EXTENDED EXPERIMENTAL PROCEDURES

Generation of PIP4K2A$^{-/-}$ Mice

PIP4K2A genomic DNA was obtained by polymerase chain reaction from murine strain 129/SvEv genomic DNA and the entire sequence was confirmed. A conditional targeting construct for deletion of the murine Type II *PIP4K2A* gene was designed using a combination of Frt (Flp recombinase sites) and loxP (Cre recombinase sites) recognition sequences as described previously (Bardeesy et al., 2002). Briefly, Frt sites (rectangles) flank a neomycin aminoglycoside 3'-phosphotransferase resistance cassette (Neo) while loxP sites (triangles) flank exon 2 of *PIP4K2A*. The presence of a diptheria toxin negative selection marker is denoted by DT. Linearized targeting construct was electroporated into murine strain 129/SvEv embryonic stem (ES) cells and neomycin resistant ES cell clones were generated using standard techniques. Clones were picked and analyzed for homologous recombination into the endogenous *PIP4K2A* locus using a combination of Southern blot and PCR analysis. Approximately 200 clones were screened and 10 positives were identified. Two of these positive clones were used for microinjection into blastocysts. Chimeric mice carrying the targeted allele were bred to wild-type 129/SvEv mice and analyzed for germline transmission of the *PIP4K2A* targeted allele. Germline transmission of the targeted allele was followed by in vivo deletion of the neomycin resistance cassette using Flp recombinase. Conditionally targeted mice were then bred to the EIIa Cre-deleter transgenic line to generate germline homozygous deficient *PIP4K2A (PIP4K2A$^{-/-}$)* mice as described previously (Bardeesy et al., 2002).

PCR

For genotyping *PIP4K2A*, primers were used to amplify regions of genomic DNA present in either wild-type samples or knockout samples. Primers used were: KO1-5′-CCTTCTGCACCACCCAGGCTC-3′, KO3-5′-GGATATCACTTAATGTATAAGACAC-3′. The KO1/KO3 primer pair amplifies a fragment of 500 bp (wild-type) and a fragment of 200–300 bp (knockout). We used the recommended cycling conditions for the GoTaq Green Master Mix (Promega) with an annealing temperature of 55°C. Genotyping of *PIP4K2B* was performed as previously described (Lamia et al., 2004). *TP53* (*Trp53$^{tm1Tyj/J}$*) mice were purchased from The Jackson Laboratory and the recommended genotyping protocol was followed.

Weight Measurements

Mice were weighed daily at 10am between the ages of 9 and 21 days, every 3 days between 21 and 54 days, and weekly between the ages of 8 to 26 weeks.

PI5P Assay

Cellular phosphoinositides were extracted as described previously (Serunian et al., 1991). The dried lipids were resuspended and sonicated in 40 μl of buffer containing 50 mM HEPES pH7.4 and 1mM EGTA. In order to measure relative PI5P level against PI4P level, the lipids were aliquoted and subjected to in vitro PI5P4K and PI4P5K assays. Kinase reaction was carried out in total 50 μl of kinase buffer containing 20 μl of the resuspended lipids, 50mM HEPES pH7.4, 10 mM $MgCl_2$, 10 uM nonradiolabeled ATP, 10uCi [g-32P]-ATP with 1 μg of GST-PI5P4Kalpha or 0.2 μg of PI4P5Kalpha (Novus H0008394) for 20 min at room temperature. The reaction was terminated by adding 20 μl of 4N HCl. Phosphoinositides were extracted by adding 70 μl of methanol/chloroform (1:1, vol:vol) mix and subjected to TLC (thin-layer chromatography) assay using heat activated 2% oxaloacetate-coated silica gel 60 plate (20 cm × 20 cm, EMD Chemicals) and 1-propanol/2M acetic acid (65:35, vol:vol) solvent system. The radiolabeled product, PI-4,5 P_2 was quantified with a phosphoimager (Molecular Dynamics, STORM840).

Immunohistochemistry for PI5P4K

Formalin-fixed paraffin-embedded (FFPE) tissue sections were deparaffinized, rehydrated, and heated with a pressure cooker to 125°C for 30 s in citrate buffer for antigen retrieval. After cooling to room temperature, sections were incubated in 3% hydrogen peroxide (Dako, Carpinteria, CA) for 5 min to quench endogenous peroxidase. Sections were then incubated in avidin block for 15 min to quench endogenous avidin, followed by incubation in biotin block for 15 min to quench endogenous biotin (Vector, Burlingame, CA). The sections were then incubated with serum-free protein block for 10 min (Dako, Carpinteria, CA). The anti-PI5P4Kβ antibody (Cell Signaling Technologies) was applied at a 1:100 dilution to sections for 1 hr at room temperature. The anti-PI5P4Kα antibody (Abgent) was applied at a 1:25 dilution to sections for 1 hr at room temperature. Detection was performed by incubation with Dako EnVision+ System HRP labeled polymer anti-rabbit (Dako, Cat# K4003) for 30 min, followed by incubation with biotin-labeled tyramide (Perkin-Elmer, Cat #SAT700001EA) at a 1:50 dilution for 10 min. The slides were then incubated with LSAB2 Streptavidin-HRP (Dako, Cat # K1016) for 30 min. DAB chromogen (Dako, Cat # K3468) was then applied. Slides were slightly counterstained with hematoxylin. The specificity of the immunoassay was validated by staining FFPE cells that expressed stable shRNAs targeting PI5P4Kα or PI5P4Kβ (negative control) or the vector control shRNA (positive control) (Figure S1).

