Supporting Information

PTEN arginine methylation by PRMT6 suppresses PI3K-AKT signaling and modulates pre-mRNA splicing

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SI Experimental Procedures

Mass-spectrometry Analysis. The purified protein in solution was digested with sequencing grade trypsin (Promega) overnight at 37 °C. After protein digestion, peptides were separated online with the Easy nLC 1000 system (Thermo Fisher Scientific) which was equipped with a C18 reverse phase column. The column was eluted with a linear gradient of 5-32% acetonitrile in 0.1% formic acid at a rate of 300 nL/min for 50 min. Mass spectra were acquired with a LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) equipped with a nanoelectrospray ion source (Proxeon Biosystems). The twenty most intense ions per scan were selected for collision induced dissociation (CID) fragmentation in linear ion trap. All raw files were processed with the Proteome Discoverer software package (Version 1.4.1.14). Raw files were searched against the UniProt human protein sequence database (released 2018.01). The search parameters were set as follows: the enzyme was trypsin; up to two missed cleavages; carbamidomethyl cysteine as a fixed modification; oxidation methionine, methyl lysine, dimethyl lysine, trimethyl lysine, methyl glutamate, methyl Aspartic acid, methyl arginine and dimethyl arginine as variable modifications. MS

tolerance was 10 ppm while MS/MS tolerance was 0.5 Da.

Cell Culture and Antibodies. HEK293FT cells were obtained from Thermo Fisher Scientific; Human cancer cell lines U2OS, U-87 MG, H4 and PC-3 were obtained from the American Type Culture Collection (ATCC); SF763 cells were obtained from Academic Lab. *PTEN*$^{-/-}$ U2OS cells were described previously (1). All cell lines were maintained according to recommendations from their sources. Antibodies which were used included anti-AKT (#9272), anti-pAKT (S473) (#4060), anti-mono-methylated arginine motif (α-MMA, #8015), anti-symmetrical dimethylated arginine motif (α-sDMA, #13222) and anti-asymmetrical dimethylated arginine motif (α-aDMA, #13522) from Cell Signaling Technology; anti-PTEN (sc-7974) and anti-PTEN agarose resin (sc-7974 AC) from Santa Cruz; anti-FLAG and anti-GAPDH from TransGen Biotech; and anti-PRMT6 (ab190902) from Abcam. The PTEN R159me2a antibody was generated by Willget Biotech Co., Ltd. (Shang Hai). The peptide CDFYGEVR^{me2a}TRDKK was synthesized and utilized as an antigen for rabbit immunization. The anti-serum was first immune-depleted with the unmethylated peptide CDFYGEVRTRDKK, and was then affinity-purified by use of the antigen

peptide.

Plasmids. The lentivirus overexpression vector was obtained from System Biosciences and modified in our laboratory. The EcoR I- Kozak sequence (GCCACC)-HA-S-tag-PTEN-Not I (the HA-S-tag-PTEN fragment was from pSA-N-ter-PTEN) and the EcoR I- Kozak sequence (GCCACC)-FLAG-PRMTs-Not I (the FLAG-PRMT fragments were from pCMV-tag2B-PRMTs) were generated by overlap PCR and were inserted into the pCDH-CMV-MCS-EF1-Puro plasmid (from System Biosciences). The pSA-N-ter plasmid was a kind gift from Dr. Y. Huang (SUNY Upstate Medical University) and was described previously (1). The pCMV-tag2B plasmid was obtained from Stratagen. pFastBac1 (Invitrogen) was used for insect expression of His-PRMT6 and His-PTEN. Mutants were created by one step PCR followed by Dpn I digestion.

S-protein Pulldown, Immunoblotting, Immunoprecipitation. For S-protein pulldown, cells were lysed in NP-40 lysis buffer as described previously (1) at 4 °C for 30 min. Lysates were incubated with S-protein beads at 4 °C for 2 hours. S-protein beads were washed 5 times with NP-40 lysis buffer and boiled with 40 μl 2 x SDS-PAGE loading buffer for 10 min. For immunoblotting, whole cell extracts were

obtained by lysing cells in RIPA buffer as described previously (1) at 4 °C for 30 min. For immunoprecipitation, cells were lysed in NP-40 lysis buffer as described previously (1) at 4 °C for 30 min. Lysates were incubated with indicated antibody at 4 °C for 6 hours, and 30 μl Protein A/G (Biochem) was added followed by rotation at 4 °C for 1 hour. Protein A/G beads were washed 5 times with NP-40 lysis buffer and boiled with 40 μl 2 x SDS-PAGE loading buffer for 10 min. Protein concentrations were measured with the BCA kit (Thermo Scientific Pierce).

