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Supplemental Information

Phosphorylation and Ubiquitination Regulate Protein Phosphatase 5 Activity and Its Prosurvival Role in Kidney Cancer

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Table S1. Cellular proteins associated with PP5. Related to Figure 3.

Protein	MW	Protein Score	Protein Score C.I.%	Total Ion Score	Total Ion C.I.%	Peptides	Accession Number
Serine/threonine-protein phosphatase 5-PP5	56842.2	95	100	80	100	FYSQAIELNPSNAIYYGN TECYGYALGDATR AFLEENNLDYIIR AEGYEVAHGGR AASNMALGKFR TQANDYFK	gi|324021715
Actin, cytoplasmic 2 -ACTG1	41765.8	351	100	303	100	TTGIVMDSGDGVTHTVPIYEGYALPHAILR KDLYANTVLSGGTTMYGPADR DLYANTVLSGGTTMYPGADR VAPEEHPVLLTEAPLNPK SYELPDGQVITIGNER QEYDESGPSIVHR AVFPSIVGRPR AGFAGDDAPR GYSFTTTAER DLTDYLMK	gi|823672677
Vimentin	53619.1	49	72	29	98	VEVERDNLAEDIMRLREK STRSVSSSSYRRMFGGP SLYASSPGGVYATR ISLPLPNFSSLNLR LGDLYEEEMR QDVDNASLAR GTASRPSSSR	gi|340218
Actin, cytoplasmic 1 -ACTB	41709.7	77	100	50	100	MDDDIAALVVDNGSGMC MTQIMFETFNTPAMYVAI VAPEEHPVLLTEAPLNPK SYELPDGQVITIGNER QAVLSLYASGR AGFAGDDAPR GYSFTTTAER DLTDYLMK	gi|823671251
Von Hippel-Lindau disease tumor suppressor-VHL	24137.9	5	5	42	94	HGIADLFR	gi|319655736

Table S2. Primer table.
Restriction sites are underlined, mutated sequences are in red, epitope sequences are in blue, PreScission protease site is in green. Related to Figures 1-6.

Primer	Sequence
Ppt1-Xho1-F(Nat-prom)	ACCTTGGCTCGAGACGAATATGTATTTTATTTTA
Ppt1- SpeI-**His_6**- R	GGTTATCACTAGTCTA**ATGATGATGATGATGATG**TAAACCAAAACCACCATTAG
Hrr25-HindIII-F	GGACCTGAAGCTTATGGACTTAAGAGTAGGAAGGAAA
Hrr25- XhoI-**cMyc**- R	AGTGCTCTCGAGTT**AAAACTCTAAATCTTCTTCGGAGATTAACTTTTGTTCC**AACCAAATTGACTGGCCAGCTGG
PP5- Kpn1-**FLAG**	TATGCGGTACC**ATGGATTACAAGGATGACGATGACAAGGGA**GCGGAGGGCGAGAGGACTGAGTGTG
PP5-Xho1-R	GGATCGTCTCGAGTCACATCATTCCTAGCTGCAG
PP5-Nde1-**His_6**-**PreScission**-F	GTAGTCATATGATG**CACCATCATCACCATCATCTGGAAGTTCTGTTCCAGGGGCCC**GCGGAGGGCGAGAGGACTGAGT
PP5-T33A-F	AGCGGGCAGAGGAGCTCAAGGCTCAGGCCAATGACTACTTCAA
PP5-T33A-R	TTGAAGTAGTCATTGGCCTGAGCCTTGAGCTCCTCTGCCCGCT
PP5-T121A-F	CCGCGCTGCGAGACTACGAGGCTGTGGTCAAGGTGAAGCCCCA
PP5-T121A-R	TGGGGCTTCACCTTGACCACAGCCTCGTAGTCTCGCAGCGCGG
PP5-T171A-F	CGCTGGACATCGAGAGCATGGCTATTGAGGATGAGTACAGCGG
PP5-T171A-R	CCGCTGTACTCATCCTCAATAGCCATGCTCTCGATGTCCAGCG
PP5-T238A-F	TCAAAGAGACAGAGAAGATTGCTGTATGTGGGGACACCCATGG
PP5-T238A-R	CCATGGGTGTCCCCACATACAGCAATCTTCTCTGTCTCTTTGA
PP5-T362A-F	TGTTCAGTGAAGACGGTGTCGCTCTGGATGACATCCGGAAAAT
PP5-T362A-R	ATTTTCCGGATGTCATCCAGAGCGACACCGTCTTCACTGAACA
PP5-T362E-F	TGTTCAGTGAAGACGGTGTCGAACTGGATGACATCCGGAAAAT
PP5-T362E-R	ATTTTCCGGATGTCATCCAGTTCGACACCGTCTTCACTGAACA
PP5-K32R-F	TGAAGCGGGCAGAGGAGCTCCGTACTCAGGCCAATGACTACTT
PP5-K32R-R	AAGTAGTCATTGGCCTGAGTACGGAGCTCCTCTGCCCGCTTCA
PP5-K40R-F	CTCAGGCCAATGACTACTTCCGTGCCAAGGACTACGAGAACGC
PP5-K40R-R	GCGTTCTCGTAGTCCTTGGCACGGAAGTAGTCATTGGCCTGAG
PP5-K185R-F	GACCCAAGCTTGAAGACGGCCGTGTGACAATCAGTTTCATGAA
PP5-K185R-R	TTCATGAAACTGATTGTCACACGGCCGTCTTCAAGCTTGGGTC
PP5-K199R-F	AGGAGCTCATGCAGTGGTACCGTGACCAGAAGAAACTGCACCG
PP5-K199R-R	CGGTGCAGTTTCTTCTGGTCACGGTACCACTGCATGAGCTCCT
PP5-K320R-F	ACGGTTTCGAGGGTGAGGTGCGTGCCAAGTACACAGCCCAGAT
PP5-K320R-R	ATCTGGGCTGTGTACTTGGCACGCACCTCACCCTCGAAACCGT
CK1δ- EcoR1-F	GTCAGCATGAATTCATGGAGCTGAGAGTCGGGAACAG
CK1δ- Xho1-**c-Myc**-R	GTTCAGCTCTCGAGTC**AAAACTCTAAATCTTCTTCGGAGATTAACTTTTGTTC**TCGGTGCACGACAGACTGAAGAC
VHL_{30}-HindIII-**FLAG**-F	TATGCGAAAGCTTATG**GACTACAAGGACGACGATGACAAG**CCCCGGAGGGCGGAGAACTGGGACGAGGCCGAGGTA
VHL_{30}-HindIII-**His_6**-F	TATGCGAAAGCTTATG**CATCATCACCACCATCAC**CCCCGGAGGGCGGAGAACTGG
VHL_{30}-Xho1-R	AGTGCGCTCTCGAGTCAATCTCCCATCCGTTGATGT