Immunohistochemistry for p27

Immunohistochemistry was performed on four micron-thick, formalin fixed, paraffin-embedded tumor sections, which were initially deparaffinized, rehydrated and heated with a pressure cooker to 125°C for 30 s in citrate buffer for antigen retrieval and then incubated with peroxidase (Dako #S2003, Carpinteria, CA) and protein blocking reagents (Dako #X0909) respectively for 5 min.

Sections were then incubated with anti-p27 (1:200, BD Biosciences #610242, San Jose, CA) antibody for 1 hr at room temperature followed by incubation with the Dako EnVision+ System HRP labeled polymer anti-mouse (Dako #K4001) for 30 min. All sections were developed using the DAB chromogen kit (Dako K3468) for 2 min and then lightly counterstained with hematoxylin. Stained slides were digitalized with Scanscope XT (Aperio Technologies, Vista, CA) at 20× magnification and analyzed using a modified Nuclear Image Analysis algorithm (Aperio Technologies).

Immunohistochemistry for 8-oxo-dG

Paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in a graded ethanol series, followed by antigen retrieval in boiling citrate buffer, pH 6.0. The 8-oxo-dGuo antibody [N45.1] was obtained from Abcam and used at a 1:50 dilution. Immunohistochemistry was performed with the UltraVision LP Detection System: HRP Polymer (Thermo Fisher) according to the manufacturer's instructions with several modifications. The M.O.M. blocking reagent (Vector Labs) was used in addition to the protein blocking step, and the H2O2 blocking step was performed after the primary antibody incubation. Tissue sections were counterstained with hematoxylin (Vector Labs).

Metabolic Labeling PIs

Cells were metabolically labeled with 10 μCi/ml [^{3}H]inositol for 24–72 hr in inositol-free DMEM supplemented with dialyzed FBS and 200 mM L-glutamine.

HPLC Method for PI Analysis

Cellular PIs were extracted and deacylated as described previously (Serunian et al., 1991). Deacylated lipids were separated by anion-exchange HPLC (Agilent 1200) using two partisphere SAX columns (Whatman) in tandem and a four-step gradient of ammonium phosphate, pH 6.0 (10–40 mM over 60 min; 40–150 mM over 5 min; 150 mM isocratic for 20 min and 150–650 mM over 25 min). Radiolabelled eluate was detected by an online flow scintillation analyzer (PerkinElmer) and quantified using ProFSA software (PerkinElmer).

Xenografts

A volume of 200 μl of 1 × 10^6 BT474 cells suspended in 50% matrigel was injected into either flank of 7-week-old NU/NU mice (Charles Rivers, USA). The vector control expressing cells were injected on the left of the mouse and the shPI5P4Kα/β double knockdown cells were injected on the right side of the same mouse. When tumors surpassed 2 mm we measured them with calipers in two dimensions (width W and length L) 2–3 times a week. The average tumor volume was calculated as $V = L \times W^2 \times 0.52$. At the end of the experiment mice were euthanized and tumors were harvested and weighed. All animal care followed approved institutional guidelines of BIDMC. All animal experiments complied with National Institutes of Health guidelines and were approved by the BIDMC Animal Care and Use Committee.

Cell Proliferation and Senescence Assays

To determine cell proliferation, cells were plated at 10^3 cells per 96-well plate in triplicate. Cells were incubated and assayed at times 0, 24 hr and 62 hr using Cell Titer-Glo Luminescent Cell Viability assay (Promega). Cells were allowed to equilibrate to room temperature for 30 min. At which time an equal volume of the Cell Titer-Glo reagent is added to cells and mixed for 2 min on an orbital shaker, then incubated for 10 min at room temperature to stabilize signal and luminescence was recorded according to the manufacturer's protocol. To determine cellular senescence, cells were plated at 10^4 cells per 6-well plate in triplicate, and 16 hr later SA-β-gal activity was measured using the senescence detection kit (Calbiochem). The pan PI3K inhibitor GDC-0941 was obtained from Selleckchem and NAC from Sigma.

Measurement of Cell Death

Cell death was measured using a cytotoxicity detection kit (Roche Applied Science) according to the manufacturer's protocol. This kit is based on the measurement of lactate dehydrogenase (LDH) that is released into the medium by damaged cells. Cell death is presented as amount of LDH measured in the medium divided by the total LDH released after treatment with 1% Triton X-100. Apoptosis was detected by determining the percentage of cells that had condensed and had fragmented nuclei by staining with Hoechst 33258 stain (1 μg/ml; Sigma) as previously described (McClintock et al., 2002). All cell death results are from four independent experiments and are represented as the mean value ± standard error of the mean (SEM).

