Supporting Information

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SI Materials and Methods

Histological Analysis. Prostates were dissected out and fixed in 10% (vol/vol) neutral buffered formalin for 24 h, dehydrated, and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (H&E). For immunohistochemistry, sections were deparaffinized, rehydrated, and then treated for antigen retrieval. Endogenous peroxidase was quenched in 3% (vol/vol) H_2O_2 in water or 0.5% H_2O_2 in methanol at room temperature. After blocking in normal serum, tissues were incubated with primary antibody overnight at 4 °C, followed by incubation with biotinylated secondary antibody. Antibodies were visualized with avidin/biotin complex (Vectastain Elite; Vector Laboratories) using diaminobenzidine as the chromagen. The following primary antibodies were used: Ki67 (clone SP6, Thermo Scientific), c-Myc (clone Y69, Epitomics), phospho-S373–c-Myc (ab30643; AbCam), PKCζ (9372; Cell Signaling) and PTEN (D4.3) (9188; Cell Signaling). For immunohistochemistry to detect p63 and smooth muscle actin (α-SMA), the Vector Mouse on Mouse (M.O.M.) immunodetection kit was used according to the manufacturer's protocol (Vector Laboratories). After blocking of endogenous peroxidase activity, sections were incubated in avidin/biotin blocking solution and M.O.M. mouse Ig-blocking reagent, and then incubated with mouse monoclonal p63 (sc-8431; Santa Cruz) or α-SMA (M0851; Dako) antibodies for 30 min. Primary antibody binding was detected by using M.O.M. biotinylated anti-mouse IgG, and visualized as above. Human prostate tissue microarray (TMA) slides were obtained from US Biomax. TMA slides were scanned by Scanscope XT system (Aperio) and images were captured using the Aperio ImageScope software (v11.1.2.760).

Cell Culture. DU145 and PC3M cells were from ATCC. WT and PKCζ embryo fibroblasts (EFs) were derived from embryonic day (E) 13.5 embryos (1). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) FCS, 1% glutamine, and 1% penicillin-streptomycin, in an atmosphere of 95% air and 5% CO_2. The PTEN-P2 prostate epithelial cell line, a generous gift from Hong Wu (University of California, Los Angeles), was maintained as previously described (2). Lentiviruses for the different target genes were made as follows: shRNA-encoding plasmids were cotransfected with psPAX2 (Addgene; plasmid 12260) and pMD.2G (Addgene; plasmid 12259) packaging plasmids into actively growing HEK-293T cells by using FuGENE 6 transfection reagent. Virus-containing supernatants were collected 24, 48, and 72 h after transfection and filtered and then used to infect target cells in the presence of 8 μg/mL polybrene. Cells were selected with puromycin after infection. Retroviruses for the different target genes were made as follows: pWZL-Hygro-HA-PKCζ (HA-PKCζ), pWZL-Hygro-HA-PKCζ^{K281R} (HA-PKCζ-KD), pWZL-Blast-Myc (Addgene, plasmid 10674) (3), pWZL-Blast-Myc S373A, or pWZL-Blast-Myc S373E. Mutations were generated using the site-directed mutagenesis kit (Invitrogen). These plasmids were transfected into actively growing Phoenix cells by using Lipofectamine 2000 transfection reagent. Virus-containing supernatants were collected and infected as above. Cells were selected with hygromycin or blasticidin after infection. For viability assay and cell-cycle analysis, cells were seeded onto six-well plates in 10% (vol/vol) FCS–DMEM, washed with PBS twice after attachment, and changed to the indicated medium. Cell viability was determined by trypan blue exclusion assay at the indicated time. For cell-cycle analysis, cells were seeded as above and fixed in ethanol and analyzed by propidium iodide (PI) staining (50 μg/mL in PBS) and flow cytometry (FACScan; Becton-Dickinson).