Table S3. List of reagents and resources. Related to Figures 1-6.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-rabbit Cdc37	StressMarq Biosciences	Cat# SPC-142
Anti-rabbit FLAG tag	Thermo Scientific	Cat# PA1-984B
Anti-mouse 6x-His epitope tag (HIS.H8)	Thermo Scientific	Cat# MA1-21315
Anti-rat Hsp90 (16F1)	Enzo Life Sciences	Cat# ADI-SPA-835
Anti-mouse GAPDH (1D4)	Enzo Life Sciences	Cat# ADI-CSA-335
Anti-rabbit PP5	Cell Signaling Technology	Cat# 2289
Anti-rabbit Phospho-Akt S473 (D9E)	Cell Signaling Technology	Cat# 2289
Anti-mouse Akt (2H10)	Cell Signaling Technology	Cat# 2967
Anti-rabbit GR (D6H2L)	Cell Signaling Technology	Cat# 12041
Anti-rabbit Phospho-GR S211	Cell Signaling Technology	Cat# 4161
Anti-rabbit HA-Tag (C29F4)	Cell Signaling Technology	Cat# 3724
Anti- rabbit myc-Tag (71D10)	Cell Signaling Technology	Cat# 2278
Anti-rabbit CK1δ	Cell Signaling Technology	Cat# 12417
Anti-rabbit CK1ε	Cell Signaling Technology	Cat# 12448
Anti-rabbit HIF1α (D2U3T)	Cell Signaling Technology	Cat# 14179
Anti-rabbit HIF2α (D9E3)	Cell Signaling Technology	Cat# 7096
Anti-rabbit VHL	Cell Signaling Technology	Cat# 68547
Anti-rabbit cleaved caspase-3 (D175) (5A1E)	Cell Signaling Technology	Cat# 9664
Anti-rabbit cleaved PARP (D214)	Cell Signaling Technology	Cat# 9544
Anti-mouse Ubiquitin (P4D1)	Santa Cruz Biotech	Cat# sc-8017
Anti-rabbit Phospho-Cdc37 S13 (EPR4879)	Abcam	Cat# ab108360
Anti-mouse PP5 (2E12)	Abcam	Cat# ab123919
Anti-mouse VHL (3F391)	Abcam	Cat# ab11189
Anti-mouse Phospho-serine (PSR-45)	Sigma–Aldrich	Cat# P5747
Anti-mouse Phospho-threonine (PTR-8)	Sigma–Aldrich	Cat# P6623
Anti-goat GST	GE Healthcare	Cat# 27457701V
Anti-mouse secondary	Santa Cruz Biotech	Cat# sc-2005
Anti-rabbit secondary	Santa Cruz Biotech	Cat# sc-2004
Anti-rat secondary	Santa Cruz Biotech	Cat# sc-2006
Anti-goat secondary	Santa Cruz Biotech	Cat# sc-2020
Chemicals, Peptides, and Recombinant Proteins		
CK1δ-GST	Signalchem	Cat# C65
CK1ε-GST	Signalchem	Cat# C66
CK2α-GST	Signalchem	Cat# C70
Bortezomib	LC Labs	Cat# B-1408; CAS# 179324-69-7

IC261	Abcam	Cat# ab145189(Li et al., 2013)(6)(6)
Critical Commercial Assays		
Mirus TransIT-2020	MirusBio	Cat# MIR5405
PNPP substrate	ThermoFisher Scientific	Cat# 34045
Anti-FLAG M2 affinity gel	Sigma-Aldrich	Cat# A2220 RRID:AB10063035
Protein G agarose	ThermoFisher Scientific	Cat# 15-920-010
Ni-NTA Agarose	ThermoFisher Scientific	Cat# 88221
Experimental Models: Cell Lines		
HEK293	ATCC	Cat# CRL-1573
786-O	ATCC	Cat# CRL-1932
A49-8	ATCC	Cat# HTB-44
Caki-1	ATCC	Cat# HTB-46
Experimental Models: Organisms/Strains		
E. coli/BL21(DE3)	EMD Millipore	Cat# 69450
Yeast/BY4743	EUROSCARF	BY4743
Oligonucleotides		
PP5 specfic 27mer siRNA duplexes	Origene	Cat# SR303702 A- AGAGAACAACCUGGACUAUAUCATC B- GCUCAAGACUCAGGCCAAUGACUAC C- AGCUAGGAAUGAUGUGAGGUGACGG
HIF1α specfic 27mer siRNA duplexes	Origene	Cat# SR302102 A- GGAAGAACUAUGAACAUAAAGUCTG B- ACAAUACCCUAUGUAGUUGUGGAAG C- CCAGCAGACUCAAAUACAAGAACCT
HIF2α specfic 27mer siRNA duplexes	Origene	Cat# SR301415 A- CCUACUGACAACAUUAUAACUGUAT B- GCAGUACCCAGACGGAUUUCAAUGA C- CCUAUUCACCAAGCUAAAGGAGGAG
Universal scrambled negative control siRNA duplex	Origene	Cat# SR30004
Recombinant DNA		
HA-EglN1-pcDNA3	Addgene	Cat# 18963
HA-EglN2-pcDNA3	Addgene	Cat# 18961
HA-EglN3-pcDNA3	Addgene	Cat# 18960

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Yeast Plasmids, Strains and Growth Media

HRR25 was cloned in to 2μ yeast expression plasmid pYES2 and PPT1 including its native promoter was cloned into centromeric yeast plasmid pRS415. The yeast strain BY4743 (MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0) was used to transform and co-express HRR25-cMyc-pYES2 and PPT1-His6-pRS415.

Yeast cells were grown on YPDA (2% (w/v) Bacto peptone, 1% yeast extract, 2% glucose, 20 mg/liter adenine), YPGal (2% (w/v) Bacto peptone, 1% yeast extract, 2% galactose, 20 mg/liter adenine) and YPRaf (2% (w/v) Bacto peptone, 1% yeast extract, 2% raffinose, 20 mg/liter adenine). Selective growth was on dropout 2% glucose (DO) medium with appropriate amino acids (Adams et al., 1997). Medium pH was adjusted to 6.8 with NaOH before autoclaving.

