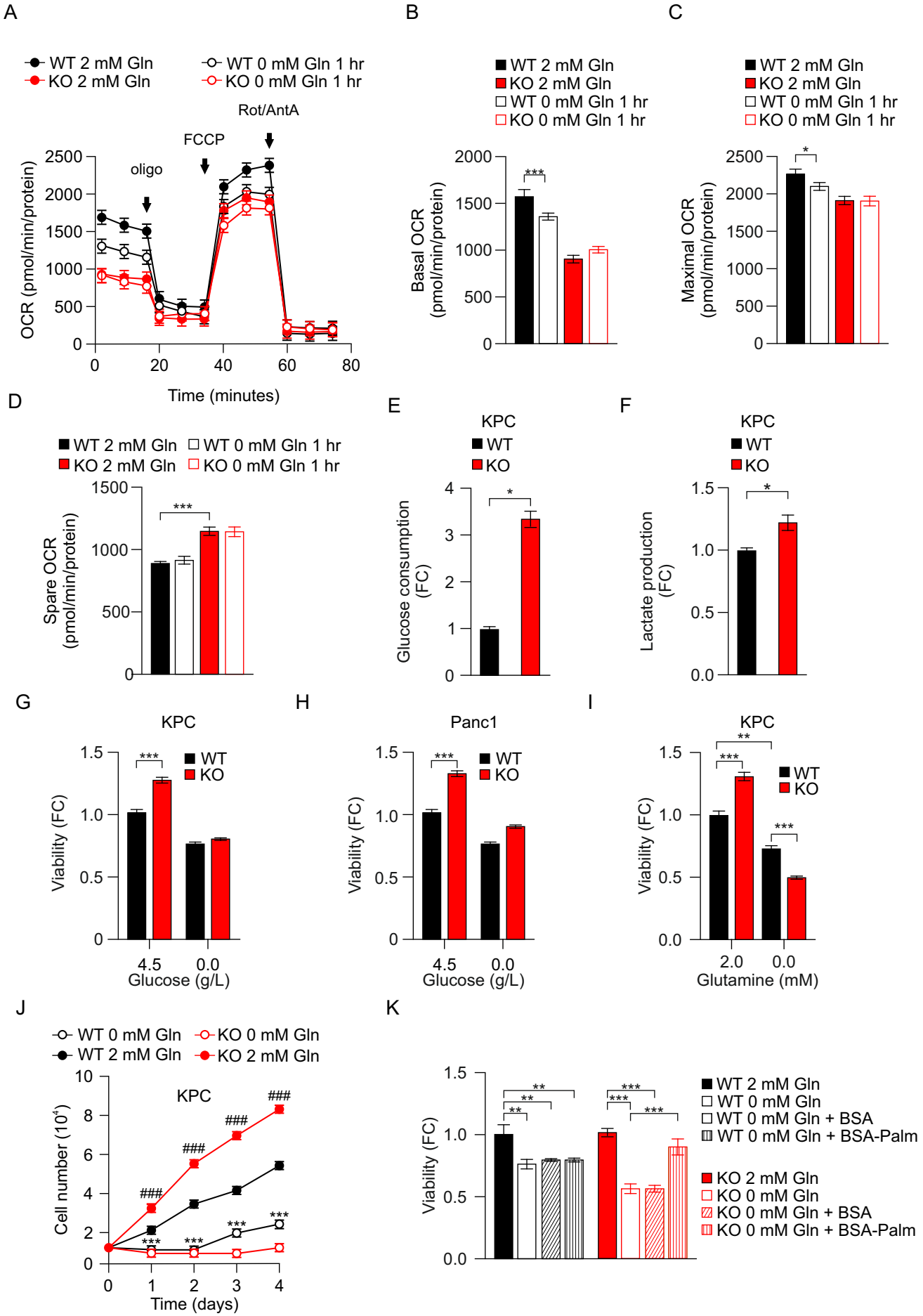
pS6K
S6K
Arf1 (total)
TL

Figure S7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KO 2 mM Gln
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Rot/AntA
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oligo
OCR (pmol/min/protein)
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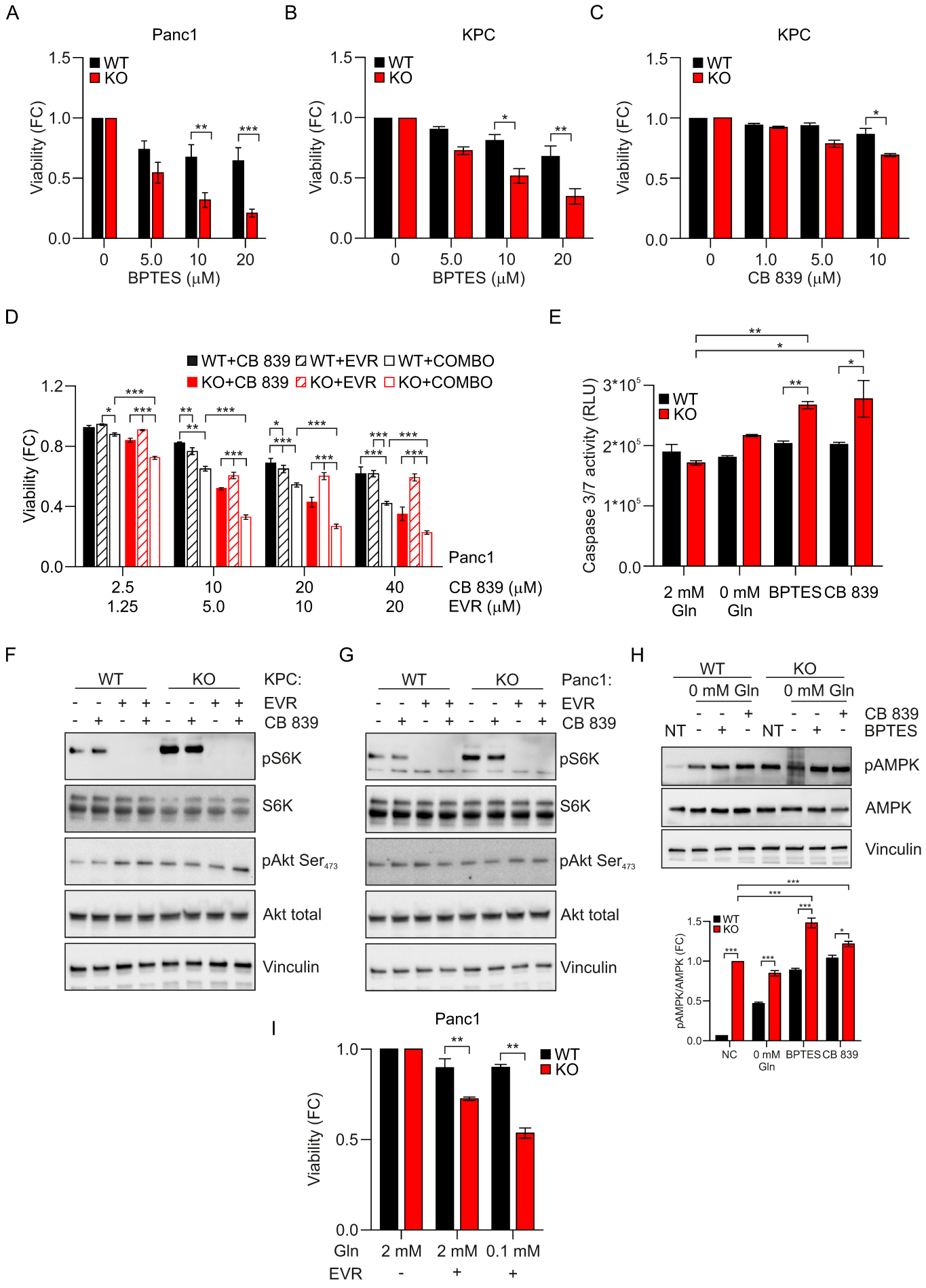
B
Basal OCR (pmol/min/protein)
C
Maximal OCR (pmol/min/protein)
D
Spare OCR (pmol/min/protein)
E
KPC
Glucose consumption (FC)
F
Lactate production (FC)
G
KPC
Viability (FC)
Glucose (g/L)
H
Panc1
I
KPC
Glutamine (mM)
J
Cell number (10^4)
Time (days)
K
Viability (FC)
WT 2 mM Gln
WT 0 mM Gln
WT 0 mM Gln + BSA
WT 0 mM Gln + BSA-Palm
KO 2 mM Gln
KO 0 mM Gln
KO 0 mM Gln + BSA
KO 0 mM Gln + BSA-Palm

Figure S8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Panc1
WT
KO
Viability (FC)
BPTES (μM)
B
KPC
C
KPC
CB 839 (μM)
D
WT+CB 839 WT+EVR WT+COMBO
KO+CB 839 KO+EVR KO+COMBO
Panc1
CB 839 (μM)
EVR (μM)
E
Caspase 3/7 activity (RLU)
2 mM Gln
0 mM Gln
BPTES
CB 839
F
KPC:
EVR
CB 839
pS6K
S6K
pAkt Ser473
Akt total
Vinculin
G
Panc1:
H
0 mM Gln
NT
pAMPK
AMPK
Vinculin
pAMPK/AMPK (FC)
NC
I
Panc1
Gln
2 mM
0.1 mM