Measurement of ROS

Intracellular ROS generation was assessed using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes). ROS in the cells cause oxidation of DCFH, yielding the fluorescent product 2′, 7′-dichlorofluorescein (DCF). Cells were plated on Petri dishes and incubated with DCFH-DA (10 μM) under various conditions. The media was then removed, cells were lysed, centrifuged to remove debris, and the fluorescence in the supernatant was measured using a spectrofluorometer (excitation, 500 nm; emission, 530 nm). Data were normalized to values obtained from untreated controls.

Oxygen Consumption

Cellular O_2 consumption rates were measured in aliquots of 1-3 X 10^6 sub-confluent cells removed from flasks and studied in a magnetically stirred, water-jacketed (37°C) anaerobic respirometer fitted with a polarographic O^2 electrode (Oxytherm system, Hansatech Instruments). Oxygraph Plus software was used to determine oxygen consumption rate.

Microarray

The BT474 breast cancer cell line was infected with lentivirus encoding either PI5P4Kα and β or the pLK0.1 vector control and each microarray experiment was performed in duplicate. Total RNA samples were isolated using the RNeasy mini kit (QIAGEN) and hybridized according to the standard protocol for Affymetrix U133 Plus 2.0 arrays at the BIDMC Genomics and Proteomics Core. Data preprocessing and quality control were performed in R (http://www.r-project.org/) and Bioconductor. Prior to any statistical computation, data were normalized using the gc-Robust Multi-array Average (gcRMA) algorithm. The differentially expressed genes were computed by an empirical Bayes (eBayes) shrinkage of the standard errors toward a common value approach (Smyth, 2004) embedded within the Limma package (Smyth, 2004). p values were adjusted for multiple comparison using the false discovery rate approach implemented by Klipper-Aurbach et al. (1995) (Reiner et al., 2003). Unsupervised hierarchical clustering using the Euclidean distance metric and the complete linkage algorithm was used to plot the genes with the lowest p value (adjusted p value < 0.001). Gene ontology and pathway (BioCarta) enrichment analysis was performed using a classical hypergeometric test and the gene ontologies or pathways with the highest negative log(10) p value were plotted. Microarray data are available at the Gene Expression Omnibus (GEO) of the NCBI (GSE number - TBA).

Targeted Mass Spectrometry

The BT474 breast cancer cell line was infected with lentivirus encoding either PI5P4Kα and β or the pLK0.1 vector control. 2.5 × 10^4 cells were plated in 6-well dishes. Metabolites were extracted by aspirating media and quickly adding prechilled 80% methanol to the cells on dry ice. Plates with 80% methanol were placed in −80°c for 15 min. Cells were scraped on dry ice and collected in precooled tubes. Tubes were spun at max speed for 15 min at 4°c and supernatants collected. Samples were dried down completely using a speed vac at room temperature. All samples were done in triplicate. Samples were then re-suspended using 20 μl HPLC grade water for mass spectrometry. 10 μl were injected and analyzed using a 5500 QTRAP triple quadrupole mass spectrometer (AB/SCIEX) coupled to a Prominence UFLC HPLC system (Shimadzu) via selected reaction monitoring (SRM) of a total of 254 endogenous water soluble metabolites for steady-state analyses of samples. Some metabolites were targeted in both positive and negative ion mode for a total of 285 SRM transitions using positive/negative ion polarity switching. ESI voltage was +4900V in positive ion mode and –4500V in negative ion mode. The dwell time was 4 ms per SRM transition and the total cycle time was 1.89 s. Approximately 9-12 data points were acquired per detected metabolite. Samples were delivered to the mass spectrometer via hydrophilic interaction chromatography (HILIC) using a 2.0 mm i.d x 15 cm Luna NH_2 column (Phenomenex) at 300 μL/min. Gradients were run starting from 85% buffer B (HPLC grade acetonitrile) to 42% B from 0-5 min; 42% B to 0% B from 5-16 min; 0% B was held from 16-24 min; 0% B to 85% B from 24-25 min; 85% B was held for 7 min to re-equilibrate the column. Buffer A was comprised of 20 mM ammonium hydroxide/20 mM ammonium acetate (pH = 9.0) in 95:5 water: acetonitrile. Peak areas from the total ion current for each metabolite SRM transition were integrated using MultiQuant v1.1 software (AB/SCIEX).

Statistical Analysis

An ordinal scoring system was used to grade the degree of immunohistochemical staining for each biomarker. PI5P4Kα and PI5P4Kβ expression were not normally distributed. The associations between PI5P4Kα and PI5P4Kβ and cancer status were assessed using Fisher's exact test statistic. All statistical analyses were performed using STATA 12.0 software.

Reverse Phase Protein Array

The Stand Up to Cancer cBio Cancer Genomics Portal (http://cbio.mskcc.org/su2c-portal/) was used to retrieve protein and phosphoprotein levels measured by reverse-phase protein arrays (RPPA) and correlate to *PIP4K2B* gain/amplification in breast tumors.