N-terminally FLAG tagged human PP5 mammalian expression plasmid pCDNA3.1 and their mutants were derived using by site-directed mutagenesis (Table S2). PP5 was subcloned into bacterial expression plasmid, pRSET-A, with His6 tag followed by a PreScission protease site at the N-terminal of human PP5. Site-directed mutagenesis was performed to mutate indicated residues (Table S2). C-terminally tagged CK1δ-cMyc was cloned into pcDNA3.1 (Table S2). N-terminally tagged VHL30-FLAG and VHL30-His6 were cloned into pcDNA3.1 (Table S2). VHL mutations were either subcloned from HA-VHL Y98H-pRc/CMV or HA-VHL C162F-pRc/CMV constructs from addgene or performed by site-directed mutagenesis into the VHL30-His6 template (Table S2). Mutations were checked by DNA sequencing.

Transient transfection and siRNA knock-down

HEK293 cells were transiently transfected with each construct using TransIT®-2020 (Mirus) transfection reagent according to company protocol and incubated at 37ºC for 16 hours. Cells were washed with cold 1X PBS (Dulbecco's Phosphate Buffered Saline, PBS, without calcium or magnesium) (Sigma) on ice. Total cell lysate was collected in cold lysis buffer (20 mM Tris (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, 0.1% NP40, protease inhibitor cocktail (Roche) and PhosSTOP (Roche)) on ice. Short interfering RNA (siRNA) scramble control and *PPP5C* (PP5), *HI1FA* (HIF1α) or *EPAS1* (HIF2α) targeting duplexes were purchased from OriGene (SKU: SR303702, SR320469, or SR301415) and suspended in provided buffer. In a 6-well plate, indicated cells were transiently transfected with the siRNA using TransIT®-2020 (Mirus). For PP5 knock-down, either 30 nM of control siRNA or 10 nM of each PP5 siRNA duplex (A, B and C) were mixed prior to transfection. For HIFα knock-down experiments, 90nM of control or 30 nM of each HIF1α or HIF2α siRNA duplex (A, B and C) were mixed prior to transfection. Cells were incubated at 37ºC for 72 hours, then harvested for protein extraction (see below).

Protein extraction, immunoprecipitation and immunoblotting

Protein extraction from both yeast and mammalian cells was carried out using methods previously described (Mollapour et al., 2010). Cell lysates were quanified using 1X Bradford reageant (Biorad). For immunoprecipitation, cell lysates were incubated with anti-FLAG antibody conjugated beads (Sigma) for 2 hr at 4ºC. Pulldowns were achieved by incubating lysate with Ni-NTA agarose (Qiagen) for 2 hr at 4ºC, or with anti-PP5 antibody (2E12, Abcam or Cell signaling), or VHL antibody (3F391, Abcam) for 1 hr followed by protein G agarose (Invitrogen) for 2 hr at 4ºC. Immunopellets were washed 4 times with fresh lysis buffer and eluted in 5x Laemmli buffer. For purification, immunopellets were washed 4 times with fresh high salt lysis buffer (20 mM HEPES (pH7.0), 500 mM NaCl, 1 mM $MgCl_2$, 0.1% NP40, protease inhibitor cocktail (Roche), and PhosSTOP (Roche). Proteins bound to Ni-NTA agarose were washed with 50 mM imidazole in lysis buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, protease inhibitor cocktail (Roche), and PhosSTOP (Roche)) and eluted with either 300 mM imidazole in lysis buffer or with 5X Laemmli buffer. Precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotted proteins were detected with indicated dilutions of antibodies recognizing 1:8000 FLAG, 1:8000 6x-His (ThermoFisher Scientific), 1:8000 Hsp90-835-16F1, 1:8000 GAPDH (ENZO Life Sciences), 1:4000 Cdc37 (StressMarq), 1:2000 GR, 1:1000 p-GR (S211), 1:1000 myc, 1:2000 HA, 1:1000 PP5, 1:4000 Akt, 1:2000 p-Akt (S473), 1:1000 VHL, 1:500 CK1δ, 1:500 CK1ε, 1:1000 cleaved caspase-3, 1:2000 cleaved-PARP, 1:500 HIF1α, 1:500 HI2α (Cell Signalling), 1:1000 Ubiquitin (Santa Cruz Biotechnology), 1:12000 p-Cdc37 (S13), 1:1000 PP5, 1:1000 VHL(Abcam), 1:1000 phosphothreonine-P6623, 1:1000 phosphoserine-P5747 (Sigma–Aldrich), and 1:10000 GST (GE

Healthcare). Secondary antibodies raised against mouse, rabbit, rat and goat (Santa Cruz Biotechnology) were used at 1:4000 dilution.

Bacterial expression and protein purification

PP5-His_6 WT and mutants were expressed and purified from *E. coli* strain BL21 (DE3). Transformed cells were grown on agar plates containing Luria Broth (LB) with 50 mg/L ampicillin at 37˚C overnight. Colonies were picked and grown in 10 mL of LB + ampicillin overnight. The following day, cultures were diluted to 50 mL with fresh LB + amplicilin and grown with continuous 200 RPM shaking at 37˚C until OD_{600} = 0.6. The cultures were then cooled to 30˚C and induced with 100 mg/L IPTG until OD_{600} = 1.2. Cells were harvested by centrifugation and lysed by sonication in fresh lysis buffer without detergent (20 mM Tris (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, protease inhibitor cocktail (Roche) and PhosSTOP (Roche)). Supernatant was collected and PP5-His_6 expression was assessed by immunoblotting. Isolation of PP5-His_6 was carried out by two sequential Ni-NTA agarose (Qiagen) pulldowns, followed by imidazole competition after each pulldown (see above). The pure protein was then washed in a 30K Amicon® Ultra, 500 µL centrifugal filter unit (Millipore) with lysis buffer 3 times. Concentrations were determined by the Micro BCA™ Protein Assay Kit (Thermo Scientific) as per manual protocol. Then, 50 ng of the purified proteins were run on an SDS-PAGE gel and coomassie stained to confirm purity prior to use in assays.

***In vitro* kinase assay**

Wild-type human PP5 and its non-phospho-T362A-mutant were N-terminally His_6 tagged using pRSETA plasmid for bacterial expression. PP5 and its phospho-mutant were isolated by incubating 2 mg of protein extracts with 50 µl of Ni-NTA agarose (Qiagen) for 2 hr. The Ni-NTA agrose beads was washed with 30 µM imidazole and then incubated with 20ng of baculovirus expressed and purified of either active CK1δ-GST or CK1ε-GST (SignalChem) for *in vitro* kinase assay. The assay was carried out in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ and 0.2 mM ATP, at 30˚C for 15 min. The Ni-NTA agrose beads were washed twice with 30 µM imidazole and then eluded with 300 µM imidazole. Samples were then dialyzed with Amicon Ultra-0.5 centrifugal filter unit with Ultracel-30 membrane (Millipore), quantified and then analyzed by immunoblotting with anti-hexahistidine (Thermo Scientific), pan-anti-phosphothreonine-P6623 (Sigma-Aldrich), anti-phospho-serine-P5754 (Sigma-Aldrich), and anti-GST (GE Healthcare) antibodies.