PTEN Enzyme Reactions. According to the manufacturer's protocol for diC8-PIP3 (Echelon), wild-type or mutant PTEN proteins were precipitated with S-protein beads, and the beads were washed 3 times with NP-40 lysis buffer with 500 mM NaCl. The washed beads were resuspended in PTEN reaction buffer (25 mM Tris-HCl pH 7.4, 140 mM NaCl, 2.7 mM KCl and 50 mM β-Mercaptoethanol), and incubated with diC8-PIP3 at 37 °C for 2 hours. Alternatively, we purified wild-type and mutant PTEN protein from SF9 insect cells and used 150 ng of purified protein in each PTEN lipid phosphatase reaction. Free phosphate in these reactions was evaluated with Green Reagent (Biomol). Peptide substrate phosphorylated polyGluTyr (4:1) was prepared

based on previous studies (2, 3). Briefly, 1 mg of polyGluTyr (4:1) (Sigma) was incubated with 4 μg of purified insulin receptor kinase domain at 32 °C for 1 hour before incubation with addition of 4 μg further kinase for another hour. The phosphorylated polyGluTyr (4:1) peptide was purified by size exclusion chromatography (Superdex 200) and the concentration of the purified peptide was determined by use of theoretical molar extinction coefficients at 280 nm. 2 μg of wild-type or mutant PTEN protein purified from SF9 insect cells was incubated with 4 μg of phosphorylated polyGluTyr (4:1) peptide in PTEN reaction buffer at 37 °C for 2 hours. Free phosphate in these reactions was evaluated with Green Reagent (Biomol).

In Vitro **Methylation Assay.** His-PRMT6 and His-PTEN were purified from SF9 cells. As described in the figure legends, indicated amounts of His-PRMT6 were incubated in Histone methyltransferase (HMT) buffer (25 mM Tris-HCl pH 8.8, 25 mM NaCl, 2 μM adomet) with indicated amounts of His-PTEN. The reaction systems were incubated at 37 °C for 2 hours.

CRISPR-based Gene Editing. LentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid # 52961). The procedure for target guide sequence cloning has been

described previously (4) and is provided on the Addgene website. After infection with the lentivirus, cells were selected with puromycin for more than 5 days. To confirm the *PRMT6* genomic editing, cell pools were analyzed with PCR and western blotting.

Mouse Xenograft. Cells as indicated (5 x 10^6/100 μl) were injected subcutaneously into nude mice (Balb/c Nude; female, 6 weeks of age). Six weeks after inoculation, mice were euthanized and tumor weight was measured.

RNA Sequencing Library Preparation. A total amount of 3 μg RNA per sample was used for the RNA sample preparations. Sequencing libraries were generated using NEBNext UltraTM RNA Library Prep Kit (NEB, USA), following the manufacturer's recommendations. In order to preferentially select cDNA fragments of 250~300 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). 3 μl USER Enzyme (NEB, USA) was incubated with processed cDNA at 37 ºC for 15 min followed by incubation at 95 ºC for 5 min. PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. PCR products were purified with the AMPure XP system and library quality was measured on the Agilent Bioanalyzer 2100 system. All of these experiments were

performed at the Novogene Bioinformatics Institute, Beijing, China.

RNA Sequencing and Analysis of Alternative Splicing Events. 125 bp/150 bp paired-end reads were sequenced on an Illumina Hiseq platform. rMATS software (3.2.1 beta) (http://rnaseq-mats.sourceforge.net/) was used to determine differentially expressed splicing events in pairwise comparisons (5). Splicing events were considered to be significant if the IncLevelDifference index was >0.2 or < -0.2, and FDR<0.01.

Gene Ontology Term Analysis. Functional annotations regarding biological processes were analyzed using the ClueGO version 2.3.4, and then visualized using the Cytoscape version 3.5.1 (http://cytoscape.org/).

Coexpression Analysis. There are 220 known or putative splicing factors as reported in a previous study (6). We filtered the TCGA GBM expression dataset into three groups, including [1] a group of normal tissues and tissues adjacent to GBM, [2] a PTEN-proficient group containing samples with the highest 20% of wild-type PTEN expression levels (based on the FPKM values) and [3] a *PTEN*-deficient group containing samples with the lowest 20% of wild-type PTEN expression levels, and samples with deep deletion or truncation or mutations of the PTEN gene. The TCGA

sample IDs in these 3 groups are shown in Dataset S7. The 220 splicing factors and the TCGA expression dataset of indicated samples were used for coexpression analysis. Correlation coefficients (R value) and corresponding P values for these genes were then calculated with the Pearson method. The Pheatmap (version 1.0.10, R version 3.5.0) was used to draw the heatmap. The correlation matrices were ordered by hierarchical clustering and the clustering distance was determined by the Correlation method.

References

1. Feng J, *et al.* (2015) PTEN Controls the DNA Replication Process through MCM2 in Response to Replicative Stress. *Cell Rep* 13(7):1295-1303.
2. Swarup G & Subrahmanyam G (1989) Purification and characterization of a protein-phosphotyrosine phosphatase from rat spleen which dephosphorylates and inactivates a tyrosine-specific protein kinase. *J Biol Chem* 264(14):7801-7808.
3. Leslie NR, Yang X, Downes CP, & Weijer CJ (2007) PtdIns(3,4,5)P(3)-dependent and -independent roles for PTEN in the control of cell migration. *Curr Biol* 17(2):115-125.
4. Sanjana NE, Shalem O, & Zhang F (2014) Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* 11(8):783-784.
5. Shen S, *et al.* (2014) rMATS: robust and flexible detection of differential alternative splicing from replicate RNA-Seq data. *Proc Natl Acad Sci U S A* 111(51):E5593-5601.
6. Han H, *et al.* (2013) MBNL proteins repress ES-cell-specific alternative splicing and reprogramming. *Nature* 498(7453):241-245.