Figure S9

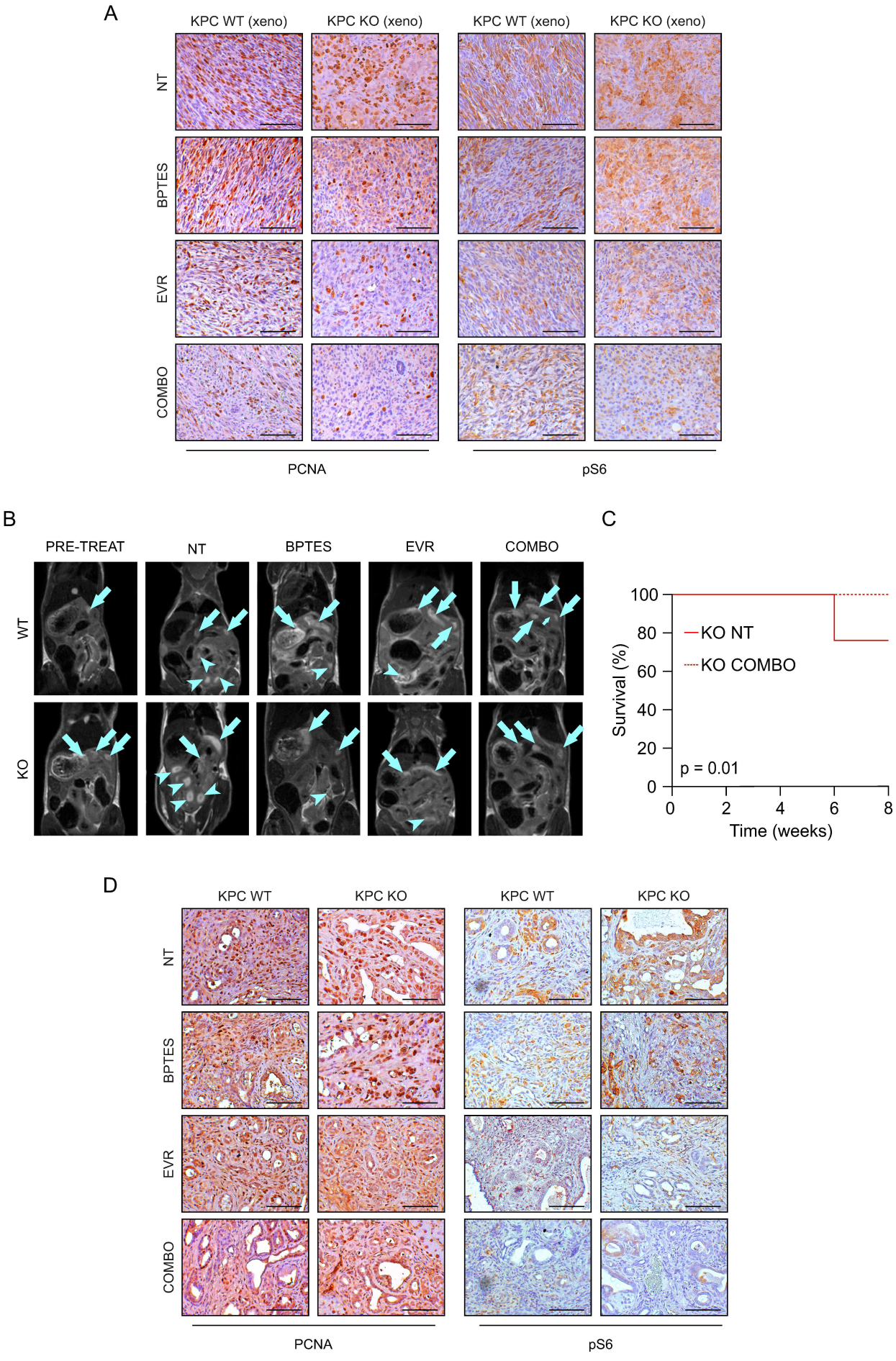
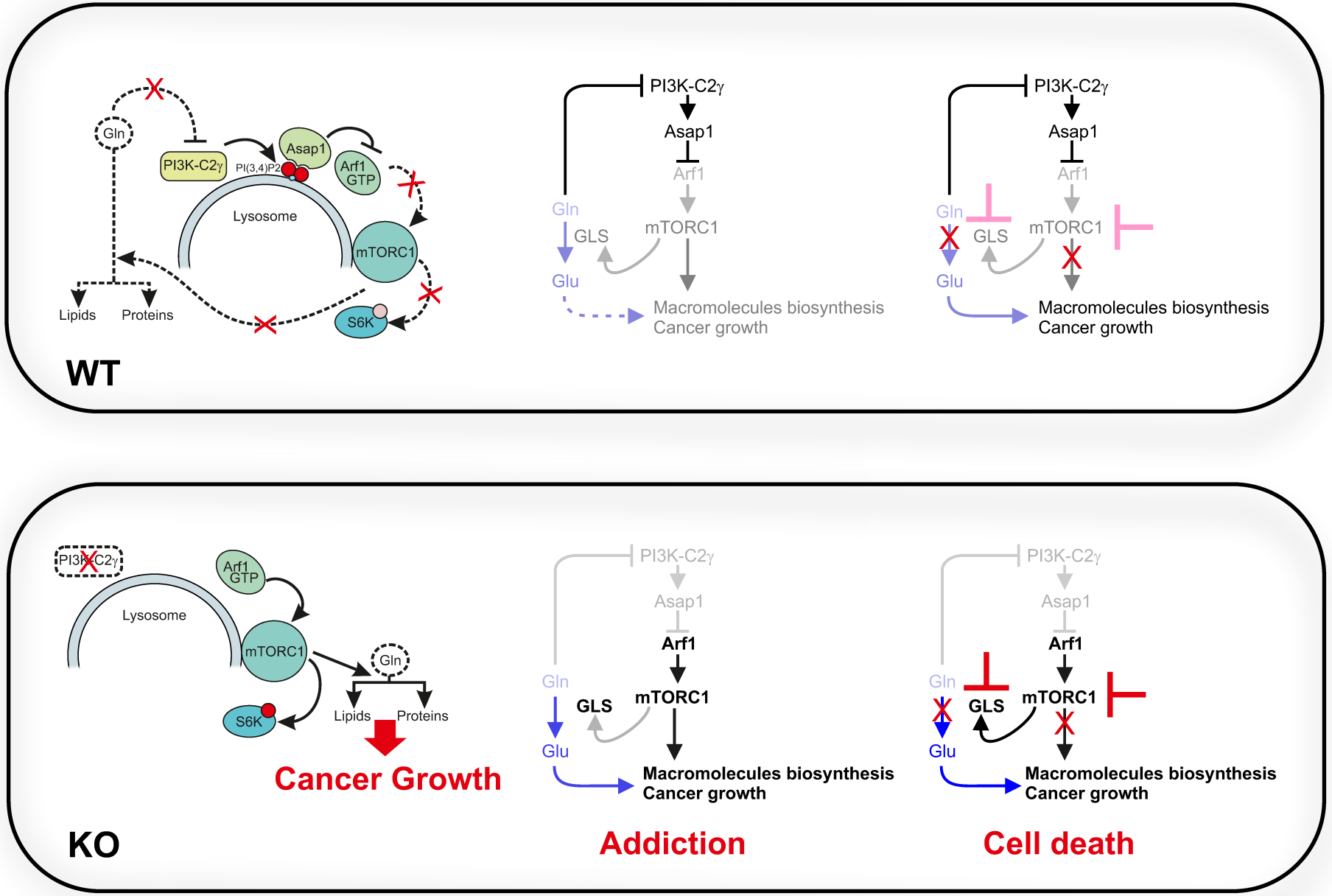