PP5 activity assay with pNPP

PP5 activity was assayed by monitoring hydrolysis of the non-specific phosphatase substrate, *para*-Nitrophenyl Phosphate, pNPP. Samples were assayed in 96-well plate in triplicate. Proteins were incubated together at 30°C for 30 min prior to addition of indicated amount of pNPP substrate. Each well contained 0.15 µM PP5-His_6 WT or mutant and when indicated, 1.5 µM Hsp90α (proteins were purified into a buffer containing 0.5 mM $MnCl_2$, 50 mM NaCl and 100 mM Tris, pH 8.0). After substrate was added, the absorbance at 405 nm was measured over 10 mins at 30 °C. Data is presented as a kinetic curve of absorbance over time (seconds) and the fold change in PP5 activity was calculated from the final data point of each curve (30 mM pNPP substrate).

***In Vitro* Ubiquitination of PP5**

50 ng WT-PP5-His_6 or the K185R/K199R-PP5-His_6 mutant were bound to Ni-NTA agarose and incubated with VHL complex (Millipore), containing 25 mM MOPS pH7.5, 0.01% Tween 20, 5 mM MgCl2, 10 µM ATP, 1 ng RBX1 (Millipore), and 2 ng GST-ubiquitin. The reaction was initiated with the addition of GST-ubiquitin. After 30 minutes at 30˚C the Ni-NTA agarose was washed with lysis buffer. The Ni-NTA agarose was resuspended in 5X Laemmli buffer, boiled, separated by SDS-PAGE and transferred to nitrocellulose membranes. Ubiquitination was detected with by immunoblotting using anti-ubiquitin antibody (Santa Cruz Biothech).

Annexin V/PI Apoptosis Analysis

Apoptosis was detected by Annexin V/PI immunostaining. Caki-1, 786-O and A-498 cells were cultured in complete medium until 80% confluency and different concentrations of CK1δ IC261 were added in the culture medium. Percentage of unstained negative (-), single stained early apoptotic cells Annexin V-FITC positive (+), PI positive (+) necrotic cells, double positive Annexin V-FITC (+)/PI (+) late apoptotic/necrotic and total apoptotic cells (Annexin V+/PI+) at 24hrs post treatment was determined by flow cytometry. Staining with Annexin-V-FITC and propidium iodine (PI) was performed with the Apoptosis Detection Kit

(BioRad/AbD Serotec) in accordance with the manufacturers' protocol. Fluorescence was measured immediately using LSRFORTESSA (BD) collected from 10,000 events and analysis was performed using FlowJo software.

MTT Assay

MTT assay was performed as described in the Quick Cell Proliferation Colorimetric Assay Kit Plus (BioVision, Cat# K302-500). A-498, 786-O and Caki-1 cells were plated at 10,000 cells per well in 96-well plates. Cells were treated with 0.1, 0.2, 0.3, 0.5, 1.0 and 2.0 μM IC261. After 72 hours, 10μl WST was added to each well and returned to the 37°C incubator. After 60 minutes, absorbance at 450nm was measured on a Tecan Infinite M200 Pro and proliferation rate was calculated. For transfected cells, transfections were incubated for 48 hours prior to trypsinization and plating in the 96-well plate.

Soft Agar Colony Formation Assay

Soft agar colony formation assay was performed similar to previously described method (Borowicz et al., 2014). In brief, 1.2% and 2% agar was dissolved in deionized water, autoclaved, and cooled to 42°C in an equilibrated water bath. To prepare the bottom layer, 1.5 ml of 1:1 mixture of 2% agar and tissue culture medium was plated into each well of a 6-well plate and allowed to solidify. To plate 5,000 Caki-1, A-498 and 786-O cells/well, sub-confluent cells were trypsinized, counted and re-suspended to an approximate concentration of 6,667 cells/ml. The cells were then aliquoted into six tubes, and the appropriate concentration of IC261 or DMSO was added to each aliquot. To prepare the top layer, 1.5ml of a 1:1 mixture of 1.2% agar and cultured cells (at 6,667 cells/ml) were plated into each well of a 6-well plate on top of the bottom layer. Upon solidification, the plates were maintained in a 37°C incubator at 5% CO_2. Twice weekly, 100μl of culture media was added to the top layer to prevent desiccation. After 21 days, plates were imaged on a Nikon Ci-L microscope for two observers equipped with an IDEA camera using SPOT software.

Mass spectrometry analysis

Visible bands were excised from the gel manually and cut into small pieces approximately 1 mm x 1 mm. These gel pieces were destained using 1:1 30 mM potassium ferricyanide: 100 mM sodium thiosulfate for 10 minutes. The destained gel pieces were then washed with 25 mM ammonium bicarbonate and acetonitrile alternatively for 5 minutes each wash. This cycle of 5 minute 25 mM ammonium bicarbonate wash followed by 5 minute acetonitrile wash was repeated 3 times. To further prepare the gel pieces for digestion, the gel pieces were then dehydrated in 100% acetonitrile. After removing all acetonitrile, 25 μl of porcine trypsin (Promega) dissolved in 25 mM ammonium bicarbonate at a concentration of 4 μg/ml was added to the gel pieces. The gel pieces were then kept at room temperature overnight (approximately 12-16 hours). Following digestion, the supernatant was transferred to a second tube, and acetonitrile was added to the gel pieces to complete the extraction of digested peptides. This extract was added to the first supernatant and this combined solution, containing the extracted peptides was frozen and lyophilized. The peptides were resuspended in 5 μl of 100:99:1 acetonitrile: water: trifluoroacetic acid immediately prior to spotting on the MALDI target. For MALDI analysis, the matrix solution consisted of alpha-cyano-4-hydroxycinnamic acid (Aldrich Chemical Co. Milwaukee, WI) saturating a solution of 1:1:0.01 acetonitrile: 25 mM ammonium citrate: trifuoroacetic acid. Approximately 0.15 μl of peptide solution was spotted on the MALDI target immediately followed by 0.15 μl of the matrix solution. This combined solution was allowed to dry at room temperature. MALDI MS and MS/MS data was then acquired using the ABSCIEX TOF/TOF® 5800 Mass Spectrometer. Resultant peptide mass fingerprint and peptide sequence data was submitted to the UniProt database using the Mascot search engine to which relevance is calculated and scores are displayed. Data are presented in (Table S1).