SUPPLEMENTAL REFERENCES

Reiner, A., Yekutieli, D., and Benjamini, Y. (2003). Identifying differentially expressed genes using false discovery rate controlling procedures. Bioinformatics *19*, 368–375.

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Smyth, G.K. (2004). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. *3*, Article3.

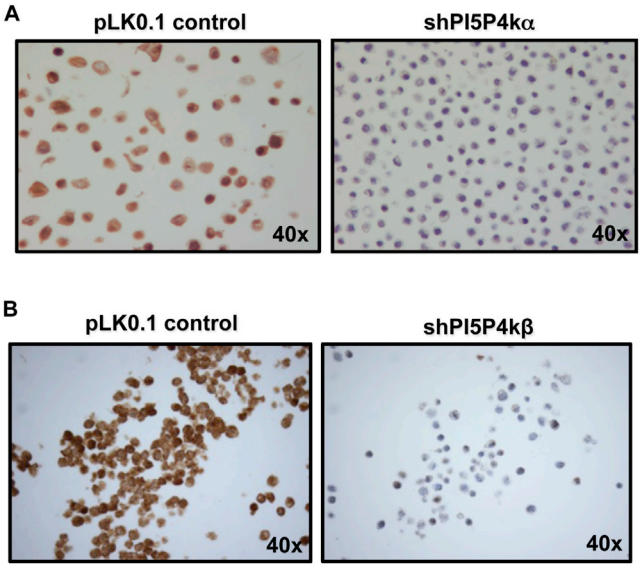
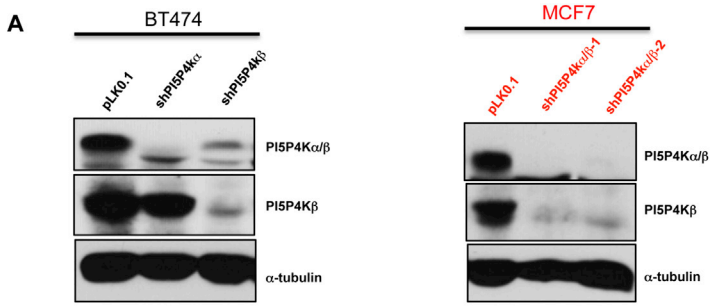
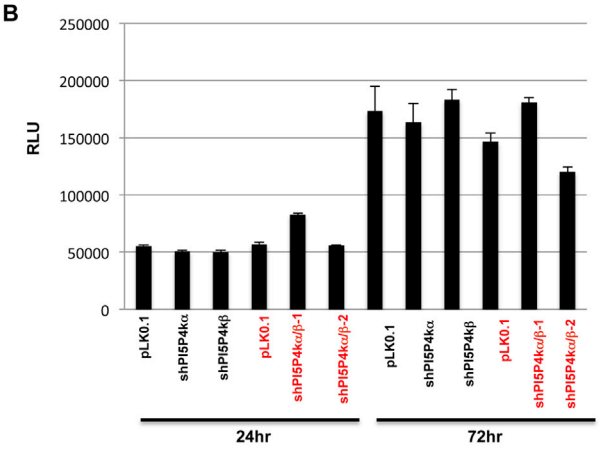


Figure S1. Validation of Specificity of the Immunoassay for Total PI5P4K, Related to Figure 2

(A) Cellblocks were prepared from BT474 cells expressing shRNA-pLK0.1 vector control (left) or shPI5P4kα (right) and stained for total levels of PI5P4kα.
(B) Cellblocks were prepared from BT474 cells expressing shRNA-pLK0.1 vector control (left) or shPI5P4kβ (right) and stained for total levels of PI5P4kβ. Pictures were taken under 40x magnification on a Leica microscope (Leica Microsystems).





