Supplementary Materials and Methods

Reagents

PHLPP1 and PHLPP2 antibodies were from Bethyl Laboratories (Montgomery, TX); antibodies against total Akt, Akt1, Akt2, pAkt (Ser473), and pAkt (Thr308) were from Cell Signaling (Beverly, MA); and Nox4 and p22*phox* antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The siRNAs against PHLPP1 and PHLPP2 were SMARTpool siRNA from Dharmacon (Chicago, IL) with the following sequences. PHLPP1: (1) GAT CTA AGG TTG AAC GTA A; (2) TGA TCT AGA TGC TAT GAT T; (3) GAT ATT GGC CAT AAT CAA A; and (4) GAA CGC CTC TGC GAA CAA A. PHLPP2: (1) GGA AAG ACC CAG CTG CAT A; (2) GAA CTT GTC CCA TAA TAA A; (3) GCT ATA ATC TTC TCA CAG A; and (4) GTA CAG CAG TCA ACT AAT G. siRNA against p22*phox* was from Santa Cruz Biotechnology (sc-36149); siRNA against Nox4 was from MWG-Biotech (Huntsville, AL) (AAA CCG GCA GAG UUU ACC CAG). Akt siRNA were SMARTpool siRNA from Dharmacon (Akt1 M-003000-03-0005 and Akt2 M-003001-02-0005). Plasmids for PHLPP1 and PHLPP2 overexpression were a gift from A. Newton (San Diego, CA). LC3-GFP constructs were a gift from T. Yoshimori (Osaka, Japan). Human IGF-I was from R&D Systems (Minneapolis, MN), Akt 1/2 Inhibitor VIII from Calbiochem (La Jolla, CA), and pNPP from BioAssay Systems (Hayward, CA). All other reagents were from Sigma (St Louis, MO).

Cell Culture

Human pancreatic adenocarcinoma cell lines MIA PaCa-2 (poorly differentiated) and PANC-1 (moderately differentiated) were obtained from American Type Culture Collection (Manassas, VA). Cells were grown in 1/1 Dulbecco's modified Eagle medium/F12 (Gibco Invitrogen Corp, Grand Island, NY) supplemented with 15% FBS, 4 mmol/L L-glutamine, and antibiotic/antimycotic solution (Omega Scientific, Tarzana, CA). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and were used between passages 3 and 12. Cells were cultured without growth factors or with FBS or IGF-I as indicated. To measure the effects of short-term incubation with growth factors, cells were grown without serum for 48 hours and then FBS or IGF-I was added for indicated times (eg, 5 or 15 minutes) before cells were collected and lysed for analyses.

Transfections

Transient transfections of MIA PaCa-2 and PANC-1 cells were performed using the electroporation system Amaxa Nucleofector (Amaxa Inc, Gaithersburg, MD) according to the manufacturer's protocol. The measurements were performed at 48 hours post-transfection.

Immunoblotting

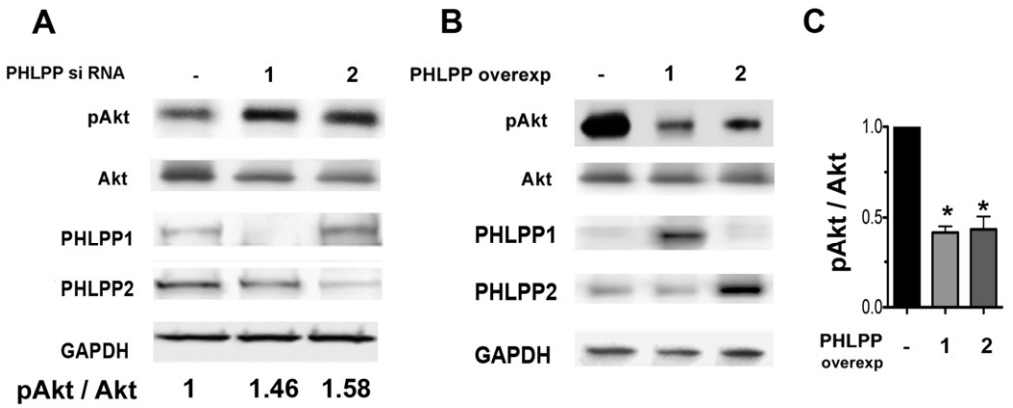
Cells were lysed on ice in RIPA phosphorylation buffer (50 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.2, 1% deoxycholic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 10 mmol/L Na_2HPO_4 + NaH_2PO_4, 100 mmol/L NaF, 2 mmol/L Na_3VO_4, 80 μmol/L glycerophosphate, 20% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL each of pepstatin, leupeptin, chymostatin, antipain, and aprotinin) for 10 minutes and centrifuged for 15 minutes at 16,000*g* at 4°C. Proteins were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Nonspecific binding was blocked with 5% bovine serum albumin or 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20. Membranes were incubated with primary antibody overnight at 4°C and then with peroxidase-conjugated secondary antibody for 1 hour. Blots were developed using SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL). For detection and densitometric quantification, we used Fluo Chem HD2 (Alpha Innotech, San Leandro, CA).