A
KPC WT (xeno)
KPC KO (xeno)
KPC WT (xeno)
KPC KO (xeno)
NT
BPTES
EVR
COMBO
PCNA
pS6
B
PRE-TREAT
NT
BPTES
EVR
COMBO
WT
KO
C
Survival (%)
100
80
60
40
20
0
KO NT
KO COMBO
p = 0.01
0
2
4
6
8
Time (weeks)
D
KPC WT
KPC KO
KPC WT
KPC KO
NT
BPTES
EVR
COMBO
PCNA
pS6

Figure S10


Gln
PI3K-C2γ
PI(3,4)P2
Asap1
Arf1 GTP
Lysosome
mTORC1
Lipids
Proteins
S6K
WT
PI3K-C2γ
Asap1
Arf1
Gln
GLS
mTORC1
Glu
Macromolecules biosynthesis
Cancer growth
PI3K-C2γ
Asap1
Arf1
Gln
GLS
mTORC1
Glu
Macromolecules biosynthesis
Cancer growth
PI3K-C2γ
Arf1 GTP
Lysosome
mTORC1
Gln
S6K
Lipids
Proteins
Cancer Growth
KO
PI3K-C2γ
Asap1
Arf1
Gln
GLS
mTORC1
Glu
Macromolecules biosynthesis
Cancer growth
Addiction
PI3K-C2γ
Asap1
Arf1
Gln
GLS
mTORC1
Glu
Macromolecules biosynthesis
Cancer growth
Cell death

Supplemental Material and methods

Gene expression analysis

For the integration of scRNA-seq datasets from PDAC tissues, we used the Harmony algorithm [1] via the R package *harmony* in order to account for the technical differences of the four datasets. The datasets (Peng et al. (primary PDAC=24, ncells=41964) [2], Lin et al. (primary PDAC=10, ncells=7752)[3], Chan-Seng-Yue et al. (primary PDAC=13, ncells=33970)[4] and Steele et al. (primary PDAC=16, ncells=42844)[5])) were first preprocessed individually using Seurat [6] for quality control and filtering (percent_mt_max=20, nFeature_min=500, nCount_min=500, nCount_max=50000) and then integrated using *harmony* function with default parameters and grouping by dataset variable. Cells were annotated with singleR package using the preloaded dataset HPCA from the celldex package [7] to stratify gene expression by cell population.

Histopathological analysis

For histological analysis, pancreata and tumors were fixed overnight in 4% paraformaldehyde (PFA), embedded in paraffin and cut into 3 μm thick sections. Sections were stained with hematoxylin and eosin (HE) following standard protocols and with specific antibodies: phospho-S6 Ser235/236 (#4858, Cell Signaling), PCNA 6D645 (sc-71858) and phospho-4E-BP1 Thr37/46 (#2855, Cell Signaling). Slides were dehydrated and mounted with moviol. Randomly selected areas were investigated under the light microscope (Olympus BH2), micro-photographed through digital imaging system then analyzed with ImageJ software. Two pathologists double-checked all the histological sections analyzed to exclude sarcomatoid pancreatic cancers that can be occasionally found in this model. Briefly, PanIN is a lesion that arises in native pancreatic ducts measuring <1 mm and not on a background of acinar-ductal metaplasia. PanIN lesions are graded as PanIN-1, 2 or 3 according to the cytological and architectural characteristics as described previously [8]. Quantitation of PanIN number was done on 5 20X fields of view from at least 4 mice.

Human pancreatic samples

PI3K-C2γ protein expression was evaluated in three independent cohorts of pancreatic ductal adenocarcinoma tumor samples. The first cohort (cohort #1) is composed by 73 pancreatic ductal adenocarcinoma tumor samples arranged in 4 TMA (Tissue MicroArray, Ethics approval number 1885 from the Integrated University Hospital Trust (AOUI) Ethics Committee (*Comitato Etico Azienda Ospedaliera Universitaria Integrata*) approved in their meeting of 17 November 2010 and documented by the ethics committee 52070/CE on 22 November 2010 and formalized by the Health Director of the AOUI on the order of the General Manager with protocol 52438 on 23 November 2010). The second cohort (cohort #2) consists of 76 pancreatic ductal adenocarcinoma tumor samples arranged in 3 TMA. The study protocol was approved by the local Ethics Committee at University Hospital of Pisa, Italy (Comitato di Bioetica, Azienda Ospedaliero-Universitaria Pisana, protocol number: 3909, July 3rd, 2013).