SUPPLEMENTAL REFERENCES

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SUPPLEMENTAL DATA

Figure S1

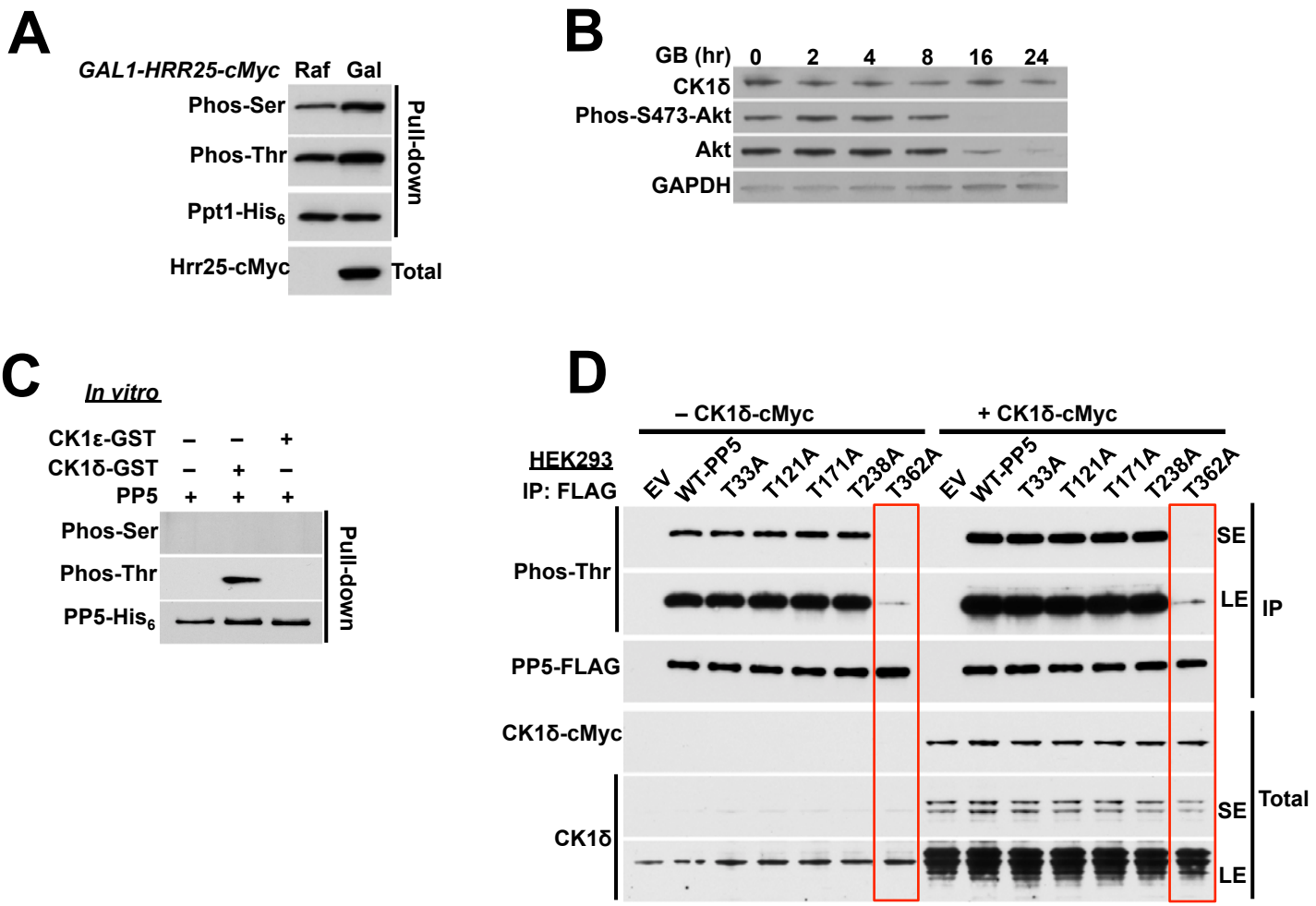


Figure S1. CK1δ mediated phosphorylation of PP5. (Related to Figure 1).

A) GAL1-HRR25-cMyc-pYES2 and PPT1-His_6-pRS415 were co-transformed and expressed in BY4743. Cells were grown on raffinose overnight at 28˚C. The cells were then shifted to galastose media for 2hr. PPT1-His_6 was isolated from cell lysate by pull-down with Ni-NTA agarose. Serine and threonine phosphorylation of Ppt1 was detected by immunoblotting with anti-phosphoserine or phosphothreonine antibodies.

B) HEK293 cells were treated with 1 μM Hsp90 inhibitor ganatespib (GB) at the indicated time points. CK1δ protein stability was assessed by immunoblotting. Akt and Phospho-S473-Akt were used as positive controls. GAPDH was used as a negative and loading control.

C) Bacterially expressed and purified human PP5-His_6 was used as a substrate of CK1δ-GST and CK1ε-GST in an *in vitro* kinase assay. Phosphorylation was assessed by immunoblotting with anti-phosphoserine or phosphothreonine antibodies.

D) PP5-threonine residues within the CK1δ consensus sequence were mutated individually to alanine (A), transiently expressed, and immunoprecipitated (IP) from HEK293 cells. Threonine phosphorylation was detected by immunoblotting with anti-phosphothreonine antibody and endogenous CK1δ with anti-CK1δ-antibody, (SE, short exposure, and LE, long exposure).