Soft-Agar Assay. HA-PKCζ or HA-PKCζ-KD–overexpressing PTEN-P2 cells (2×10^4) were suspended in 0.35% agar in DMEM containing 20% (vol/vol) FCS and overlaid on 0.7% agar in DMEM containing 10% (vol/vol) FCS. Cells were fed with 10% (vol/vol) FCS-containing DMEM twice a week for 3 wk.

Invasion Assay. Cell invasion was evaluated by using 8-μm pore size Matrigel Invasion chambers (BD Biosciences), according to the manufacturer's instruction. Cells (2.5×10^4) were seeded in serum-free medium into the upper chamber and 15% (vol/vol) FCS-containing medium was used as the chemoattractant in the lower chamber. After 22 h incubation, noninvading cells on the upper surface of the chamber were removed with a cotton swab, and cells on the lower surface of the chamber were fixed in methanol and stained with crystal violet. Cells in at least five random fields from each of three experiments were counted.

Luciferase Assay. DU145 non-targeting shRNA (shNT) and shPKCζ cells were plated and transfected with pBV-luc (Addgene, plasmid 16539) or pBV-luc c-Myc binding sites (MBS) 1–4 (Addgene, plasmid 16564) (3) along with *Renilla* luciferase for normalization using Lipofectamine 2000. Luciferase activity was measured by using the Promega Dual-Luciferase reporter assay system.

Air–Liquid Interface Organotypic Cultures. Organotypic gels were prepared as previously described (5) with a few modifications. The gels were prepared by using 1 mL of a mixture of 5.25 volumes of collagen type I, 1.75 volumes of Matrigel, 1 volume of 1× DMEM, 1 volume of 10× DMEM, and 1 volume of filtered FCS. A total of 5×10^5 (DU145 and WPMY-1, 50:50) cells were seeded on top of the gels. Gels were lifted onto collagen-coated nylon sheets resting on sterile steel grids. Organotypic cultures were harvested at day 14. Images were taken with a Zeiss light microscope supplemented with Axiovision40 software. Quantification and invasion analysis was carried out, as described, using ImageProPlus software (6).

Real-Time PCR (Quantitative Real-Time PCR). Total RNA was extracted by using RNeasy mini kits and treated with DNase I (Qiagen) following the manufacturer's protocol. Reverse transcription was performed with 1 μg of total RNA. Quantitative real-time PCR (Q-PCR) was used to evaluate expression levels from the cDNA by using Absolute SYBR Green Real-Time PCR Mastermix (Thermo Fisher Scientific) and a Real-Time thermocycler (CFX96; Bio-Rad). Expression levels of each gene were measured and then normalized with hypoxanthine phosphoribosyltransferase 1 (HPRT1).

Western Blot. Cell lysates were resolved by SDS/PAGE before transferring to nitrocellulose or PVDF membranes and probing with specific antibodies. Blots were then incubated with horseradish peroxidase-conjugated anti-IgG, and proteins were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

In Vitro Kinase Assay and MS/MS Phosphopeptide Identification. For in vitro phosphorylation assays, 1 μg of recombinant c-Myc (Sigma) was incubated at 30 °C for 60 min in kinase assay buffer containing 25 mM Tris·HCl pH 7.5, 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM DTT, 100 μM ATP, and 50 μCi of [γ-^{32}P] ATP in the

presence of recombinant PKCζ (Upstate). Protein digestion, TiO_2-based phosphopeptide enrichment, electrospray ionization-liquid chromatography tandem mass spectrometry, and MS/MS analysis was performed as previously described.