Four 1 mm tissue cores per case were included. Additional normal pancreatic parenchyma cores were used as controls and were integrated in the TMAs. Briefly, 4 μm FFPE sections were subjected to antigen retrieval in 10 mM citrate buffer (pH 6.0) for 5 min in pressure cooker at low pressure (106-110°C). Sections were than incubated overnight at 4°C with primary antibodies anti-PIK3C2G (1:300, ThermoFisher, Rockford, PA5-15239). Primary antibody was detected by incubation with HRP-labeled secondary antibody (Vector Laboratories, MP-7401) following by detection with 3,3' diaminobenzidine (Vector Laboratories, SK-4105). All slides were counterstained with Harris hematoxylin and then were scanned at 20x magnification and digitalized using the Aperio Scan-Scope XT Slide Scanner (Aperio Technologies). The immunolabeled slides were reviewed and scored blinded to any histopathological or clinical variables. The intensity of cytoplasmatic staining was scored in a three-tiered manner (scale:0=negative, 1+=weak staining, 2+=strong staining).

PI3K-C2γ protein expression was also evaluated in a third cohort (cohort #3) of 45 formalin-fixed and paraffin-embedded tissues of pancreatic ductal adenocarcinomas arranged in single TMA. The requirement for informed consent was waived because of the anonymous nature of the data and the descriptive nature of the immunohistochemical study. Briefly, 5 μm sections were stained with a rabbit polyclonal antibody raised against

93-123 amino acids from the N-terminal region of the human PI3K-C2γ protein (1:150; overnight incubation; cat. number PA5-15239; ThermoFisher, Rockford, IL). Antigen retrieval was performed by microwave treatment at 750 W (10 min) in 10 mM sodium citrate buffer (pH 6.0). The anti-rabbit EnVision kit (Agilent, Santa Clara, CA) was used for signal amplification. In control sections, the specific primary antibodies were replaced with isotype-matched immunoglobulins. The immunolabeled slides were reviewed and scored to any histopathological variables. Staining was evaluated by a molecular pathologist, assessing the amount of tumor and tissue loss, background, and overall interpretability. The intensity of cytoplasmatic staining was scored in a three-tiered manner (scale:0=negative, 1+=weak staining, 2+=strong staining). We attributed one, two, or three additional points if the percentage of positive cells was less than 25%, 25% to 50%, or greater than 50%, respectively. Neoplastic cells were always uniformly stained and positivity assessment was made by counting all the tumor cells present in three tumor cores.

Magnetic Resonance Imaging of mice

For the MRI experiments, mice were anesthetized *via* the intramuscular injection of tiletamine/zolazepam (Zoletil 100; Virbac, Milan, Italy) 20 mg/kg plus xylazine (Rompun; Bayer, Milan, Italy) 5 mg/kg using a 27-G syringe. MRI scans were acquired at 7.1 T on a Bruker Avance Neo 300 spectrometer equipped with the Micro 2.5 microimaging probe at room temperature. T_{2W} images were acquired using a standard RARE (Rapid Acquisition with Refocused Echoes) sequence with the following parameters (TR = 4000 ms, TE = 24 ms, RARE factor= 24, flip angle= 180°, number of averages= 6, FOV = 30 mm x 30 mm, slice thickness = 1 mm, matrix size 128 x 128).

Analysis of mice

Differences in survival were evaluated with Mantel-Cox log-rank test.

Serum biochemical analysis of *Pik3c2g*$^{+/+}$ and *Pik3c2g*$^{-/-}$ mice was performed using IDEXX Catalyst One instrument according to manufacturer instructions. For lipase levels, *Pik3c2g*$^{+/+}$ and *Pik3c2g*$^{-/-}$ mice were fed with Western/Fast Food diet (Envigo TD.120528) for 2 weeks and plasma lipase levels were measured with commercial kit (MAK046,

Sigma) according to manufacturer instructions. Blood glucose measurements of WT/KO KPC mice were performed as previously described in [9].

For orthotopic injections, WT/KO KPC cells (10^6 cells) were injected into 8 weeks old *Pik3c2g*$^{+/+}$ mice. About 2 weeks after inoculation, the tumors were measured twice a week using caliper and the volume was calculated according to the formula: $d*D^2/2$, where d stands for minor diameter and D for major diameter. All mice were culled at the end of treatment or when the tumor mass was >1500 mm^3. Tumor masses, lymph nodes, lungs and livers were collected for histological analysis.

For BPTES and/or Everolimus treatment, WT/KO KPC ($2*10^6$ cells/mice) and Panc1 ($4*10^6$ cells/mice) cells were injected subcutaneously in nu/nu CD1$^+$ mice. About 3 weeks after inoculation, the tumors were measured twice a week using caliper and the volume was calculated according to the formula: $d*D^2/2$, where d stands for minor diameter and D for major diameter. For treatment studies, mice with tumors in the 30–50 mm^3 size range or 5-6 weeks-old KPC mice were enrolled in a randomized fashion to either vehicle (PBS 10% DMSO) or BPTES (every 3 days, 12.5 mg/kg in PBS 10% DMSO) and/or Everolimus (daily, 1.5 mg/kg in PBS 5% Tween 20) for 2.5 weeks by intraperitoneal injection.

All experiments on mice have been performed in accordance with institutional and national guidelines and they conform to the relevant regulatory standards. The investigators were not blinded during experiments and outcome assessment.

