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Supplementary Information for

Components of the phosphatidylserine endoplasmic reticulum to plasma membrane transport mechanism as targets for KRAS inhibition in pancreatic cancer

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Supplementary Information Text

Materials and Methods

Materials. Class III PI4K alpha inhibitor Small Molecule (Tool Compound), C7, was purchased from Cancer Research UK (ximbio.com, cat. no. 153579, distributed by Ximbio) and dissolved in DMSO. Simeprevir was purchased from MedChem Express (Cat No. HY-114277). Trametinib, Copanlisib, and LY3214996 were generously provided by Dr. Scott Kopetz at MD Anderson Cancer Center, Houston, TX. For *in vitro* experiments, Simeprevir, Trametinib and LY3214996 were dissolved in DMSO, and Copanlisib was dissolved in 1M HCL. For *in vivo* experiments, Simeprevir (SelleckChem, cat# TMC-435350), Trametinib (SelleckChem, cat# GSK1120212), and Copanlisib (MedChem Express, cat# BAY 80-6946) were purchased. Cell culture media were purchased from HyClone and GIBCO. FBS was purchased from GIBCO. Puromycin was purchased from Thermo Fisher Scientific (BP2956-100). Ki67 (12202) and cleaved caspase 3 (9661 antibodies were purchased from Cell Signaling Technology. Normal rat IgG (sc-2026) was purchased from SantaCruz Biotechnology. Rabbit anti-mGFP antibodies for immunogold labeling were generated in house. Agarose-low melting point (CAS 39346-81-1) was purchased from Sigma-Aldrich.

Cell lines. MDCK, MiaPaCa-2 and PANC-1 cells were purchased from American Type Culture Collection. BxPC3 and MOH were kindly provided by Dr. Craig Logsdon at MD Anderson Cancer, Houston, TX. KPC cells were generously provided by Dr. Jennifer Bailey, McGovern Medical School, Houston, TX. MDCK, PANC-1 and KPC cells were grown in DMEM supplemented with 2 mM L-glutamine and 10% FBS. MiaPaCa-2 cells were cultured in DMEM supplemented with 2 mM L-glutamine and 10% FBS and 2.5% horse serum. BxPC3 and MOH cells were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% FBS. All cell lines were grown at 37°C in 5% CO2.

***C. elegans* strains and growth conditions.** *C. elegans* strain MT2124 was used in the study. The genotype *let-60*(n1046) IV of this strain expresses a multivulval (Muv) phenotype in the worms. The worms were grown at 20^{o}C on Nematode Growth Medium (NGM) plates (2.5 g of peptone, 3 g of NaCl, 20g Agar, 25 mL of 1 M potassium phosphate buffer (pH = 6.0), 1 mL of 1 M MgSO4, 1 mL of 1 M CaCl2, 1 mL of (5 mg/mL in 95% ethanol) cholesterol, 1 mL of (10% v/w in ethanol) nystatin, and 1 mL of 25 mg/mL streptomycin) containing *E. coli* OP50.

Preparation of a synchronous *C. elegans* culture. Using a sterile worm pick 10 -12 gravid adult worms were transferred from a previously established plate to newly seeded NGM plates containing *E. coli* OP50. Plates were incubated at 20^{O}C overnight. Subsequently, the adult worms were removed using a sterile worm pick and the embryos were allowed to develop to gravid adult worms (~ 3 days). When the worms reached the gravid adult stage, 4 - 6 plates containing adult worms were washed with sterile M9W (5 g of NaCl, 6 g of Na_2HPO_4, 3 g of KH_2PO_4 and 1 mL of 1 M $MgSO_4$). The worms were collected in 15 ml conical tubes and centrifuged at 1500 rpm for 1 minute. The supernatant was replaced with worm lysis solution (400 µl of 8.25% sodium hypochlorite and 100 µl of 5 N NaOH). The worms were allowed to lyse to release the embryos by periodically flicking the tube. The reaction was stopped when 70% of the adult worms lysed by diluting the medium with M9W. Subsequently, the tubes were centrifuged at 1500 rpm for 1 minute and the supernatant decanted. The embryos were washed 3 times with M9W and were resuspend in 3 – 5 ml of M9W. The embryos were incubated overnight at a speed of 18 rpm on a tube rotator at room temperature (RT). The resulting L1 larvae were used in the drug assay.