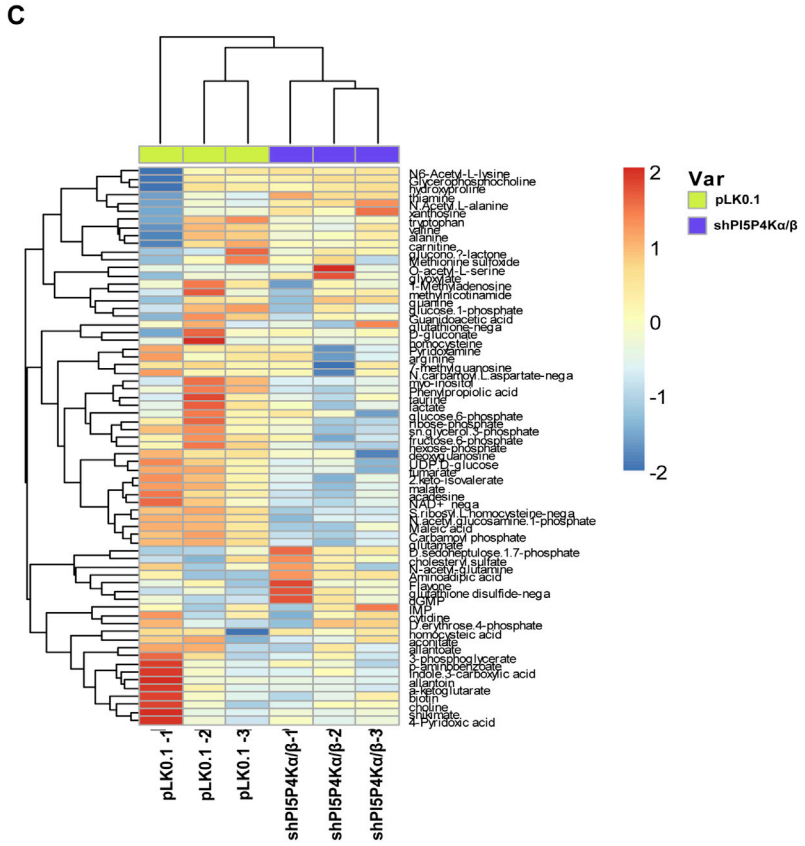


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Figure S2. Knocking Down PI5P4Kα and β Has No Effect in MCF7 Cells PI5P4K, Related to Figures 3 and 4

(A) Stable knockdown of PI5P4Kα or β in BT474 cells *(left)*, and double PI5P4Kα/β knockdown in MCF7 cells *(right)*. The PI5P4Kα/β antibody recognizes both the α and β isoforms. PI5P4Kβ antibody only recognizes the β isoform. shPI5P4Kα/β-1 (sequence 1) and shPI5P4Kα/β-2 (sequence 2) are two independent hairpins targeted against PI5P4Kα and PI5P4Kβ. All single knockdowns are sequence 1 *(See experimental procedures)*. An anti-α-tubulin antibody was used as loading control.

(B) Luminescent Cell Viability Assay in stable knockdown cells; BT474 cell lines (*black*) and MCF7 cell lines (*red*). Results are from four independent experiments and are represented as the mean value ± standard error of the mean (SEM).

(C) AKT phosphorylation at threonine 308 (pT308) and serine 473 (pS473) and total AKT protein levels in pLK0.1 vector control cells and in shPI5P4Kα/β double knockdown cells. Cells were serum starved overnight and then treated with 10 nM insulin for the indicated time points.

(D) Metabolomics analysis of MCF7 PI5P4Kα/β double knockdown cells. Heat-map of the most differentially expressed metabolites between control (pLK0.1 empty vector) and PI5P4Kα/β knockdown MCF7 cells (shPI5P4Kα/β). Orange indicates high and blue low metabolite expression relative to the median expression.

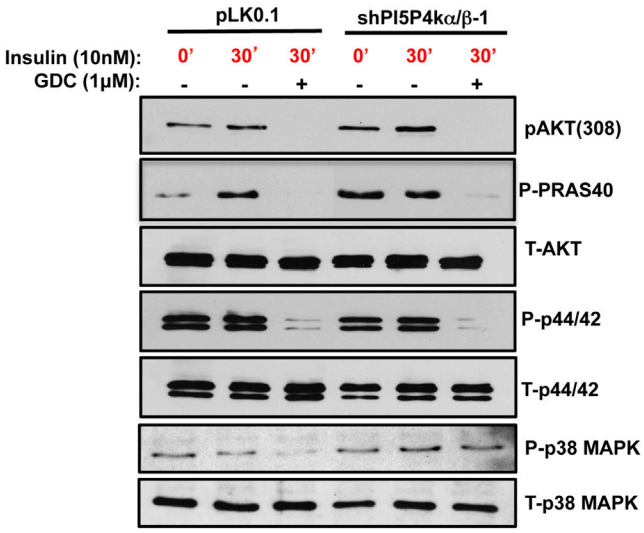

Figure S3. Signaling in Stable BT474 Cells, Related to Figure 3

AKT phosphorylation at threonine 308 (pT308), phosphorylation of AKT target gene PRAS40, total AKT protein levels, phosphorylation of ERK (P-p44/42), total ERK (T-p44/42), phosphorylation of p38 MAPK (P-p38 MAPK) and total p38 MAPK in pLK0.1 vector control cells and in shPI5P4Kα/β double knockdown cells. Cells were serum starved overnight and then treated with 10 nM insulin for the indicated time points ± GDC0941 (1 μM).