Supplemental Figures

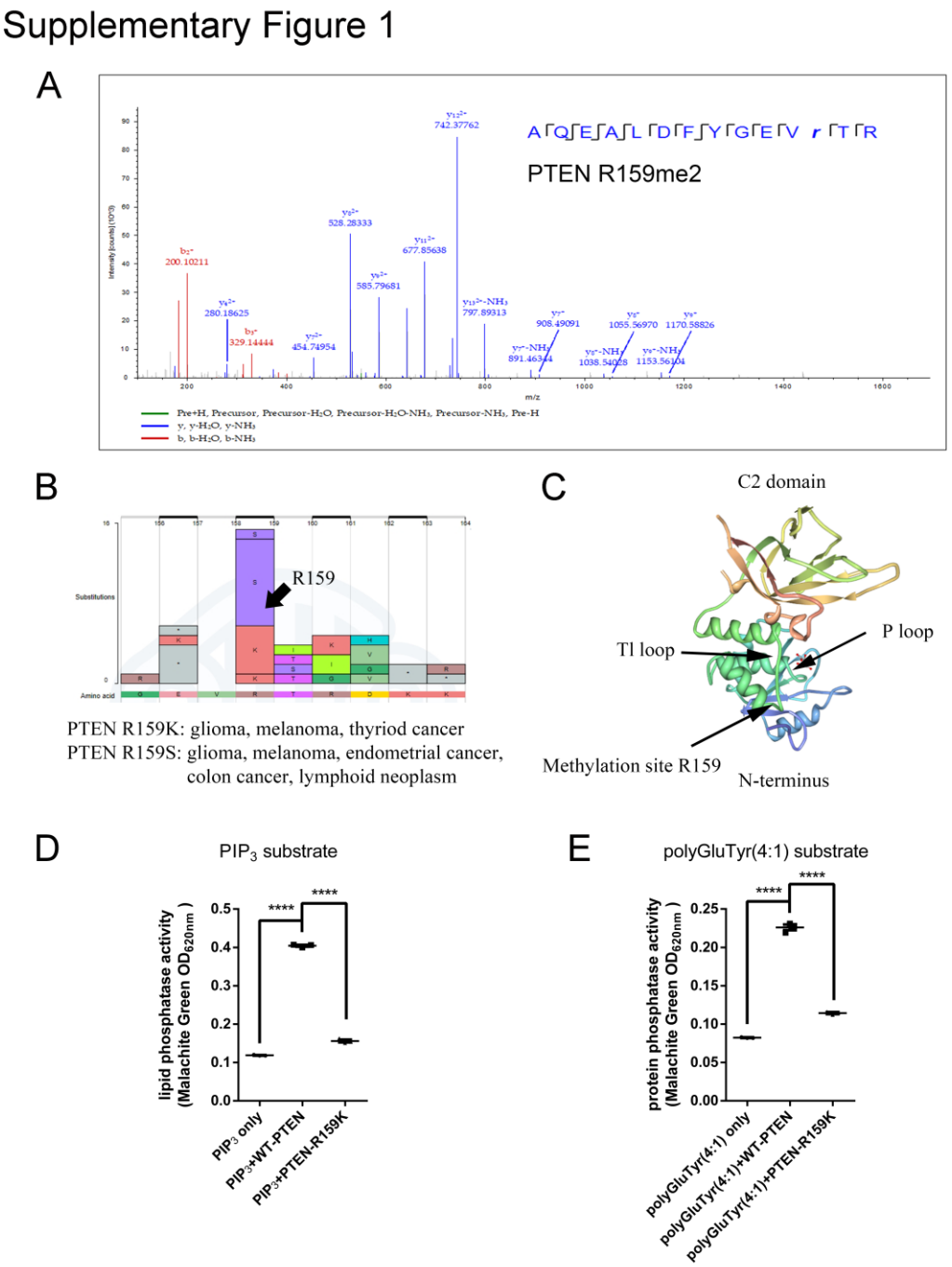

Figure S1. Analysis of the methylation site of PTEN. (A) Ectopic expression of exogenous FLAG-tag-PTEN in HEK293FT cells. FLAG-tag-PTEN protein was immunoprecipitated with an anti-FLAG M2 affinity gel (sigma, #A2220), then

eluted with 0.1 M glycine HCl, pH 3.5, and then neutralized with Tris-HCl, pH 8.8. Mass-spectrometry analysis showed that PTEN R159 was dimethylated. (B) Data from the COSMIC cancer database showed PTEN R159 is mutated in different types of human cancer. (C) The PTEN R159 residue resides in the PTEN phosphatase domain. Black arrows indicate the location of the P loop (activity center), the TI loop and the R159 residue (Methylated site). (D, E) Wild-type PTEN and PTEN R159K proteins were purified from SF9 insect cells and subjected to PTEN enzyme reactions. (D: PIP_3 substrate; E: phosphorylated polyGluTyr peptide substrate). OD_{620nm} reflects the quantity of PO_4 released from these reactions. Data were analyzed with the paired two-tailed Student's test. Error bars indicate SEM (n=3). ****p<0.0001.