Preparation *C. elegans* of drug assays. *E. coli* OP50 was grown in an orbital shaker at 150 rpm and 37^{o}C overnight and thereafter centrifuged at 4000 rpms for 10 to pellet the cells. The

supernatant was removed, and the pellet was resuspended in 3 ml of M9W to concentrate the culture. Prior to preparing the drug working solutions, 50 ml of M9W was supplement with 0.05 ml of cholesterol (5 mg/mL in 95% ethanol). The working solution for each drug was prepared to a final volume of 4.8 ml M9W supplemented with cholesterol. Thereafter, 200 ml of concentrated *E. coli* OP50 was added to each drug working solution and the vehicle control. 2 mL of each working drug solution or vehicle control was added to the wells in a 12-well tissue culture plate. Approximately 100 L1 larvae contained in 20ml was added to each well using a sterile micropipette. The plates were incubated at 20^{o}C. The drug concentrations and vehicle control were tested in duplicates.

Observation of the MUV phenotype in the *let-60* strain. When the worms reached the adult stage, they were collected and washed two times with M9W. Thereafter, the supernatant was removed without disturbing the pellet and 500 ml of 2 mM sodium azide was added to anesthetize the worms. 10ml of anesthetized worm suspension was added to glass slides containing agarose pads. A no. 1.5 coverslip was gently placed over the worm suspension. The samples were imaged using a DIC/Nomarski microscope at 10X and 20X magnifications. The adult worms were scored based on the presence or absence of the Muv phenotype.

Confocal microscopy. Cells were seeded onto coverslips and allowed to grow for 48 hr before fixation with 4% PFA and quenching with 50 mM NH4Cl. Coverslips were mounted in Mowiol and visualized by confocal microscopy (Nikon A1R) using a 60X objective.

EM and spatial mapping. Basal PM sheets of MDCK cells were prepared, fixed with 4% PFA and 0.1% glutaraldehyde, and labeled with affinity-purified anti-GFP antisera conjugated to 4.5-nm gold as described previously (1). Digital images of immunolabeled membrane sheets were taken with a transition electron microscope at 100,000× magnification and intact 1-μm2 areas were identified with ImageJ. (x, y) coordinates of the gold particles were determined as described in (1). Univariate K function (2) was calculated and standardized on a 99% confidence interval (3-5), whereby an L(r)-r value greater than the confidence interval is indicative of significant clustering. The extent of clustering is represented by the (L_{max}) value, the maximum value of the K function. Bootstrap tests were used to analyze differences between replicated point patterns as described previously (3), and statistical significance was determined by evaluation against 1,000 bootstrap samples.

XTT proliferation assay. For Simeprevir and C7 combination studies, BxPC-3, PANC-1, MiaPaCa-2, and MOH (6000, 5000, 2500, 1500 cells/well, respectively) were seeded in 96-well plates in triplicate and treated 24 hours later with drugs or control solvent (DMSO or HCL) for 72 hours in phenol-red free media. XTT reagent/activator solution was added to the cells as per the manufacturer's instructions (TACS XTT Cell Proliferation Assay, Trevigen, cat # 4891-025-K), and incubated in a 37°C 5%CO2 incubator for 30 minutes before reading the absorbance at 490 nm with a reference at 630 nm on a Synergy2 plate reader (BioTek) to correct for any background.

Anchorage-independent growth assay. BxPC-3 (10^3), PANC-1 (5×10^3), MiaPaCa-2 (5×10^3), and MOH (5×10^3) parental and knockdown cells were seeded in soft agar in six-well plates, with a base layer of 1% agar–media mixture, and a top layer of 0.6% agar–cell suspension mix as performed in (Borowicz et al, 2014). After 2–3 weeks, colonies were stained with 0.01% crystal violet and imaged and quantified by ImageJ.