Microarray Data Analysis. Microarray studies were performed in the Genomics and Microarray Laboratory at the Department of Environmental Health, University of Cincinnati Medical Center. Briefly, total RNA was extracted from three independent cultures of P2–PKCζ-KD and P2–PKCζ-WT cell lines and hybridized on Affymetrix Mouse Gene 1.0 ST Array. Scanning of the images and the first-pass processing of probe-level fluorescence intensities was performed using the Microarray Suite 5.0 software (MAS 5.0; Affymetrix). The data were normalized, and the calculation of the gene-specific summary measures was performed by the robust multiarray average (RMA) procedure (8) based on the Entrez gene-centric probeset definitions provided by the University of Michigan "brainarray" group (9). Statistical significance of genes differentially expressed was assessed using the empirical Bayes linear model (10). Transcriptional profiles of human prostate cancer samples (11) were established by processing raw data downloaded from Gene Expression Omnibus database (GSE21034) by the same procedure used for our experimental data. Average-linkage hierarchical clustering based on the Pearson's correlation as the similarity measure was used for unsupervised clustering of prostate cancer samples. Patient transcriptional profiles were classified as PKCζ-KD–like or PKCζ-like based on the correlation between the expression levels of signature genes for a given sample and their average expression levels in PKCζ-KD and PKCζ-WT samples, respectively. If the Pearson's correlation between the expression levels of signature genes in a given patient sample and their average expression level in PKCζ-KD samples was higher, the sample was classified as PKCζ-KD–like, and vice versa. The statistical significance of the association between PKCζ-KD–like samples and disease status was assessed using Fisher's exact test. To correlate differentially expressed genes with Myc regulatory targets, we downloaded the dataset with Myc-binding peaks in mouse embryonic stem cells from the GEO database (GSE11431) (12). Gene-specific Myc cumulative binding scores (CBSs) were calculated as the weighted sum of peak intensities within a 2-Mb window (−1 Mb, +1 Mb) around the transcriptional start site (TSS) (13). Weights used were a function of the distance of the peak from the TSS. To calculate weights in an unbiased fashion, we approximated the distribution of distances for all peaks by a mixture of exponential and uniform random variables. Distances of random binding events were assumed to be distributed uniformly throughout the 2-Mb window, whereas distances of "functional" binding peaks were assumed to be distributed as the exponential random variable. Thus, each peak was associated with a weight equal to the probability of it belonging to the exponential component of the mixture model. The differences in the distribution of CBSs for differentially expressed and nondifferentially expressed genes were assessed using the Kolmogorov–Smirnov test. The subsequent analysis was done for human array GSE21034 from the GEO database (National Center for Biotechnology Information). For 103 differentially expressed mouse genes (PKCζ-KD vs. PKCζ-WT condition), we found orthologous human genes in the GSE21034 array. We obtained 83 human genes that are orthologous to mouse genes differentially expressed between KD vs. Control conditions. The GSE21034 array data were then log2 transformed and quantile normalized in Partek Genomics Suite (GS). Using these 83 human genes, we developed a predictive model of human disease (Partek GS). For variable selection, the ANOVA method was used; as the classification methods, we used K-nearest neighbor, nearest centroid, and discriminant analysis. To generate variables, we used multiple groups with sizes from 1 to 20 variables. One-level cross-validation was used to evaluate multiple models and pick the best model. Two-level cross-validation was used to get the accuracy estimate. Accuracy was estimated to be 88% (on 80 models, with no preselected variables—each time ANOVA selects variables from the whole set). Twenty models produced correct rates ranging from 86 to 90%. The best model had only two variables and it used linear discriminant analysis with equal prior probability as a classification method. This predictive model identified c-Myc. Analysis of PTEN and PKCζ correlations were done in the human prostate cancer dataset (GEO GSE6919) as above. The statistical significance of differences in PTEN expression levels was assessed using one-way ANOVA of PTEN expression levels on a log scale. The statistical significance of ratios between PKCζ expression levels in primary and metastatic tumors for different PTEN expression cutoff levels was assessed by *t* test comparisons of expression levels on the log scale. All analyses were performed using R software and Bioconductor packages.

Tail Vein Injections of Human Prostate Cancer Cells. DU145 cells were infected with pWZL-Blast Myc, pWZL-Blast Myc S373A, or pWZL-Blast Myc S373E. A total of 5×10^6 cells in 200 μL of DMEM containing 10% (vol/vol) FCS were administrated to 6-wk-old male SCID mice via tail vein injection. After 10 wk, the lungs were collected, fixed, and stained with H&E.