Supplementary Figure 2

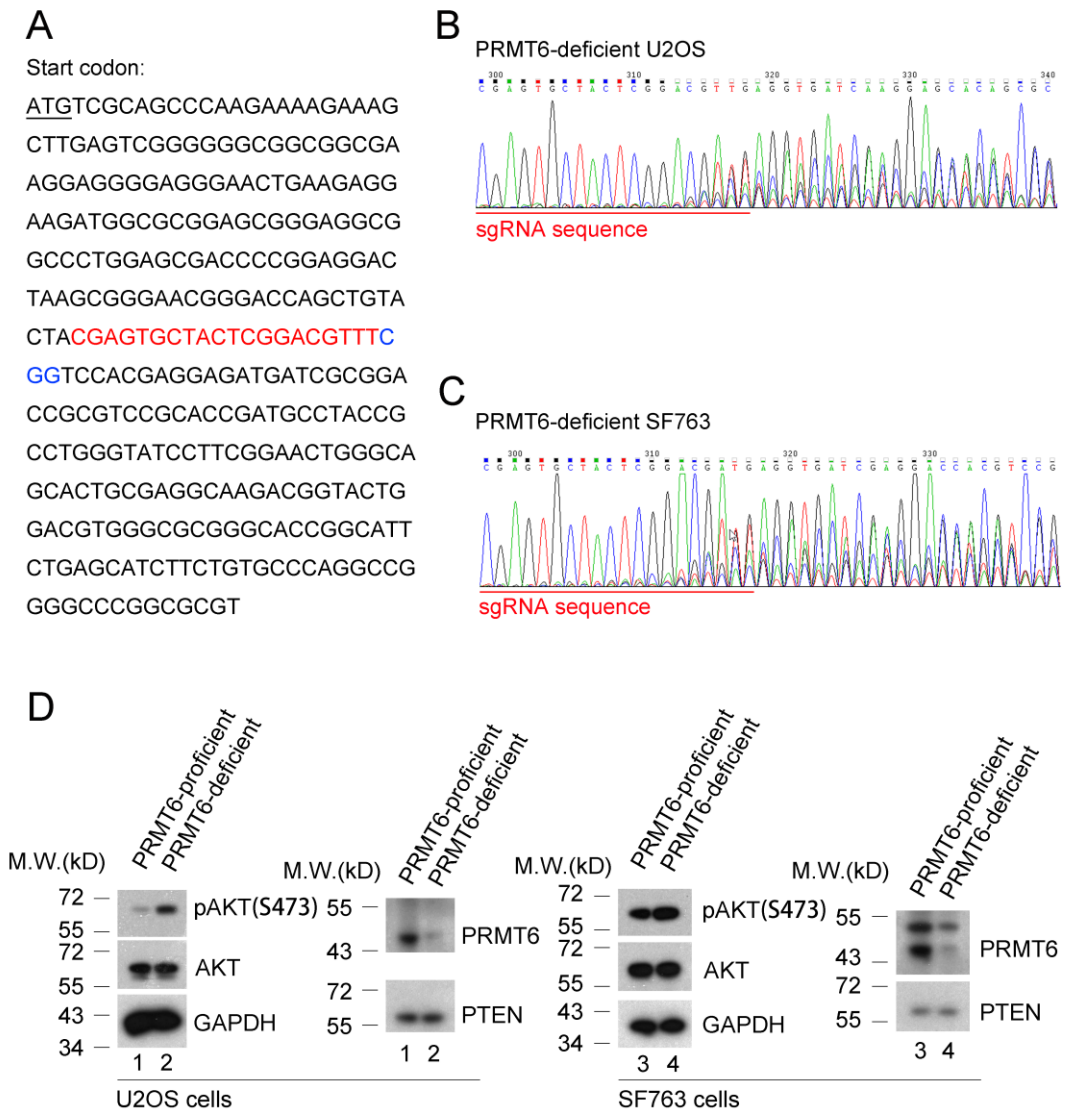

Figure S2. PRMT6 deficiency leads to activation of PI3K-AKT signaling. (A) Sequence initiated by the ATG start codon of PRMT6 EXON 1. The sgRNA sequence for CRISPR targeting is labeled in Red and the NGG PAM is labeled in Blue. (B, C) PTEN-proficient U2OS and SF763 cells were infected with the

lentiCRISPR v2-sgPRMT6 lentivirus. After selection with puromycin, genomic DNA derived from cell pools was used as a template for PCR of the targeted region of the PRMT6 genome. DNA sequencing data in (B) and (C) show gene targeting was achieved. (D) pAKT (S473) levels were analyzed in *PRMT6*-deficient cells and *PRMT6*-proficient cells as indicated. The expression levels of PRMT6 and total AKT were analyzed. GAPDH served as a loading control.