Luciferase Assay. KPC cells were seeded in a 6-well plate and The Dual-Luciferase Reporter Assay System (Promega, cat# E1910) was used to generate cells constitutively expressing Luciferase. Luciferase-expressing plasmid was generously provided to us by Dr. Jeffrey Chang at McGovern Medical School, Houston TX. Single colonies were then selected for by 250ug/uL Hygromycin B (Invitrogen, PN 10687-010) and luminescence intensity was measured using a Tecan Infinite M200 Pro microplate reader.

Generation of CRISPR/Cas9 cell lines. KPC cells were transduced with human OSBPL8 sgRNA (3′-TGCAAATCTTTGGTTGGCGT-5′) cloned into pLenti6.3-V5-TOPO vector (K5315-20;

Invitrogen) and packaged into viral particles. 24 hours after infection, cells were selected by 4μg/ml puromycin (BP2956-100, Fisher Scientific) 3 cell passages. Single colonies were generated from the pool of polyclonal KO cells. KPC cells were similarly infected with mouse OSBPL5 sgRNA (F: CACCGATGTACCTCTCCAACGACCA, R: AAACTGGTCGTTGGAGAGGTACATC) and mouse OSBPL8 sgRNA (F: CACCGCTGAGGCAGATCTCTAGTTG, R: AAACCAACTAGAGATCTGCCTCAGCGGTGC) followed by puromycin selection (4 μg/ml) after 24 h for 2 days only to avoid integration of the lentiviral plasmid into the host DNA to prevent an immunogenic response in animals to Cas9. Single colonies were chosen, their DNA extracted and amplified by PCR (OSBPL5 F: ATTCTGGGACCCCTGCTTT, R: AATATGTGGGTCATGGAGTGC; OSBPL8 F: TCATCGAAAAGGCACATTAGG, R: AGGCAGAAATGCAATGTGG) using the MangoMix PCR kit (Bioline, cat# BIO-25033) which was a generous gift from Dr. Guangwei Du at McGovern Medical School, and sequenced by Sanger sequencing to verify gene knockout.

Animal Experiments. All animal studies were performed under an Institutional Animal Care and Use Committee (IACUC) approved animal protocol, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Please refer to Supporting Information S1 for detailed experimental procedures. Female nu/nu (#007850-Outbred athymic nude) and male C57BL/6 (#000664-C57BL/6J inbred) mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Mice xenograft tumor initiation assay. Early passage BxPC-3 or MiaPaCa-2 cells were harvested, and $2.5X10^6$ cells expressing an empty vector were implanted subcutaneously into the right flanks nu/nu while cells expressing shRNA of *OSBPL5*, *OSBPL8* or both were injected into the left flank, rendering each animal its own control. Tumor volume was measured twice a week by an external caliper and calculated as $V = (\text{Length} \times \text{Width}^2)/2$.

IVIS imaging. $0.5x10^6$ luciferase-expressing KPC cells with or without gene knockout were resuspended in 50μl of 1XPBS and injected directly into the pancreas after performing a laparotomy on C57BL/6 mice. Mice were randomized into control, *OSBPL8* knockout, and *OSBPL5* knockout groups, with each group containing 8 mice. Mice were imaged 1-2 times a week for 3 weeks. Prior to imaging, 150mg/Kg of D-luciferin (# MB102, Syd Labs) was injected intraperitonially into the mice and allowed to spread for 2 minutes before anesthetizing the mice for 3 minutes followed by image acquisition. Images were taken by IVIS Lumina XR Imaging System (Caliper Life Sciences).

Mice xenograft drug treatments. Simeprevir and Trametinib stocks were prepared in DMSO at 100mg/ml and 22mg/ml of DMSO, respectively. Copanlisib was dissolved in PEG100 in acidified water. Simeprevir and Copanlisib were administered via intraperitoneal injection while Trametinib was administered via oral gavage. Working solutions of Simeprevir and Trametinib were administered in corn oil. Treatment was started 4 days post inoculation. For Simeprevir single treatment, 3 and 10mgs/kgs were administered 5X/week (daily for 5 days with a 2-day break) for 6 weeks. For combination experiments the following dosages and scheduling was followed for 6 weeks: 3mgs/kgs Simeprevir 5X/week, 0.5mgs/kgs Trametinib 3X/week, and 5mgs/kgs Copanlisib 3X/week. Tumor volume was measured twice a week by an external caliper and calculated as $V = (\text{Length} \times \text{Width}^2)/2$.