Cell lines and primary cultures

Whole pancreas was collected from WT/KO KPC mice at late stage of tumor development. Tumors were finely chopped and then digested in DMEM medium (Gibco®), containing 1 mg/ml collagenase A (Roche Applied Science, Indianapolis, IN, USA). After 30 min at 37 °C, a mixture of epithelial-enriched fragments and non-epithelial single cells was obtained. The epithelial-enriched fraction, resulting from filtration through a 70 μm mesh (BD), was centrifuged at 1200 rpm for 5 min and plated on 10 cm dish. Cells were treated with 0.025% trypsin-EDTA (Gibco®) until fibroblasts elimination and cultured in DMEM High Glucose (Gibco®) 10% dialyzed Fetal Bovine Serum (dFBS, #26400-036, Invitrogen) supplemented with 2 mM glutamine (Invitrogen) and 5000 U/ml

Penicillin-Streptomycin (Gibco®). Three independent primary cell lines per genotype were used. Cells were detached using 0.1% trypsin-EDTA (Gibco®).

The human PC cell lines Capan1 (HBT-79), MiaPaca2 (CRL-1420), Panc1 (CRL-1469) and Cos7 (CRL-1651) cell lines were purchased from American Tissue Cell Culture (ATCC, Manassas, VA, USA) and maintained at 37 °C and in a 5% CO2 atmosphere in complete growing medium composed as follows: DMEM (Gibco®) High Glucose containing 2mM glutamine (Invitrogen), 5000 U/ml Penicillin-Streptomycin (Gibco®) and 10% heat-inactivated dialyzed fetal bovine serum (FBS) (dFBS, Invitrogen). Glucose free DMEM (containing 2 mM Gln) and glutamine-free DMEM were obtained from Invitrogen. A passage number of 20 was assumed for each cell line; after these 20 passages, a new culture was started with the stock constituted after the reception of the cells from ATCC. Human cell lines authentication was performed by BMR Genomics. All cell lines were routinely tested for Mycoplasma contamination using PCR.

Cell culture and growth

For growth assays via cell counts, KPC and Panc1 cells were plated into 96-well plates in quadruplicate at $5 * 10^3$ cells/well and counted with CellTiter-Fluor® Cell Viability Assay (Promega). Fluorescence was recorded at indicated time point.

For drug treatment, cells were seeded in quadruplicate at $5 * 10^3$ cells per 96-well plate; vehicle, Everolimus (11597, Cayman Chemical), CB-839 (22038, Cayman Chemical) or BPTES (S7753, Aurogene) were added the day after seeding in complete growing medium. Cells were counted at the indicated time point with CellTiter-Fluor® Cell Viability Assay (Promega). Cell survival was considered as the ratio of live cells after treatment compared with WT live cells treated with vehicle alone.

For glutamine deprivation, cells were seeded in complete growing medium. 16 hours after seeding, cells were washed twice with PBS and DMEM High Glucose, no glutamine containing 10% dFBS or DMEM High Glucose, no glutamine containing 10% dFBS supplemented with either 2 mM or 10 mM glutamine (Invitrogen) was added for 2 hours or otherwise indicated.

For metabolite treatment, 16 hours post seeding, complete growing medium was replaced with glutamine-free medium supplemented with 10% dFBS. After 16 hours of glutamine withdrawal, metabolites were added at the indicated concentrations. Dimethyl

α-KetoGlutarate, Dimethyl L-glutamate and BSA-conjugated palmitate were purchased from Sigma. After 24 hours, cells were counted with CellTiter-Fluor® Cell Viability Assay (Promega). Cell survival was considered as the ratio of live cells after treatment compared with WT live cells in normal growing conditions (2 mM glutamine).

Caspase activity was measured using Caspase-Glo 3/7 assay kit following the manufacturer's protocol (Promega).

Plasmids

All constructs were verified by restriction digest and automated DNA sequencing. KPC and Panc-1 cells were transfected using X-tremeGENE™ HP DNA Transfection Reagent (XTGHP-RO, Roche) and Cos7 cells with Lipofectamine® 2000 (Life Technologies) according to manufacturer's instructions.

The following plasmids were used: PIK3C2G-tGFP (OriGene Technologies, cat. n. RG217086), ASAP1-mCherry (Vector Builder, NM_001362924.1), pLAMP1-miRFP703 (a gift from Vladislav Verkhusha, Addgene plasmid #79998; http://n2t.net/addgene:79998; RRID:Addgene_79998), NES-EGFP-cPHx3 and NES-mCherry-cPHx3 (a kind gift of prof. Gerry Hammond, University of Pittsburgh).

PIK3C2G Kinase Dead (D1051A) [10, 11] mutant was generated by site directed mutagenesis (Quikchange Lightning kit, Agilent) using the following pair of primers: CGGGCCACATGTTTCATATTGCCTTTGGAAAATTC and GAATTTTCCAAAGGCAATATGAAACATGTGGCCCG and was verified by sequencing.

ASAP1 R345L mutant [12, 13] was generated by site directed mutagenesis (Quikchange Lightning kit, Agilent) using the following pair of primers: ATCCGGAAAGTATGGCAGAGGCTAAAGTGTTCAGTCAAGAATGGG and CCCATTCTTGACTGAACACTTTAGCCTCTGCCATACTTTCCGGAT and was verified by sequencing.

CRISPR/Cas9

For CRISPR/Cas9 mediated gene editing, target sequences were designed via a gRNA design tool (Feng Lab CRISPR Design Web Tool at http://crispr.mit.edu). Each sequence was cloned into the PX459-SpCas9 plasmid to express the Cas9 and single guide RNAs (sgRNAs). The following primers were used: for *PIK3C2G*

AAACTCAATTTCACTCTCGTAGTGC and CACCGCACTACGAGAGTGAAATTGA, for *Asap1*: CACCGCAGGAACACCGTCACGCTGC and AAACGCAGCGTGACGGTGTTCCTGC.