Supplementary Figure 3

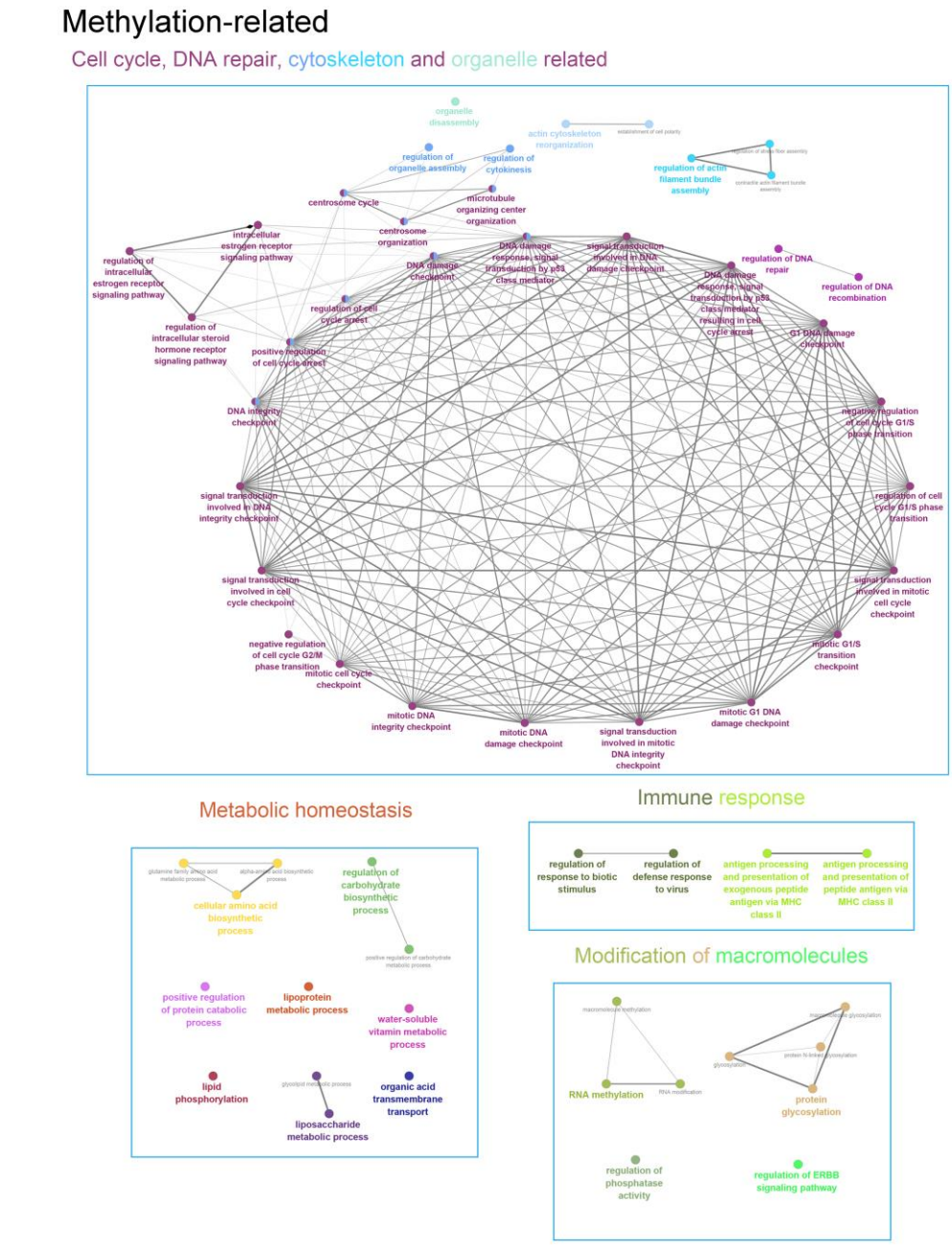

Figure S3. Gene ontology of alternative splicing products regulated by PTEN in a methylation-related manner.