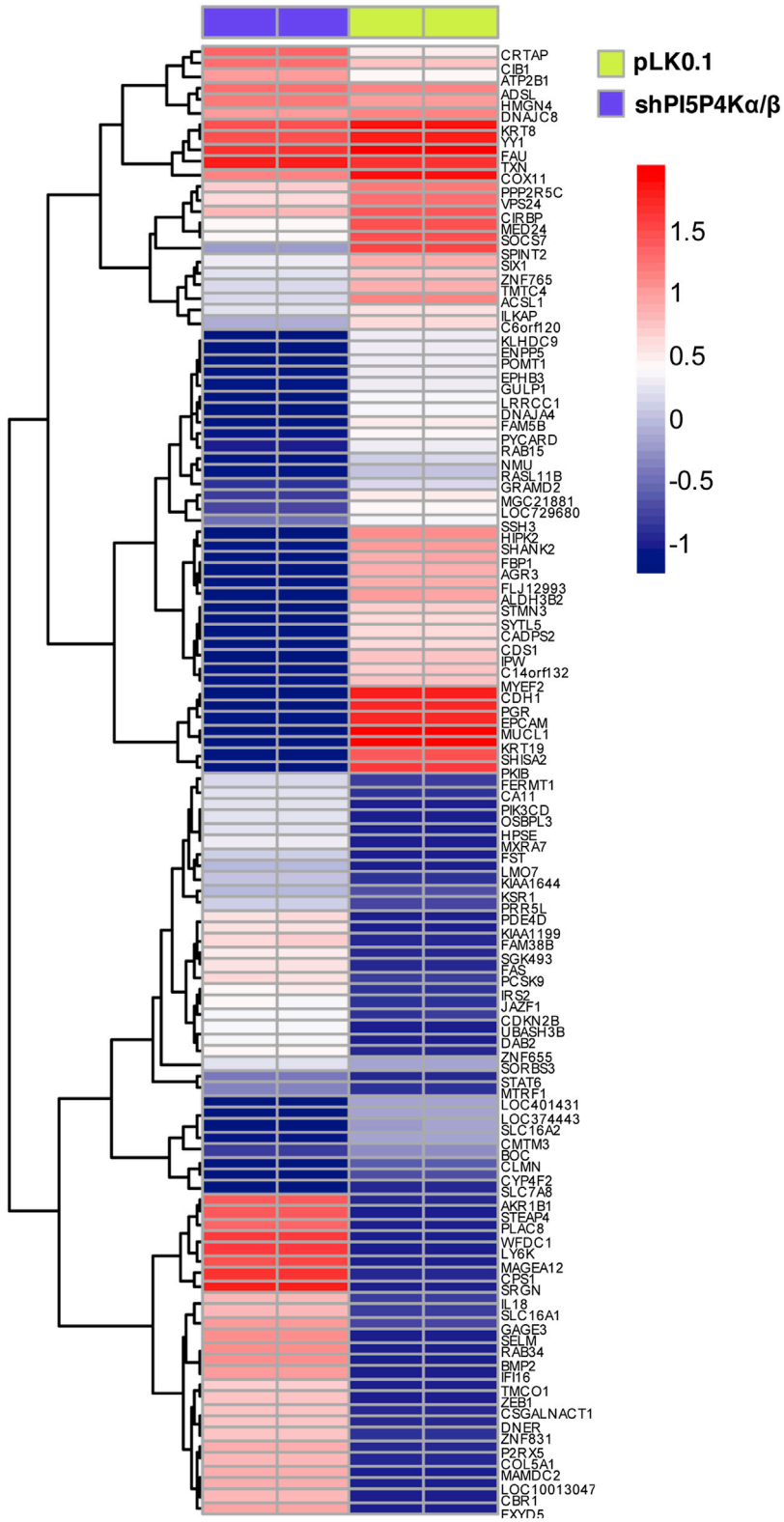

Figure S4. Distinct Gene Expression Signature the PI5P4Kα/β Double-Knockdown Cells, Related to Figure 5

Heat-map of the most differentially expressed genes (p value < 0.001) between control (pLK0.1 empty vector) and PI5P4Kα/β knockdown BT474 cells (shPI5P4Kα/β). Red indicates high and blue low gene expression relative to the median expression.