Immunoprecipitation and Coimmunoprecipitation

Immunoprecipitation and coimmunoprecipitation were performed using the Catch and Release Kit (Millipore, Billerica, MA), according to the manufacturer's protocol. The antibodies against Akt1, Akt2, pAkt, PHLPP1, and PHLPP2 were as specified in Reagents.

Measurements of Apoptosis, Necrosis, Autophagy, and Proliferation

Apoptosis was assessed as described before[21] by measuring internucleosomal DNA fragmentation with Cell Death Detection Elisa^{Plus} kit (Roche Molecular Biochemicals, Mannheim, Germany) and by flow cytometric analysis (FACScan; BD Biosciences, San Jose, CA) of phosphatidylserine externalization in cells labeled with Annexin V (AnnV) and propidium iodine (PI) using Annexin V FLUOS Staining Kit (Roche Biochemicals, Mannheim, Germany). $AnnV^+/PI^-$ cells were considered apoptotic, $AnnV^-/PI^+$ cells were considered necrotic, and $AnnV^+/PI^+$ cells were considered as advanced necrosis or apoptosis associated with secondary necrosis. Autophagy was measured as the number of LC3 dots per cell[24] using confocal microscopy on cells transfected with LC3-GFP. Quantification of LC3 dots was performed using MetaMorph and ImageJ software (Molecular Devices, Downingtown, PA). Proliferation was measured by thymidine incorporation. Cells were grown for 12 hours with 10 μCi ^{3}H-thymidine and then precipitated with trichloroacetic acid, and ^{3}H-thymidine levels in trichloroacetic acid precipitates were measured in a liquid scintillation counter and normalized to cell number.



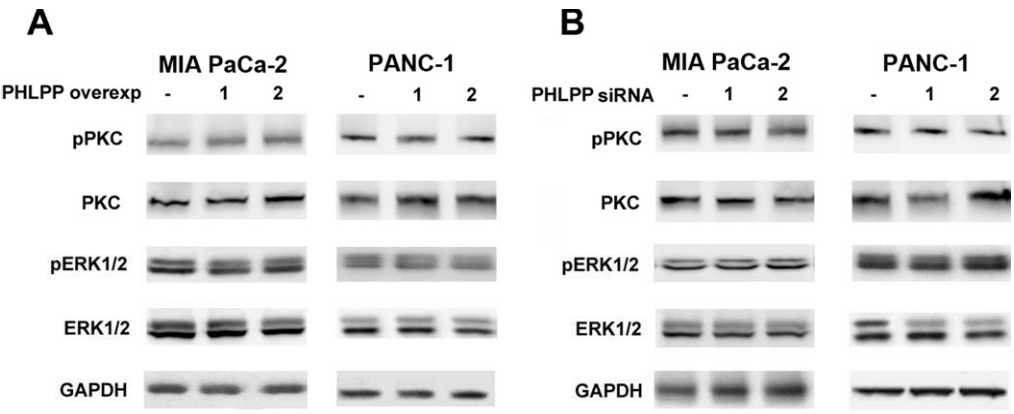
Supplementary Figure 1. The phosphatases PHLPP1 and PHLPP2 inhibit Akt phosphorylation in PANC-1 cells. Cells were transfected with (*A*) control, PHLPP1, or PHLPP2 siRNA or (*B* and *C*) control, PHLPP1, or PHLPP2 overexpression plasmid and cultured with 15% FBS. (*A* and *B*) Levels of PHLPP1 and PHLPP2 (the transfection efficiency), pAkt, and total Akt were measured by immunoblotting. In this and other figures, blots were reprobed for GAPDH to confirm equal loading. (*A* and *C*) The intensities of pAkt and Akt bands were quantified by densitometry, and their ratios were normalized to control siRNA (*A*; values below the immunoblot) or control plasmid (*C*) transfections. Values are means ± SE (n = 3). *$P < .05$ versus transfection with control plasmid.