Supplementary Figure 4

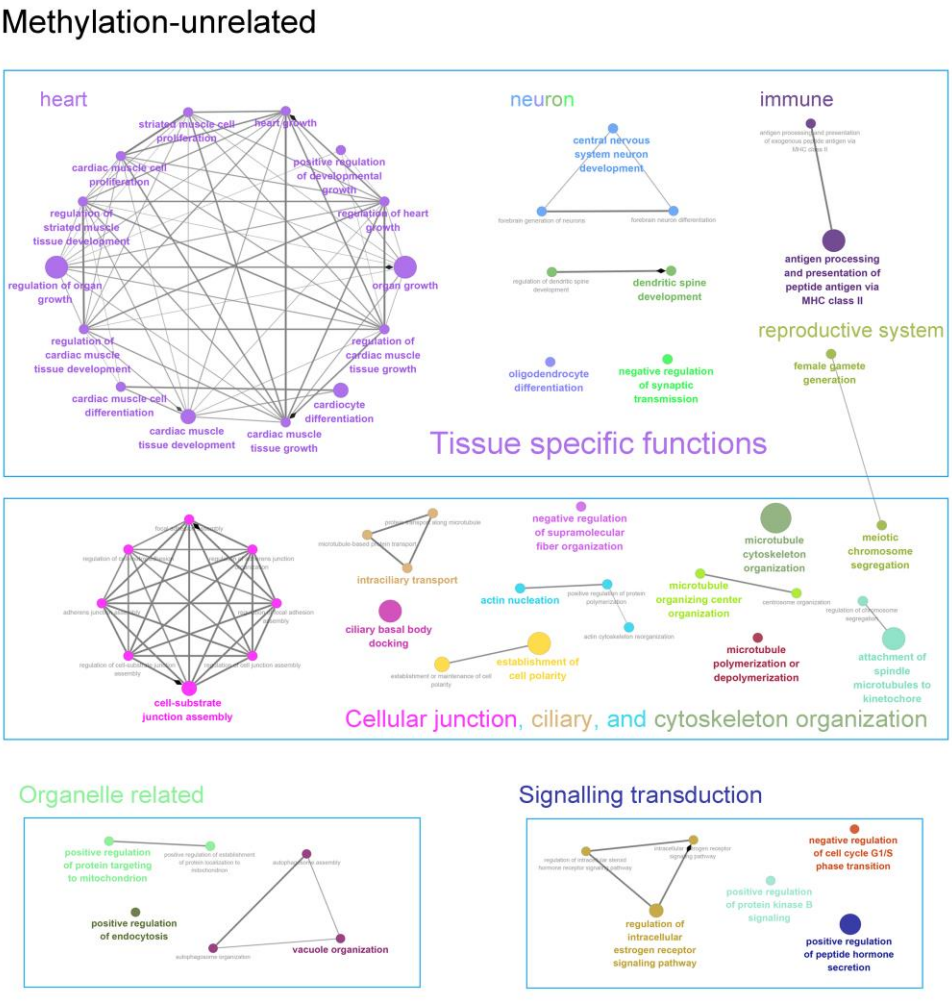

Figure S4. Gene ontology of alternative splicing products regulated by PTEN in a methylation-unrelated manner.

Supplementary Figure 5

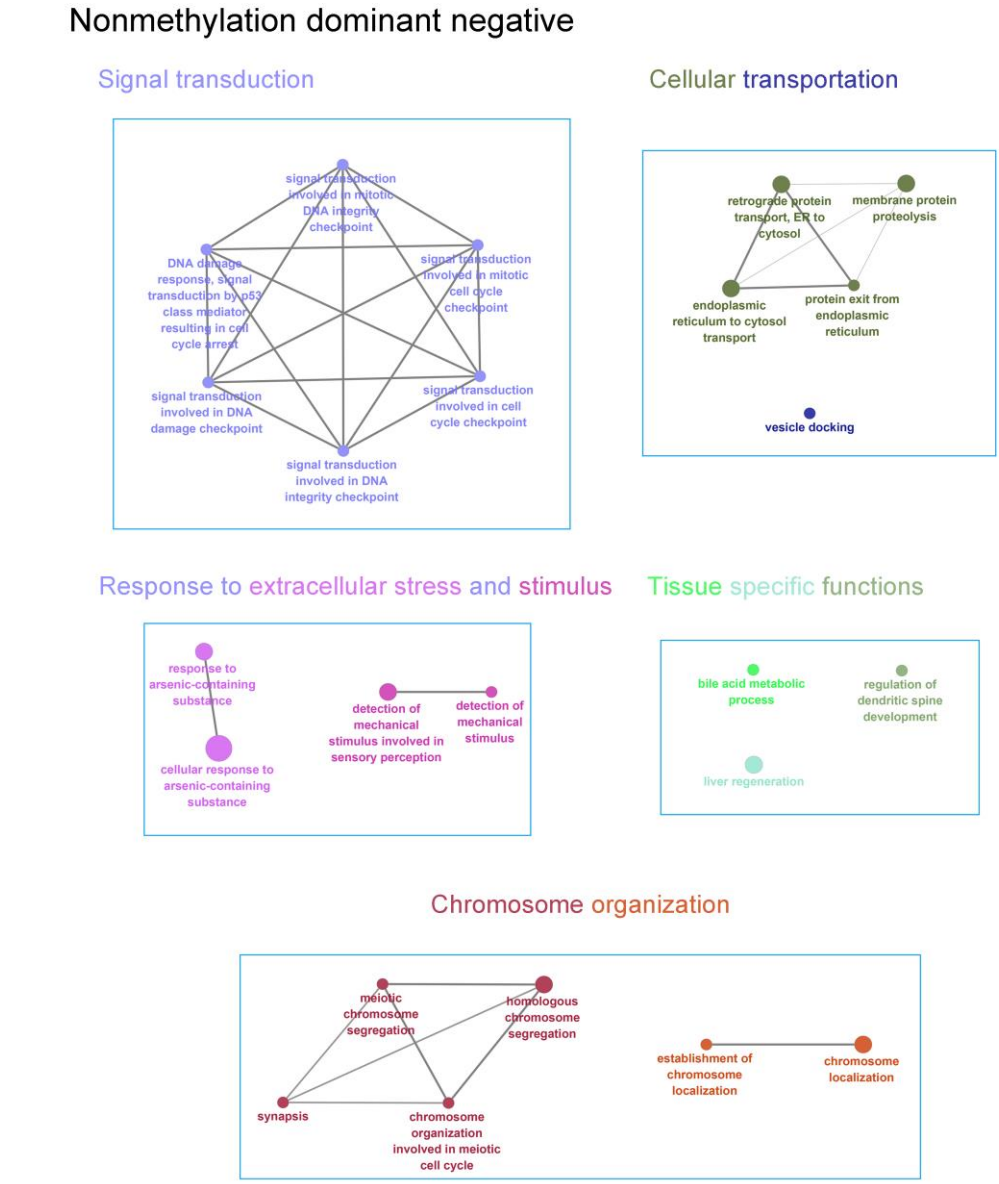

Figure S5. Gene ontology of alternative splicing products regulated by PTEN in a nonmethylation dominant negative manner.