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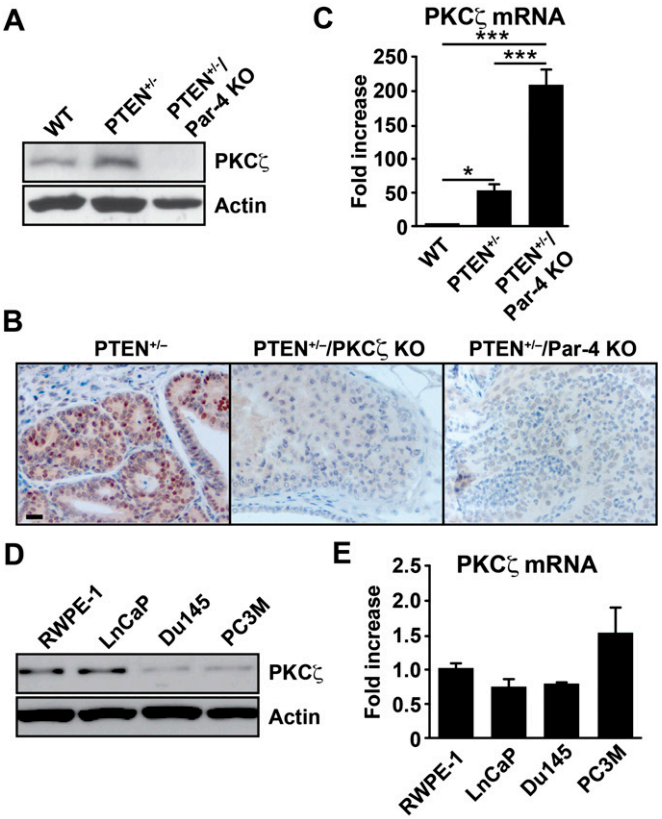


Fig. S1. PKCζ protein and mRNA levels in prostate cancer (PCa). (*A*) Immunoblot analysis of PKCζ levels in prostate tissue from mice of the indicated genotypes. (*B*) Immunostaining to detect PKCζ in prostate sections of mice of the indicated genotypes. (Scale bar, 20 μm.) (*C*) Quantification of PKCζ mRNA levels by qRT-PCR in prostate tissue of the indicated genotypes. Results are shown as mean ± SD $n = 3$. $^{*}P < 0.05$; $^{***}P < 0.001$. (*D*) Immunoblot analysis of PKCζ levels in lysates of human PCa cell lines. (*E*) Quantification of PKCζ mRNA levels in the same PCa cell lines as in *D*. Results are shown as mean ± SD $n = 3$.