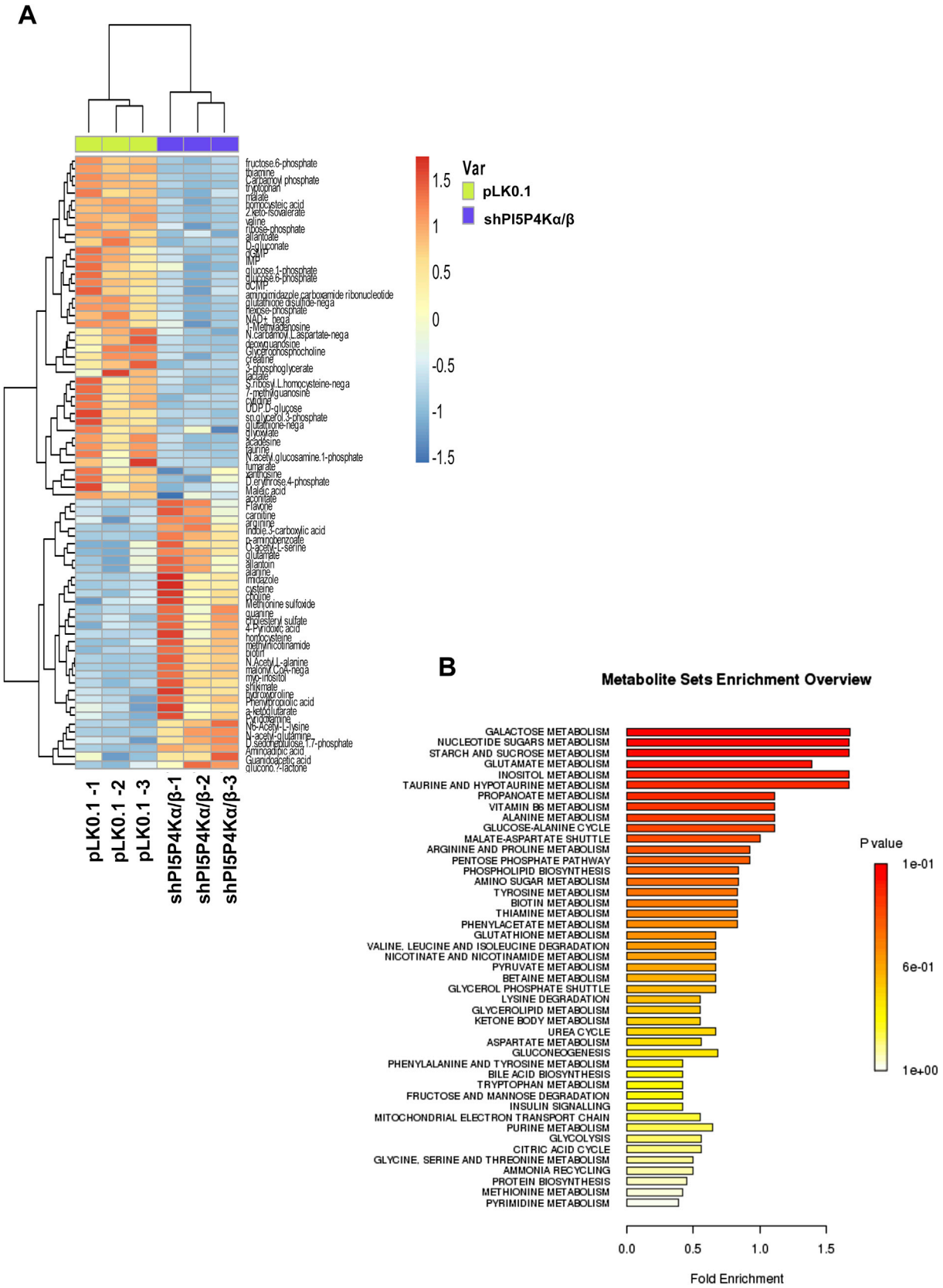

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Figure S5. Metabolomics Analysis and Enrichment Overview of BT474 PI5P4Kα/β Double-Knockdown Cells, Related to Figure 5

(A) Heat-map of the most differentially expressed metabolites between control (pLK0.1 empty vector) and PI5P4Kα/β knockdown BT474 cells (shPI5P4Kα/β). Orange indicates high and blue low metabolite expression relative to the median expression.

(B) Heat-map of the most differentially expressed metabolism pathways between control (pLK0.1 empty vector) and PI5P4Kα/β knockdown BT474 cells (shPI5P4Kα/β). Red indicates high and yellow low metabolite expression relative to the median expression.

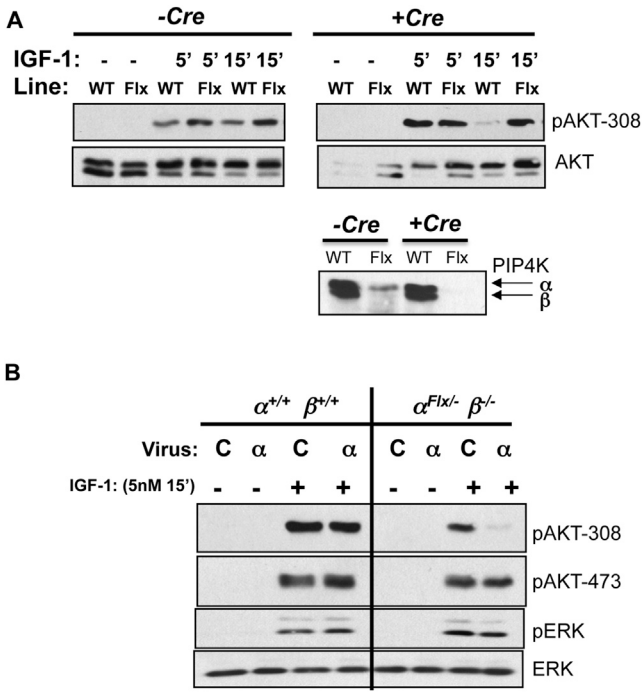

Figure S6. Endogenous PIP4K2A/B Inhibits AKT Activation, Related to Figure 6

(A) Primary MEFs were treated with or without adenoviral Cre for 72 hr, followed by treatment of IGF-1 for the indicated times. Acute knockdown of PIP4K2A leads to enhanced AKT activation at 15' post-IGF-1 treatment.

(B) Reconstitution of PIP4K2A into primary MEFs leads to inhibition of IGF-1 induced AKT activation on pT308 but not pS473 (C, control virus; a, PIP4K2A virus).