Immunohistochemistry. Tumors were fixed in 4% paraformaldehyde and incubated overnight at 25 °C then stored in 70% ethanol at 4 °C until paraffin embedding. Five-micron sections were cut, deparaffinized in xylene, followed by successive dehydration in 70%, 95% and 100%, 95% ethanol. The sections were then boiled for 20 minutes in 10 mM sodium citrate for antigen retrieval, washed in 1X PBS, and quenched with 3% H_2O_2 at room temperature for 30 minutes. Tumor sections were blocked in M.O.M. blocking buffer (BMK2202; Vector Labs) for 1 hour at room temperature and then incubated with primary antibodies 4 °C overnight. The next day, sections were washed 5 times with 1X PBS and incubated for 45 minutes with secondary antibody for 45 at room temperature. Afterwards, sections were washed again in 1X PBS, incubated for 30 minutes in ABC solution (PK7100; Vector Labs), developed in diaminobenzidine (K3468; Dako) and counterstained with hematoxylin (Thermo Fisher Scientific). Sections were then successively rehydrated in 100%, 95%

and 70% ethanol. Images were taken using Eclipse 80i microscope (Nikon) and Digital Sight DS-VI1 camera (Nikon), and staining was quantitated using NIS-Elements Basic Research software (Nikon).

Bioinformatic analysis using UCSC Xena browser. *KRAS, PI4KA, EFR3A, and SACM1L* mRNA expression in human normal and tumor samples were analyzed and visualized using data in GDC TCGA-PAAD and TCGA TARGET GTEx by Xena browser (https://xenabrowser.net/) (6).

Statistical analysis. Results are presented as the mean ± SEM. Prism 9 (GraphPad Software) was used for One-Way ANOVA and two-tailed t tests. Levels of significance are labeled as: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001