Supplementary Figure 6

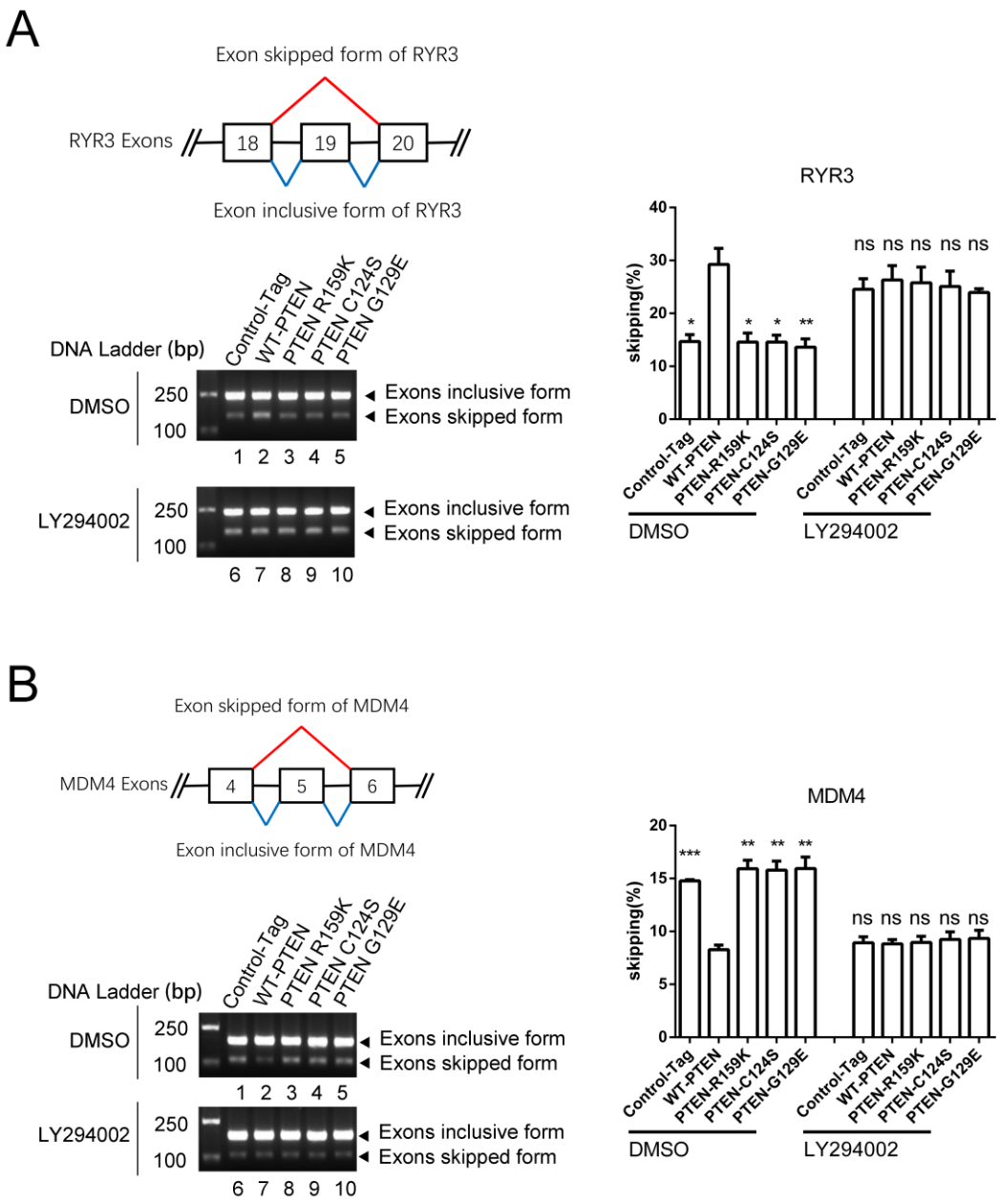

Figure S6. PTEN methylation regulates alternative spliced RYR3 and MDM4 by suppression of PI3K-AKT signaling. *PTEN*-null H4 cells expressing S-tag control (Control-Tag), wild-type PTEN (WT-PTEN), PTEN R159K, PTEN C124S or PTEN G129E were treated with DMSO or PI3K inhibitor LY294002, and

subjected to analysis of alternative splicing events by semi-quantitative PCR. (A) Schematic model of alternatively spliced RYR3 in the left upper panel. Representative result of analysis of alternatively spliced RYR3 in the left lower panel. Quantitative analysis is shown in the right panel. (B) Schematic model of alternative spliced MDM4 in the left upper panel. Representative result of analysis of alternative spliced MDM4 in the left lower panel. Quantitative analysis is shown in the right panel. Data were analyzed with the unpaired two-tailed Student's test. Error bars indicate SEM (n=3). ns, no significant difference, *p<0.05, **p<0.01, ***p<0.0001