Plasmid was transfected in Capan1, MiaPaca2 and Panc1 cells and single cell dilution was performed in order to select 3 clones per condition.

PtdIns(3,4)P2 ELISA assay

WT/KO KPC and Panc1 were plated in a 15 cm dish in complete growing media, otherwise indicated. Acidic lipids extraction and quantification of PI(3,4)P2 were performed using ELISA kits (K-3800, Echelon Biosciences), according to manufacturer instructions.

Live cell imaging

5 * 10^4 Cos7 or 7 * 10^4 Panc1 cells were plated in 8 well μ-Slide and transfected with PIK3C2G-tGFP, NES-EGFP-cPHx3, NES-mCherry-cPHx3, pLAMP1-miRFP703, pARF1-CFP or mCherry-Asap1. 48 hours post-transfection, cells were washed twice and complete growing medium was replaced with glutamine-free medium supplemented with 10% dFBS alone or with 2 mM glutamine (Invitrogen), for 2 hours before image acquisition. Image acquisition was performed with Leica TCS SP8 confocal system (Leica Microsystems) equipped with a HCX PL APO 63×/1.4 NA oil-immersion objective. Images were acquired on the three coordinates of the space (XYZ planes) with a resolution of 0.09 μm x 0.09 μm x 0.6 μm and were processed and analysed with ImageJ software (Rasband, W.S., U.S. National Institutes of Health, Bethesda, MA). In particular, images were pre-processed with background subtraction and Gaussian blur and segmented using intensity thresholding; binary images were post-processed to improve spot detection, finally, colocalization is analysed with JACoP plugin [14] using an object based method (centres-particles coincidence).

Immunofluorescence

Immunofluorescence was performed as previously described [15]. The following primary antibodies were used: CD107a/LAMP1 (H4A3) (Mouse, 555798, BD Pharmingen) and mTOR (7C10) (Rabbit, #2983, Cell Signaling). Number of colocalized

voxels (mTOR green and Lamp1 Magenta) has been calculated by using ImarisColoc software.

Protein analysis

Cells were homogenized in lysis buffer (120 mM NaCl, 50 mM Tris-HCl pH=8, 1% Triton X-100) supplemented with 25x protease inhibitor cocktail (Roche), 50 mM sodium fluoride and 1 mM sodium orthovanadate. Lysates were cleared by centrifugation at 13,000 rpm for 10 min at 4°C. Protein concentration was determined by Bradford method and supernatants were analyzed for immunoblotting with the indicated antibodies. Membranes probed with the indicated antibodies were then incubated with HRP-conjugated secondary antibodies (anti-mouse used 1:10000, anti-rabbit 1:5000, Sigma or anti-rabbit light chain specific, 211-032-171, Jackson Lab) and developed with enhanced chemiluminescence (Clarity Western ECL Substrate, 1705060, Biorad).

The following primary antibodies were used: DDEF1/Asap1 (mouse, sc-374410; Santa Cruz), Arf1 (Rabbit, 16121, Thermo Scientific), Phospho-p70 S6 Kinase (Thr389) (Rabbit, #9234, Cell Signaling), p70 S6 Kinase (Rabbit, #9202, Cell Signaling), mTOR (7C10) (Rabbit, #2983, Cell Signaling), Raptor (24C12) (Rabbit, #2280S, Cell Signaling), anti-beta Tubulin (Rabbit, ab6046, Abcam), turboGFP (clone OTI2H8, Mouse, TA150041, OriGene), mCherry (Rabbit, #43590, Cell Signaling), Phospho-Akt (Ser473) (Rabbit, #9271, Cell Signaling), Phospho-Akt2 (Ser474) (D3H2) (Rabbit, #8599, Cell Signaling), Akt (pan) (40D4) (Mouse, #2920, Cell Signaling), pAMPKalpha T172 D4D6D (Rabbit, #50081, Cell Signaling), AMPK-alpha D5A2 (#5831, Cell Signaling) and Vinculin (Mouse, sc-25336), Phospho-4E-BP1 (Thr37/46) (236B4) (Rabbit, #2855, Cell Signaling), 4E-BP1 (53H11) (Rabbit, #9644, Cell Signaling), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (20G11) (Rabbit, #4376, Cell Signaling), p44/42 MAPK (Erk1/2) (Rabbit, #9102, Cell Signaling).

mTORC1 kinase assay was performed as previously described [16]. Briefly, cells were homogenized in ice-cold lysis buffer (40 mM HEPES [pH 7.4], 2 mM EDTA, 0.3% CHAPS supplemented with 25x protease inhibitor cocktail (Roche), 50 mM sodium fluoride and 1 mM sodium. Lysates were cleared by centrifugation at 13,000 rpm for 10 min at 4°C. For immunoprecipitation, anti-Raptor antibody (Rabbit, #2280, Cell Signaling) or Rabbit IgG isotype control (#10500C, Invitrogen) was added to the lysates

and incubated with rotation for 2 hrs at 4°C. 10 μl of G Sepharose was added for an additional 30 min. Immunoprecipitates were washed five times with low salt wash buffer (40 mM HEPES [pH 7.4], 150 mM NaCl, 2 mM EDTA, 0.3% CHAPS). For kinases assay, immunoprecipitates were then washed twice in 25 mM HEPES (pH 7.4), 20 mM potassium chloride. Kinase assays were performed for 5 min at 30°C in a final volume of 15 μl consisting of mTORC1 kinase buffer (25 mM HEPES [pH 7.4], 50 mM KCl, 10 mM MgCl2, 250 μM ATP) and 100 ng GST tagged human P70S6K (SRP5055, Sigma) as substrate. Reactions were stopped by the addition of 20 μl of sample buffer, boiling for 10 min and analyzed by SDS-PAGE and immunoblotting.