Figure S2

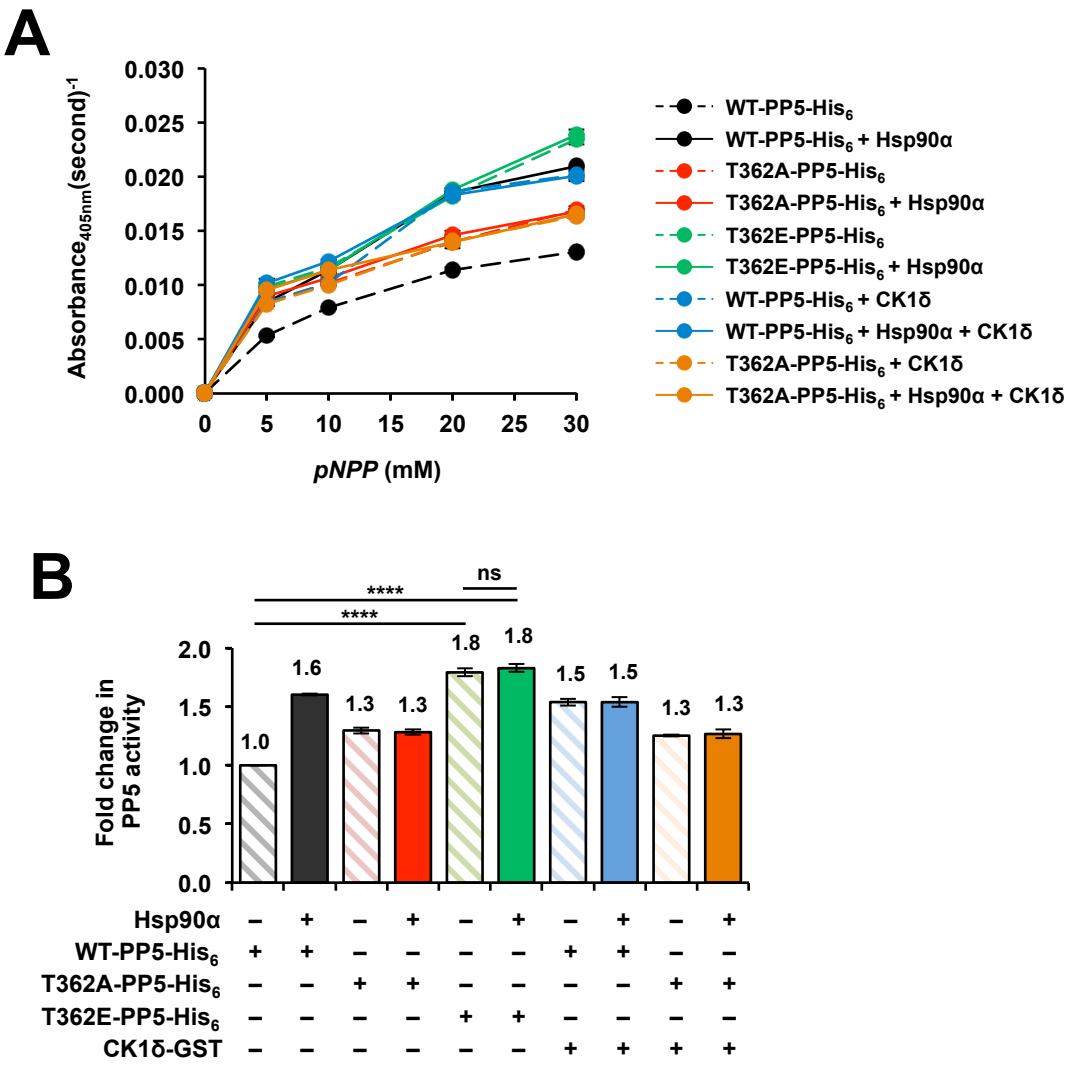


Figure S2. Phosphatase activity of the phospho-T362-PP5. (Related to Figure 2).

A) Rate of dephosphorylation of the model substrate pNPP by the wild-type PP5 (black) and PP5 mutants: T362A (red), T362E (green), CK1δ mediated phosphorylation of the wild-type PP5 (blue), and CK1δ mediated phosphorylation of the T362A (orange). The data represents three independent experiments.

B) Fold increase from wild-type PP5 basal activity at 30 mM data point from samples in (A). The error bars represent mean ± S.D. of three independent experiments. A Student's t-test was performed to assess statistical significance (****P<0.0001) or non-significant (n.s.)).

Figure S3

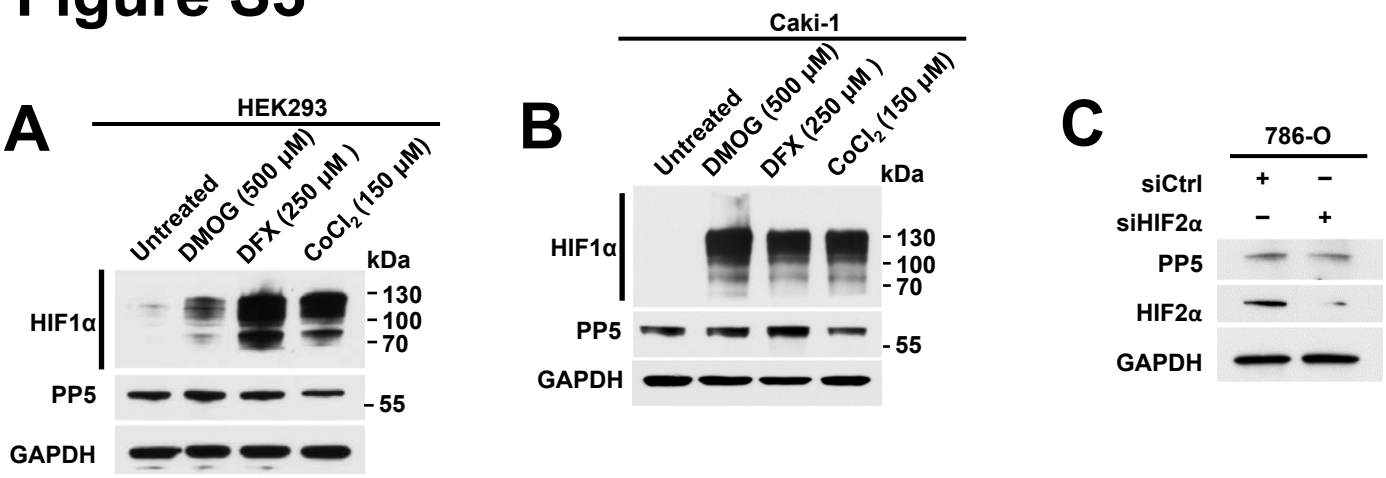


Figure S3. Ubiquitination of PP5 independent of prolyl hydroxylation. (Related to Figure 3).

A) HEK293 cells were treated with the prolyl hydroxylases (PHD) inhibitor 500 μM dimethyloxaloylglycine (DMOG) or hypoxia mimetic 250 μM deferoxamine (DFX) or 150 μM $CoCl_2$ for 18 hr. PP5 and HIF1α protein levels were examined by immunoblotting using anti-PP5 and anti- HIF1α antibodies. GAPDH was used a control.

B) Caki-1 cells were treated with the PHD inhibitor 500 μM DMOG or hypoxia mimetic 250 μM DFX or 150 μM $CoCl_2$ for 18 hr. PP5 and HIF1α protein levels were examined by immunoblotting using anti-PP5 and anti-HIF1α antibodies. GAPDH was used a control.