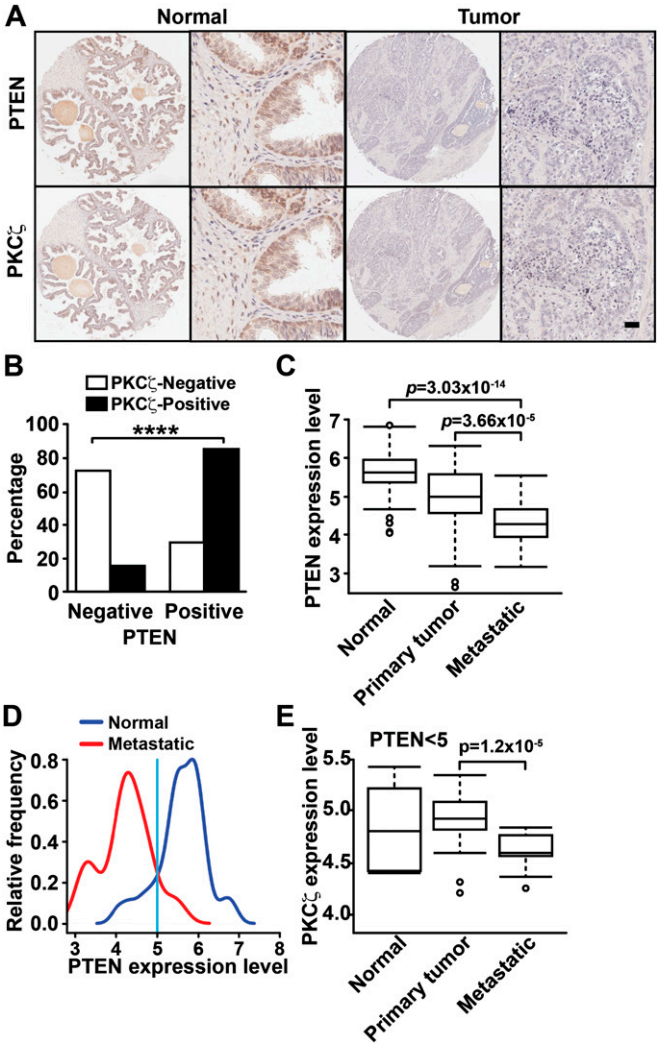


Fig. S2. PKCζ is a tumor suppressor in human PCa in the context of PTEN deficiency. (*A*) Representative examples of PTEN and PKCζ staining of normal prostate and a high Gleason score PCa (tumor). (Scale bar, 20 μm.) (*B*) Correlation of PTEN and PKCζ protein expression in human PCa tissue microarray (TMA). $n = 161$. $^{****}P < 0.001$ (Fisher's exact test). (*C*) Analysis of PTEN mRNA expression levels in human PCa dataset (GEO GSE6919). $n = 164$. (*D*) Cutoff levels for PTEN expression sorted in increasing order. (*E*) Analysis of PKCζ mRNA levels in the same PCa samples as above (*C*) with PTEN mRNA levels below cutoff (PTEN < 5).

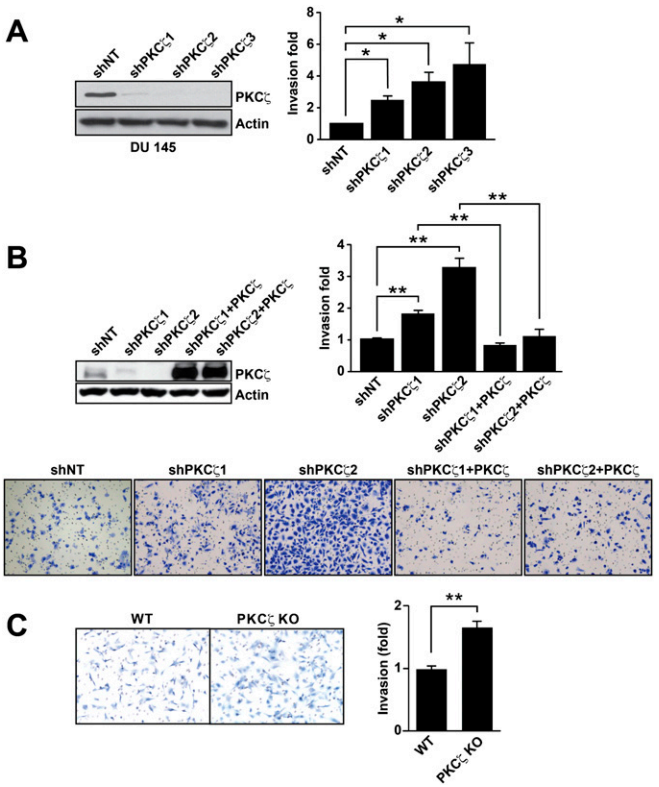
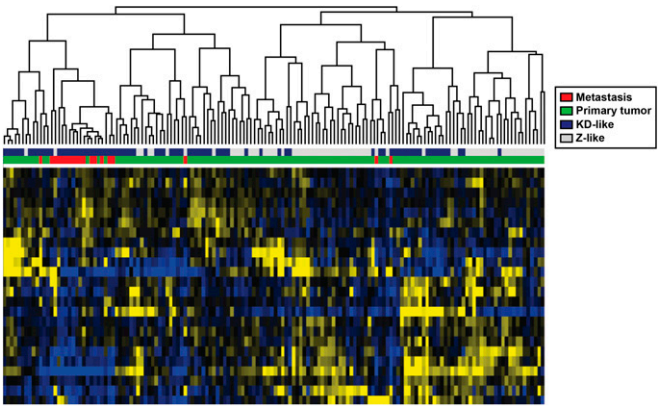