Immunofluorescence and Immunohistochemistry

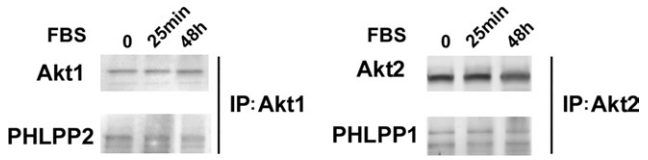
Cells were fixed for 15 minutes at −20°C in methanol/acetone (1:1), and the nonspecific binding was blocked with 5% goat or donkey serum (Abcam, Cambridge, MA) and 1% Aurion BSA in phosphate-buffered saline containing 0.2% Triton (Triton; Wageningen, The Netherlands). After incubation with primary antibody and fluorescent-labeled secondary antibody, cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI), mounted with Antifade (Invitrogen, Carlsbad, CA), and analyzed in a Nikon Eclipse 2000S fluorescent microscope (Nikon, Tokyo, Japan). Sections of paraffin-embedded human and mouse pancreatic adenocarcinoma and normal pancreas were deparaffinized with xylol for 30 minutes and then rehydrated with graded ethanol. Antigen retrieval was performed by boiling in 10 mmol/L citrate buffer (pH 6.0) for 5 minutes twice. Nonspecific binding was blocked with 5% Aurion BSA. Sections were incubated with primary antibody followed by secondary Alexa Fluor coupled antibody or with Vectastain ABC Kit (Vector Laboratories, Burlingame, CA). Slides were counterstained with DAPI or hematoxylin, mounted with Vectashield (Vector Laboratories), and analyzed by fluorescence or conventional microscopy. Secondary only controls showed no signal.

Lentiviral Transduction of MIA PaCa-2 Cells

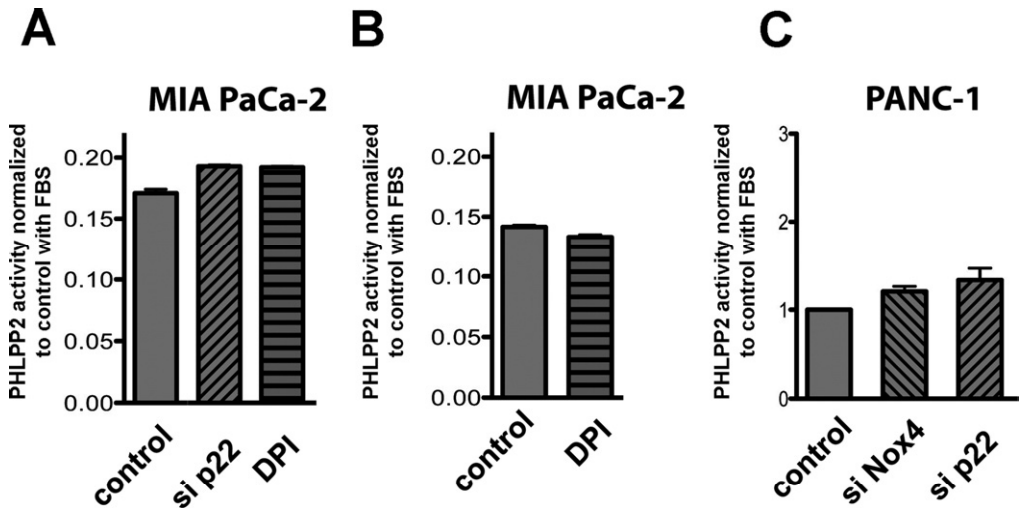
To generate stable PHLPP1 and PHLPP2 overexpressing MIA PaCa-2 cells, PHLPP1 and PHLPP2 were cloned into an IRES-GFP vector designed by the Vector Core (N.K.) at UCLA. The vectors have been sequence verified. Lentiviral particles were produced by the Vector Core in HEK 293 cells, and 5×10^6 MIA PaCa-2 cells were transduced with the particles. A transfection efficiency of >80% was determined by green fluorescent protein fluorescence.