C) *HIF2α* was silenced by siRNA in 786-O cells. HIF2α and PP5 protein levels were examined by immunoblotting using anti-HIF2α and anti-PP5 antibodies. GAPDH was used a control.

Figure S4

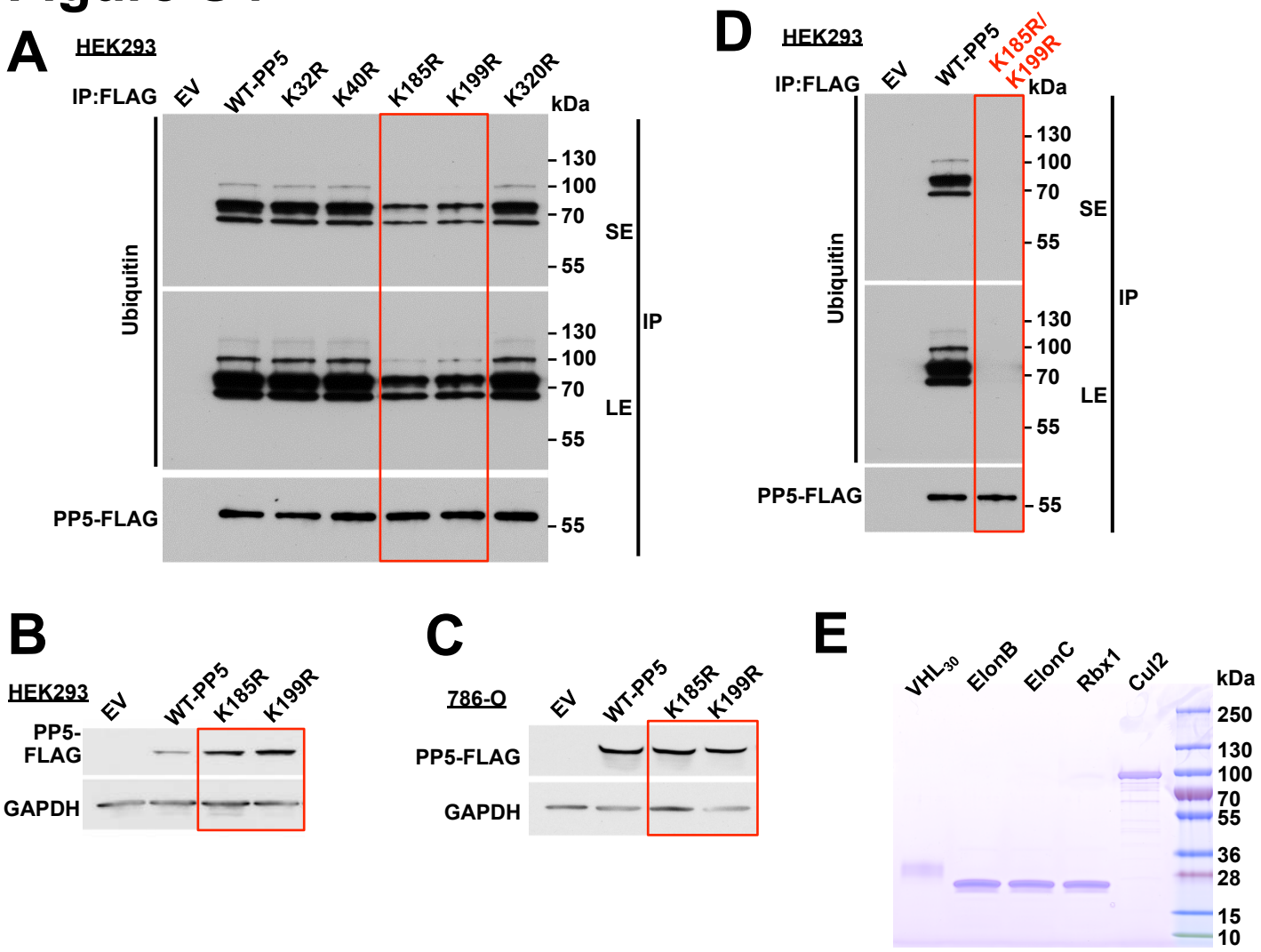


Figure S4. VHL E3 ligase multi-monoubiquitination of K185/K199-PP5. (Related to Figure 4).

A) Wild-type PP5-FLAG and its potentially non-ubiquitinating lysine mutants were transiently expressed and immunoprecipitated (IP) from HEK293 cells. Ubiquitination of PP5 was examined by immunoblotting using anti-ubiquitin antibody. Empty vector (EV) was used as a control. SE, short exposure; LE, long exposure.

B) Wild-type PP5-FLAG and its non-ubiquitinating lysine mutants, K185R or K199R mutants were individually and transiently expressed and in HEK293 cells. PP5 protein levels were examined by immunoblotting using anti-FLAG antibody. Empty vector (EV) was used as a control.

C) Wild-type PP5-FLAG and its non-ubiquitinating lysine mutants, K185R or K199R mutants were transiently expressed in 786-O cells. PP5 protein levels were examined by immunoblotting using anti-FLAG antibody. Empty vector (EV) was used as a control.

D) Wild-type PP5-FLAG and its non-ubiquitinating K185/K199R mutant were transiently expressed and immunoprecipitated (IP) from HEK293 cells. Ubiquitination of PP5 was examined by immunoblotting using anti-ubiquitin antibody. Empty vector (EV) was used as a control. SE, short exposure; LE, long exposure.

E) The multi-protein complex, VCB-Cul2 (VHL_{30}-Elongin C-Elongin B-Cullin-2) and Rbx1, were resolved on a SDS-PAGE gels and stained with coomassie blue.