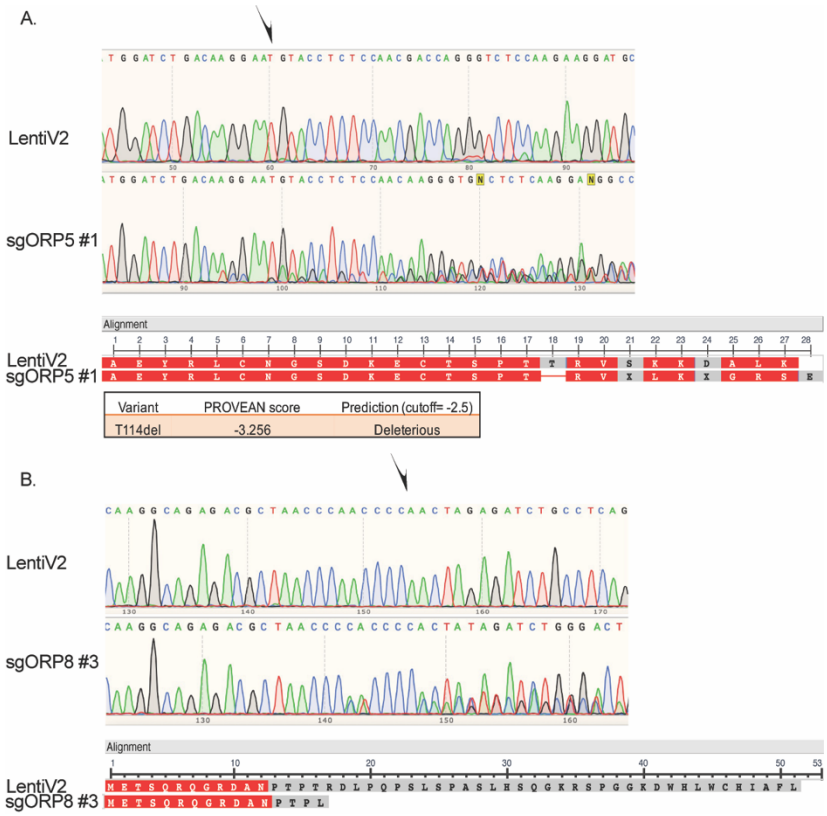

Figure S1. Validation of CRISPR-Cas9 knockout in monoclonal KPC ORP5 and ORP8 knockout cells. DNA was extracted from monoclonal KPC ORP5 and ORP8 knockout cells, amplified via PCR and sent for Sanger sequencing. Sequencing traces of ORP5 **(A)** and ORP8 **(B)** knockout cells were aligned to control cells transfected with an empty vector backbone. Arrows indicate start of designed sgRNA sequence targeting the Cas9 enzyme to the gene, after which we see overlapping peaks, indicative of DNA nicks and subsequent mutations. Below each trace is the translated peptide sequence. **(A)** A tryptophan deletion in ORP5 was assessed as deleterious to protein function. **(B)** An early stop codon in ORP8 knockout cells led to a truncated protein.

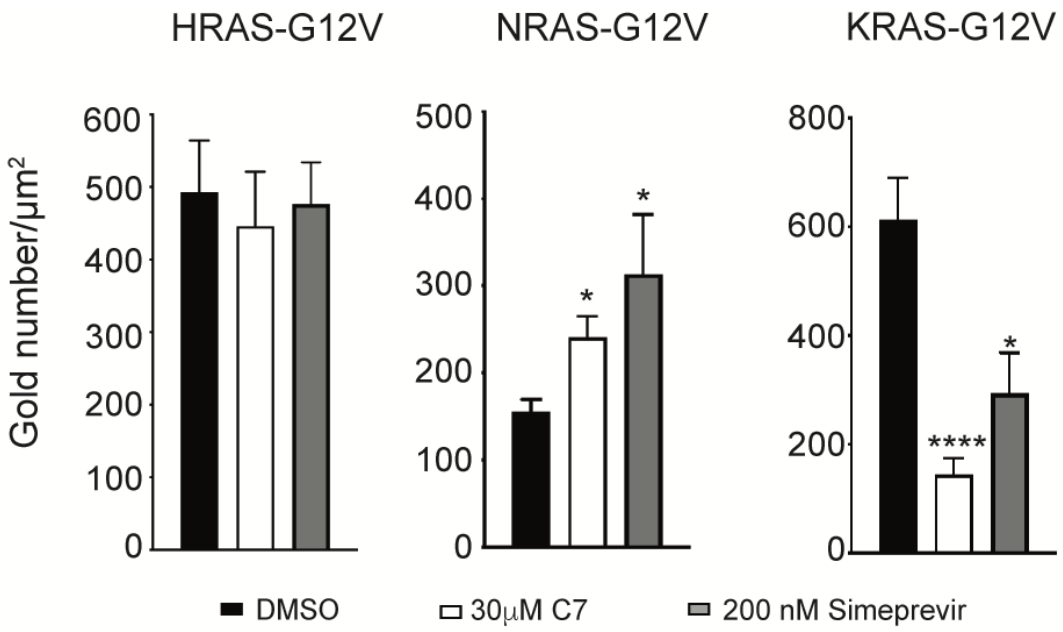

Figure S2. Functional and pharmacological inhibition of PI4KIIIα have no effect on HRAS and NRAS PM localization. Basal plasma membrane sheets from MDCK cells stability expressing GFP-HRASG12V, GFP-NRASG12V or GFP-KRASG12V treated with DMSO, 30μM C7 or 200nM of Simeprevir for 48 hours were prepared and labeled with anti-GFP antibodies coupled directly to 4.5nm gold particles and visualized by EM. The amount of HRASG12V, NRASG12V and KRASG12V on the PM was measured as gold particle labeling per μm^2, and significant differences were quantified using Student's *t* tests (± SEM, n=17) (NRAS-G12V C7 p=0.01; NRAS-G12V Simeprevir p=0.03; KRAS-G12V C7 p=2.16286e^-6, KRAS-G12V Simeprevir p=0.006915154).