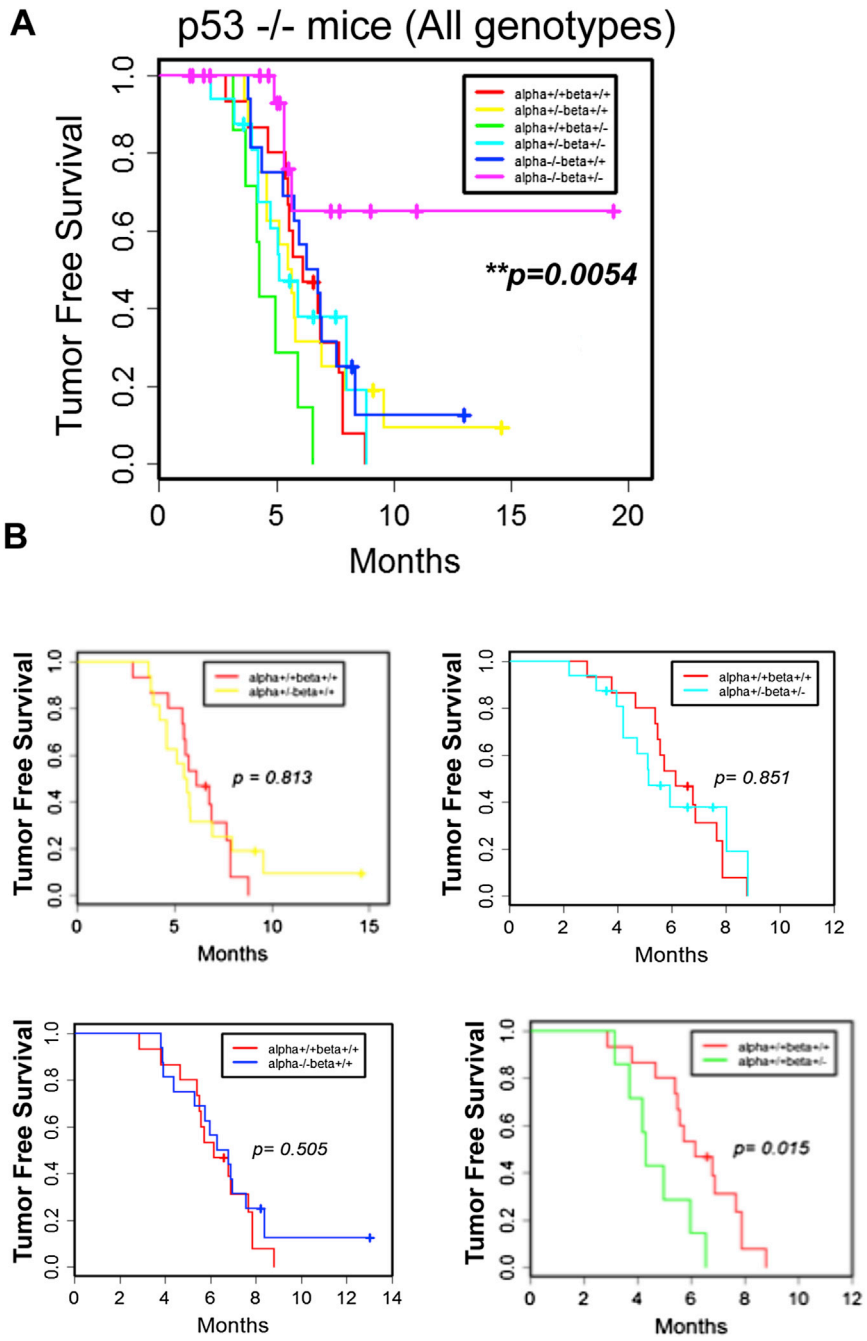

Figure S7. PI5P4K Deficiency Restricts Tumor Death after p53 Deletion, Related to Figure 7

(A) Kaplan-Meier plot analysis of tumor free survival of all mouse genotypes. Total numbers of mice: 15 PI5P4K$\alpha^{+/+}$ PI5P4K$\beta^{+/+}$, 16 PI5P4K$\alpha^{+/-}$ PI5P4K$\beta^{+/+}$, 7 PI5P4K$\alpha^{+/+}$ PI5P4K$\beta^{+/-}$, 16 PI5P4K$\alpha^{+/-}$ PI5P4K$\beta^{+/-}$, 16 PI5P4K$\alpha^{-/-}$ PI5P4K$\beta^{+/+}$, and 20 PI5P4K$\alpha^{-/-}$PI5P4K$\beta^{+/-}$. * indicates a p value < 0.05 with two-tailed Student's t test.

(B) Kaplan-Meier plot analysis of tumor free survival for PI5P4K$\alpha^{+/+}$ PI5P4K$\beta^{+/+}$ and individual genotypes PI5P4K$\alpha^{+/-}$ PI5P4K$\beta^{+/+}$, PI5P4K$\alpha^{+/-}$ PI5P4K$\beta^{+/-}$, PI5P4K$\alpha^{-/-}$ PI5P4K$\beta^{+/+}$, and PI5P4K$\alpha^{+/+}$ PI5P4K$\beta^{+/-}$. All p values are shown.