Active Arf1 pull-down experiment was performed according to manufacturer instructions (16121, Thermo Scientific). Briefly, 16 hours post-seeding or 48 hours post transfection with PIK3C2G-tGFP WT or PIK3C2G-tGFP KD or tGFP alone, Panc1 cells were washed twice with PBS and media were replaced with complete growing media or glutamine deprived media for 2 hours, then cells were harvested and homogenized in lysis buffer supplemented with 25x protease inhibitor cocktail (Roche), 50 mM sodium fluoride and 1 mM sodium orthovanadate. 500 μg of lysates were incubated with 30 μl of Glutathione Agarose Resin and 30 μg of GST-GGA3-RBD Fusion Protein for 1 hour at 4 °C on a rotating rack. Samples were collected by centrifugation (3000 g for 1 min) and washed six-times with lysis buffer. Bound protein complexes were then eluted by adding 30 μl Laemli sample buffer.

PI(3,4)P2 (P-B034a, Echelon Biosciences) and PI(4,5)P2 (P-B045a, Echelon Biosciences) pull down experiments were performed according to manufacturer instruction. Briefly, 24 hours post transfection with Asap1-mCherry WT, Asap1-mCherry R345L or mCherry alone, complete growing medium was replaced with glutamine-free medium supplemented with 10% dFBS. After 2 hours of glutamine withdrawal, Panc1 cells were harvested and homogenized in lysis buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.25% Igepal) supplemented with 25x protease inhibitor cocktail (Roche), 50 mM sodium fluoride and 1 mM sodium orthovanadate. 2 mg of lysates were incubated with 30 μl of PI(3,4)P2 or PI(4,5)P2 beads for 3 hours at 4 °C on a rotating rack. Samples were collected by centrifugation (3000 g for 1 min) and washed six-times with lysis buffer. Bound protein complexes were then eluted by adding 30 μl Laemli sample buffer.

Metabolic assay

A total of 2 * 10^3 cells per well were seeded in 96-well plates, cells counted using Cell Titer Fluor, while the supernatants of cell culture medium were collected. The media were assayed for glucose and lactate levels by using Biosen C-Line analyzer according to the manufacturer's instruction. The glucose consumption and lactate production were normalized to cell number. The experiments were performed with three replicates and repeated for three times.

Seahorse XFe96 Metabolic Assay

1.5 - 2 * 10^4 KPC WT and KO cells were seeded per well in XFe96 cell culture plates (5-10 technical replicates) in the presence or absence of 2 mM glutamine and subjected to the Seahorse XF Cell Mito Stress Test. 24 hours post seeding, routine culture medium was replaced with XF base medium supplemented with 25 mM glucose and 1 mM sodium pyruvate ± 2 mM glutamine. Cells were incubated for 1 hour at 37°C in a non-CO_2 incubator to allow cells equilibrating with the XF base medium. Mito Stress Test analysis measures basal respiration, maximal respiration, and the cells ability to exploit the mitochondrial oxidative metabolism. The analysis was performed in real-time by recording the value of OCR (Oxygen Consumption Rate) and ECAR (Extracellular Acidification Rate) after serial injections of drugs that affect the electron transport chain: oligomycin (1 μM), FCCP (carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone) (1 μM) and a mix of Rotenone/Antimycin A (0.5 μM) as previously described in [17]. Basal respiration is calculated as last rate OCR measurement before oligomycin injection – non-mitochondrial oxygen consumption rate. Maximal respiration is calculated as the maximum rate OCR measurement after FCCP injection – non-mitochondrial oxygen consumption rate. The results were normalized on protein content.

Radioactive assays

1 * 10^5 KPC WT and KO cells were seeded in 6-well culture plates in their routine culture medium. To analyze the incorporation of uniformly (U) radiolabeled ^{14}C-glutamine into proteins and lipids, culture medium was supplemented with 0.05 μCi/mL ^{14}C-glutamine (Perkin Elmer) 24 hours prior to the experiment. For radioactive incorporation into proteins, cells were washed in ice cold PBS and resuspended in 20% trichloroacetic

acid, then moved in ice for 30 minutes and centrifuged at 10,000 rpm for 10 minutes at 4°C. Pellet was resuspended in dH_2O, transferred into a scintillation vial and counted on the scintillation counter. For radioactive incorporation into lipids, cells were washed three times in ice cold PBS, lysed with methanol, and resuspended first in 4 volumes of a CHCl3:MeOH (1:1) solution and then in an additional volume of dH_2O. Samples were centrifuged at 1,000 rpm for 5 minutes at room temperature and the lower phases were collected into a scintillation vial and counted on the scintillation counter as previously described in [18]. Protein quantification was used to normalize all the radioactive signals.

Statistical Analysis

Prism software (GraphPad) was used for statistical analysis. Significance was calculated with Student t test and one- or two-way analysis of variance tests (ANOVA) followed by Bonferroni's post hoc analysis, or Mantel Cox log-rank test where appropriate. Values are reported as the mean ± standard error of the mean (SEM). $p<0.05$ was considered statistically significant (∗), $p<0.01$ very significant (∗∗) and extremely significant $p<0.001$ (∗∗∗).

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