Fig. S3. Role of PKCζ in PCa cell invasion. (*A*) PKCζ expression levels (*Left*) and invasion (*Right*) of DU145 cells infected with three different lentiviral vectors for PKCζ. (*B*) Immunoblot analysis of PKCζ expression levels (*Left*), and invasion (*Right* and *Lower*) of DU145 cells infected with two different shRNAs for PKCζ and reexpression of shRNA resistant PKCζ cDNA. (*C*) Cell invasion of embryo fibroblasts from WT or PKCζ KO mice. Results are shown as mean ± SEM $n = 3$. $*P < 0.05$; $**P < 0.01$.



	Tumor	Metastasis
PKCζ-KD-like	80% (65)	20% (16)
PKCζ-like	96% (66)	4% (3)

p< 0.01

Fig. S4. Bioinformatic analysis of differentially expressed genes regulated by PKCζ. Unsupervised patient clustering analysis of the human PCa dataset GSE21034 using genes with FDR <0.01 and fold change higher than three in PKCζ-KD vs. PKCζ-WT gene signature, comparing metastases with primary tumors. Patient signatures were classified as PKCζ-KD–like or PKCζ-like based on whether the expression levels of signature genes were similar to PKCζ-KD or PKCζ-WT mice. If the expression levels of signature genes in a patient sample correlated better with PKCζ-KD samples, the sample was classified as PKCζ-KD–like, and vice versa. The association between such classification and the disease status of the sample (primary tumor vs. metastasis) was statistically significant (Fisher's exact test $P < 0.01$).

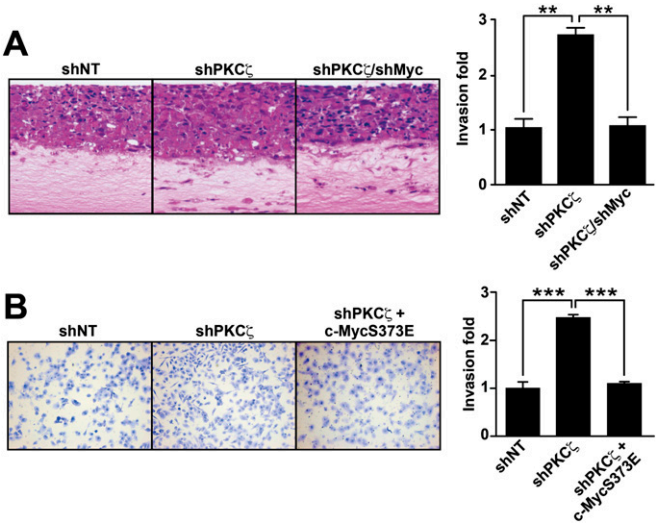


Fig. S5. Role of PKCζ and c-Myc phosphorylation in PCa cell invasion. (*A*) Organotypic cell invasion of PC3 cells infected with the corresponding lentiviral vectors. Results (*Right*) are shown as mean ± SD ($n = 3$ $^{**}P < 0.01$). (*B*) Invasion of DU145 cells infected with the corresponding lentiviral vectors. Results (*Right*) are shown as mean ± SEM ($n = 3$ $^{***}P < 0.001$).