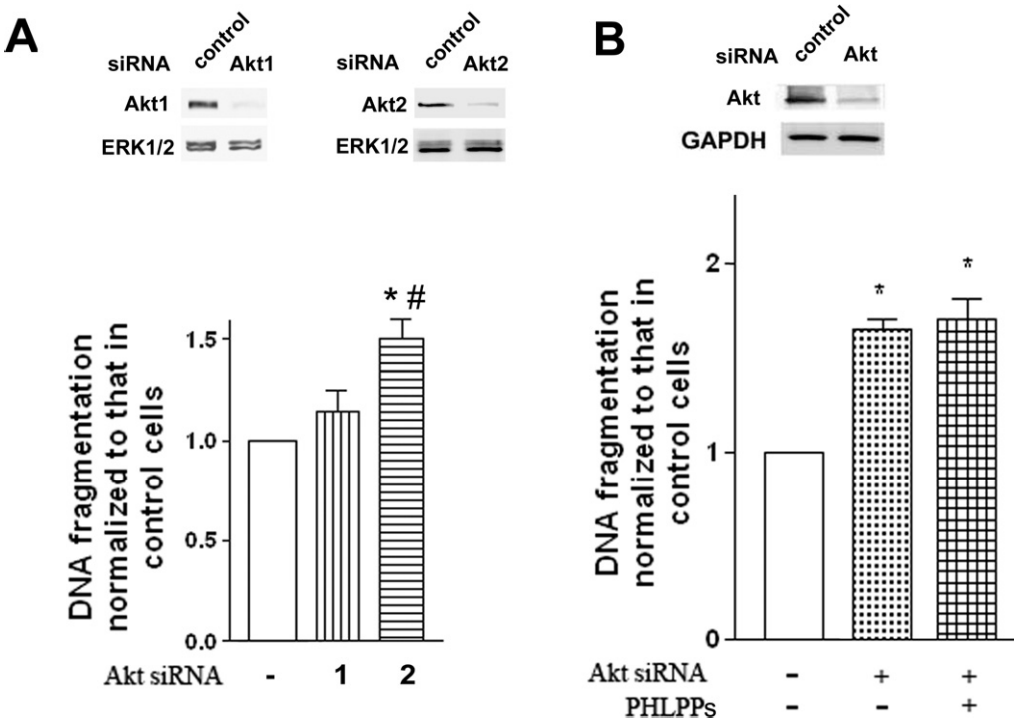
Supplementary Figure 2. PHLPP1 and PHLPP2 do not alter phosphorylation levels of PKC and ERK kinases. MIA PaCa-2 and PANC-1 cells were transfected with (*A*) control, PHLPP1, or PHLPP2 overexpression plasmid or (*B*) siRNA and cultured with 15% FBS. Transfection efficiencies are shown in Figure 1*B* and *C* (for MIA PaCa-2) and Supplementary Figure 1 (for PANC-1). Levels of pPKC, total PKC, pERK1/2, and total ERK1/2 were measured by immunoblotting. Representative of 3 independent experiments, which all gave similar results.



Supplementary Figure 3. FBS does not alter the amount of PHLPP coimmunoprecipitated with Akt. MIA PaCa-2 cells were depleted of growth factors and then cultured with and without 15% FBS for indicated times, and Akt1 and Akt2 were immunoprecipitated. The immunoprecipitates were probed for Akt1, Akt2, PHLPP1, and PHLPP2. The experiment was repeated twice with the same results.



Supplementary Figure 4. ROS have no significant effect on PHLPP2 activity. (*A* and *B*) MIA PaCa-2 and (*C*) PANC-1 cells were transfected with control, Nox4, or p22 siRNA and grown for 48 hours with (*A* and *C*) 15% FBS or (*B*) 100 ng/mL IGF-I in the presence or absence of 10 μmol/L DPI. PHLPP2 activity was measured in PHLPP2 immunoprecipitates and normalized to that in control cells (ie, cells transfected with control siRNA or cultured without DPI). Values are means ± SE (n = 3).



Supplementary Figure 5. The stimulatory effects of PHLPP overexpression and Akt knockdown on apoptotic cell death. (*A*) MIA PaCa-2 cells were transfected with siRNAs against Akt1 and Akt2. (*B*) MIA PaCa-2 cells were transfected with control RNA and control plasmid, a combination of Akt1 and Akt2 siRNAs (Akt siRNA), or a cotransfection of PHLPP1 and PHLPP2 (PHLPPs) overexpression. Plasmids with Akt siRNA. Cells were grown with 15% FBS. Apoptosis was quantified by measuring DNA fragmentation with the Cell Death ELISA Kit. *Insets* show Akt siRNA transfection efficiency (immunoblots); the efficiencies of PHLPP plasmid transfections are shown in Figure 1*C*. Values are means ± SE (n = 3). $^{*}P < .05$ versus transfection with control plasmid; $^{\#}P < .05$ versus transfection with Akt1 siRNA.

Supplementary Table 1. Data for the Analysis of the Correlation Between PHLPP Expression and Survival of Patients With PDAC

Parameters	High PHLPP1 expression	Low PHLPP1 expression	High PHLPP2 expression	High PHLPP2 expression
No. of patients	17	24	20	21
Death	8	18	13	13
Censored	9	6	7	8

NOTE. The intensities of PHLPP1 and PHLPP2 immunostaining were scored using a semiquantitative classification into 2 groups: (1) equal to or higher expression than in normal tissue and (2) lower expression. Ten representative fields per tumor (magnification 20×), with the use of ImageJ software.

Supplementary Table 2. Median Survival of Patients With PDAC With High or Low Akt1 and Akt2 Expression

	Median survival (*mo*)	
Akt1 or Akt2 expression	Akt1	Akt2
High	16	16
Low	17	44

NOTE. The intensities of Akt1 and Akt2 immunostaining were scored using a semiquantitative classification into 2 groups: (1) higher expression than in normal tissue and (2) equal or lower expression. Ten representative fields per tumor (original magnification 20×), with the use of ImageJ software.