Supplementary Figure 7

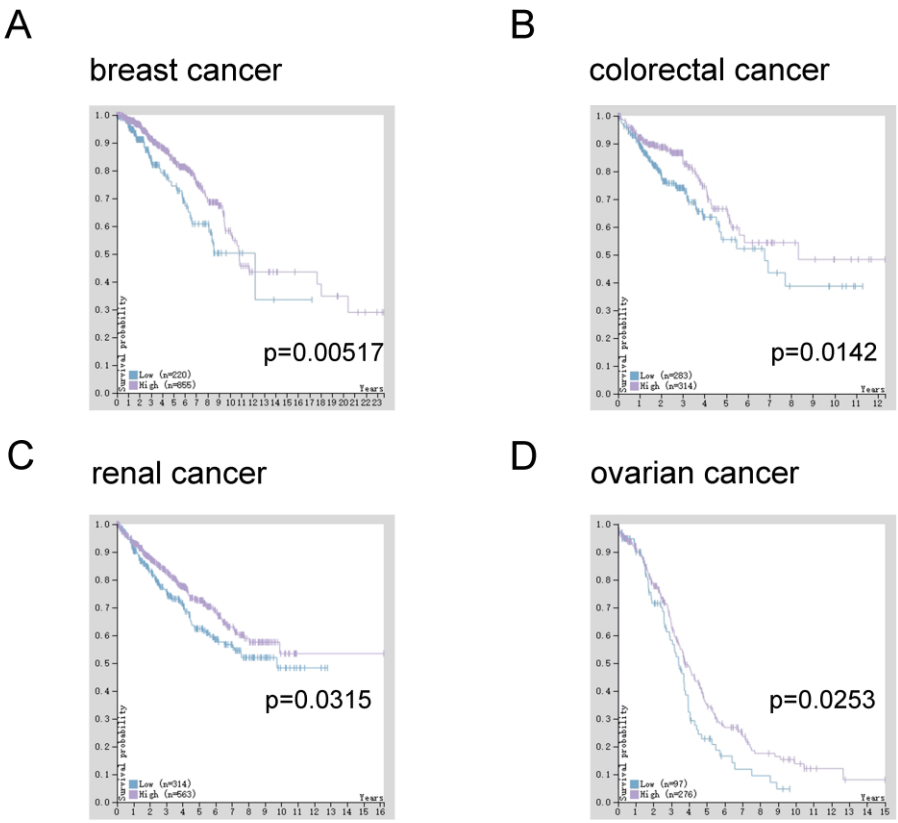

Figure S7. High expression of PRMT6 predicts better overall survival in multiple types of cancer. (A-D) Data from the Human Protein Atlas website (http://www.proteinatlas.org/). Kaplan-Meier survival analysis in breast cancer (A), colorectal cancer (B), renal cancer (C) and ovarian cancer (D). Log-rank *p* values are shown.

Supplementary Figure 8

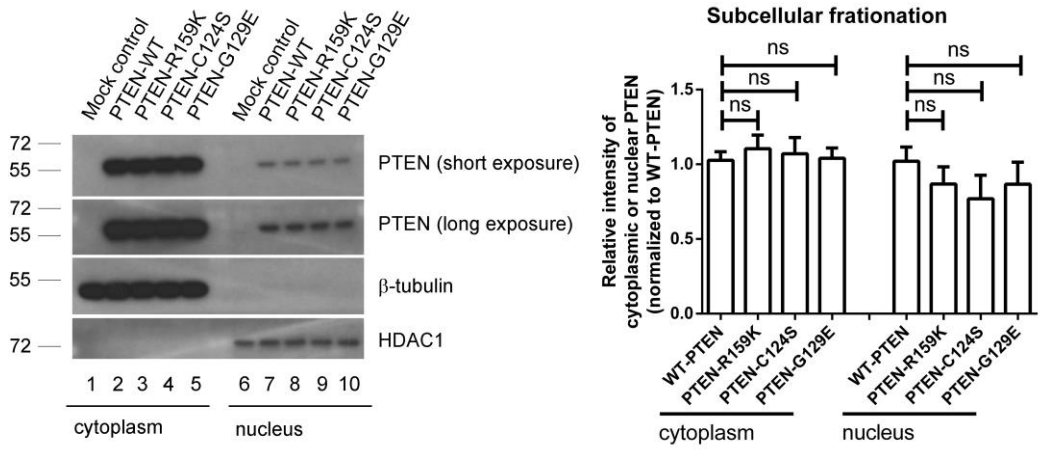

Figure S8. The protein level of the PTEN R159K mutant is similar to that of wild-type PTEN and the PTEN C124S and G129E mutants in cytoplasm or nucleus. PTEN protein levels were detected in cytoplasmic and nuclear extracts from *PTEN*$^{-/-}$ U2OS cells with re-expression of Control S-tag, wild-type PTEN (PTEN-WT), PTEN R159K, PTEN C124S or PTEN G129E. β-tubulin served as a loading control in the cytoplasmic extracts and HDAC1 served as a loading control in the nuclear extracts. Representative results are shown in the left panel. Quantitative analysis is shown in the right panel. Data were analyzed with the unpaired two-tailed Student's test. Error bars indicate SEM (n=3). ns, no significant difference.

Other Supporting Information Files

Dataset S1. Differentially expressed alternative splicing events in pairwise comparisons of PTEN vs. Control.

Dataset S2. Differentially expressed alternative splicing events in pairwise comparisons of PTEN R159K vs. PTEN.

Dataset S3. Differentially expressed alternative splicing events in pairwise comparisons of PTEN R159K vs. Control.

Dataset S4. Alternative splicing events regulated by PTEN in methylation-related manner.

Dataset S5. Alternative splicing events regulated by PTEN in methylation-unrelated manner.

Dataset S6. Alternative splicing events regulated by PTEN in nonmethylation dominant negative manner.

Dataset S7. Sample IDs of 3 groups for coexpression analysis.