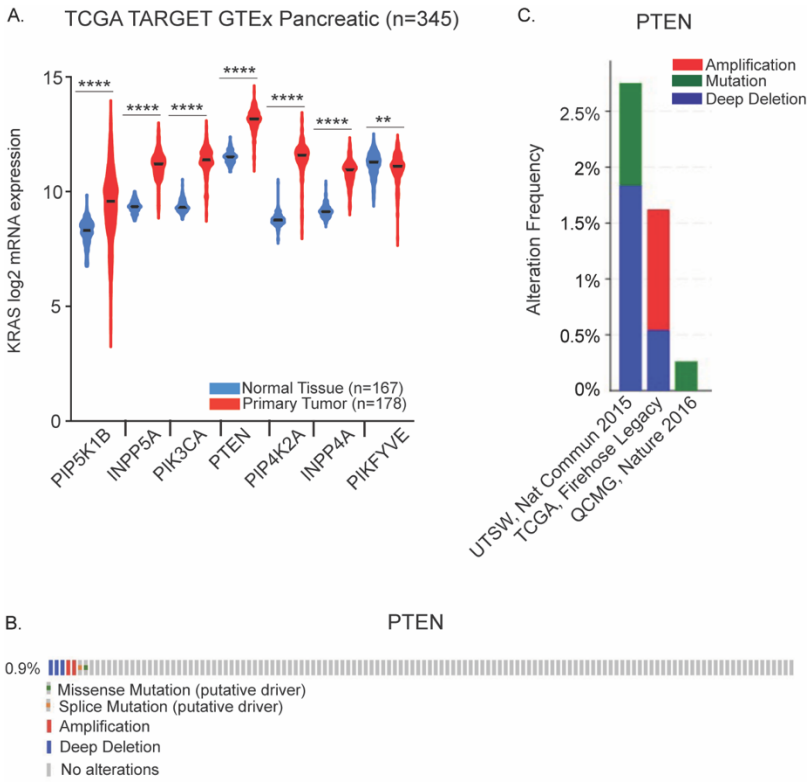

Figure S3. Analysis of mRNA expression levels of PM PIP2 PI kinases and phosphatases reveal increased expression in PDAC. (A) Violin plots indicating medians of *PIP5K1B* (*p* = 3.204e-19*)*, *INPP5A* (*p* = 3.504e-102*)*, *PIK3CA* (*p* = 1.130e-104*)*, *PTEN* (*p* = 9.123e-110*)*, *PIP4K2A* (*p* = 2.345e-111*)*, *INPP4A* (*p* = 1.589e-109*)*, and *PIKFYVE* (*p* = 0.001555*)* mRNA expression levels in normal and primary tumor human pancreatic tissue samples (TCGA TARGET GTEx, n=345). Statistical significance was analyzed with Welch's *t* test for violin plots. Plots were generated using the UCSC Xena Browser. **(B)** Oncoprint representation of PTEN genetic alterations in the multiple pancreatic cancer patient cohorts shown in **(C)** Plots were generated in cBioPortal (n=776) and the percentage of genetic alterations was calculated as altered samples/profiled samples: (7/776)*100=0.9%. **(C).** Bar graph showing the extent and nature of genetic alterations occurring in PTEN in pancreatic cancer patient cohorts. Plots and statistical analyses were generated in cBioPortal (n=776).

Table S1. IC50 values for several growth inhibitors in proliferation and clonogenic assays

	IC50 value (uM)			
Proliferation Assay				
Drug/Cell line	**BxPC-3**	**PANC-1**	**MiaPaCa-2**	**MoH**
C7	27	2.3	10	13.8
LY3214996	4.4	10	4	3.2
Trametinib	0.0037	2.5	0.0094	0.005
Copanlisib	0.94	0.0486	0.104	0.051
Clonogenic Assay				
Simeprevir	N/A	6	6	6
LY3214996	N/A	5	2	1.6
Trametinib	N/A	5	0.005	0.0026
Copanlisib	N/A	0.024	0.052	0.0256

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