Figure S5

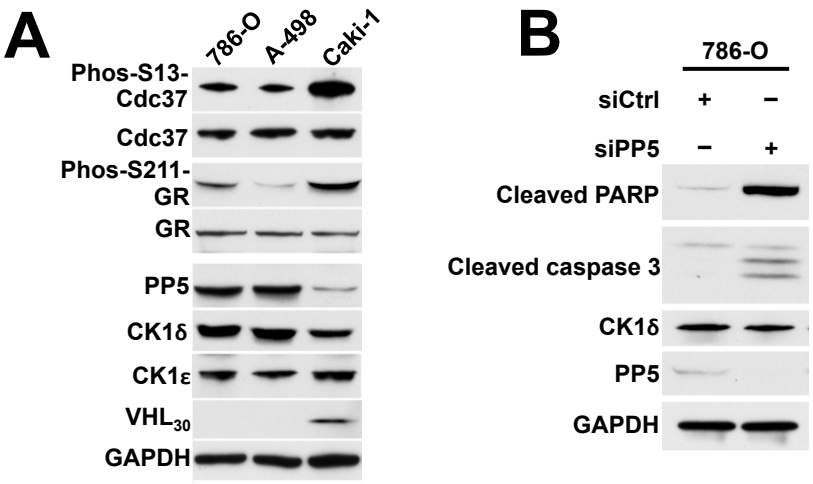
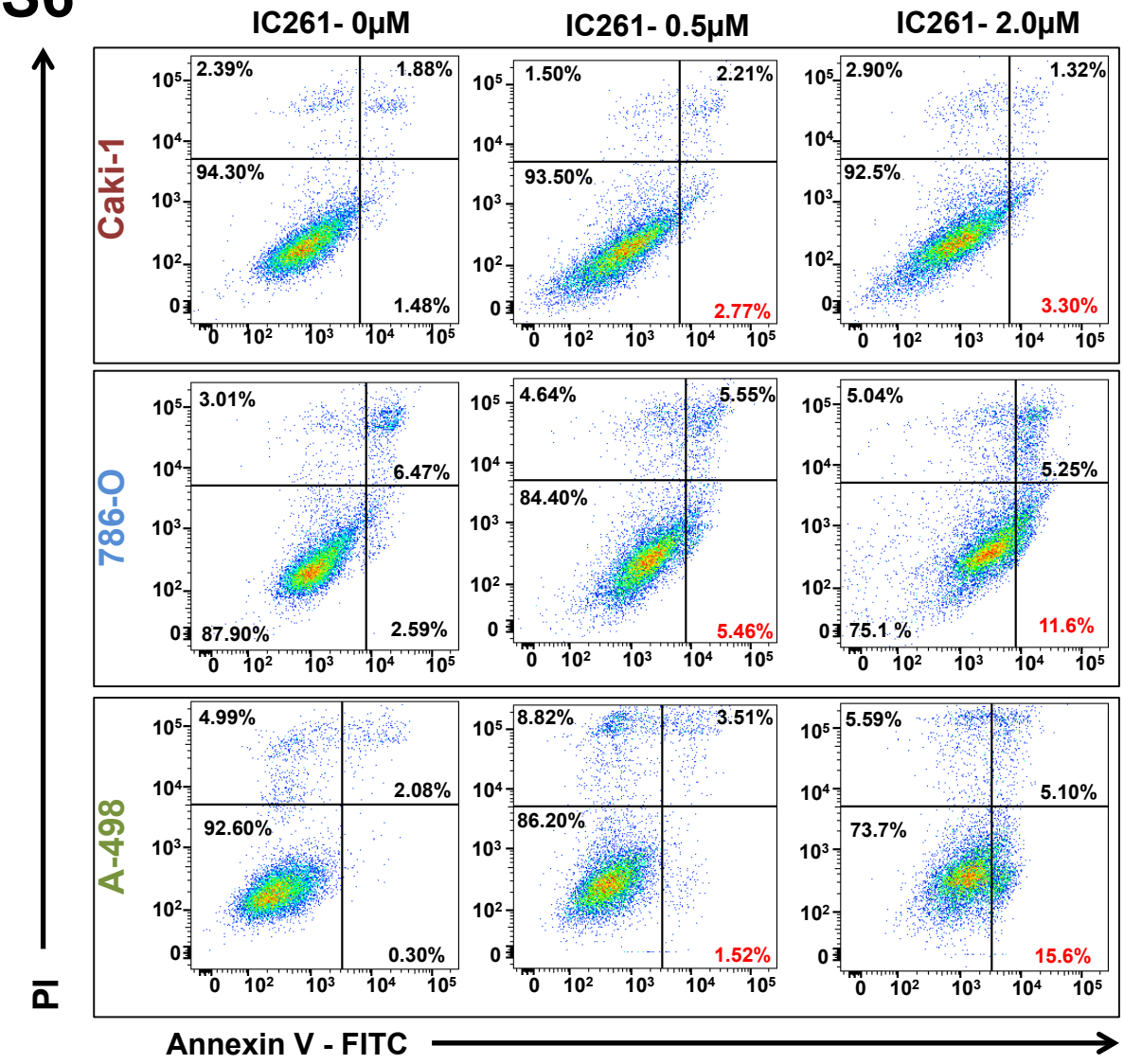


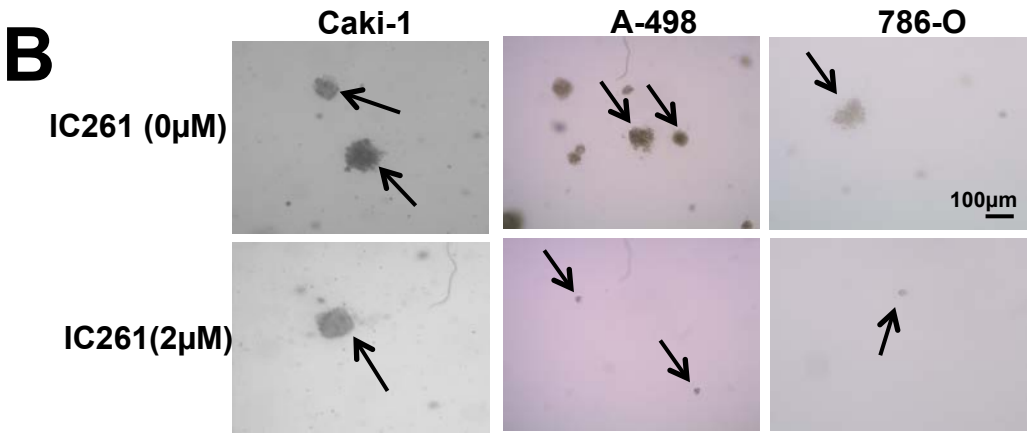
Figure S5. Down-regulation of PP5 caused apoptosis in VHL null kidney cancer cells. (Related to Figure 5).

A) PP5, Cdc37 and phosphorylated S13-Cdc37, GR and phosphorylated S211-GR, CK1δ, CK1ε and VHL_{30} proteins from ccRCC cells lines 786-O, A-498 and Caki-1 were assessed by immunoblotting. GAPDH was used as a loading control.

B) Silencing of *PP5* by siRNA in 786-O cells. Induction of apoptotic markers shown by immunoblotting using anti-cleaved caspase-3 and cleaved-PARP antibodies. CK1δ and PP5 protein levels were also examined by immunoblotting. GAPDH was used as a control. siCtrl represents the non-targeting siRNA control.

Figure S6


A
IC261- 0μM
IC261- 0.5μM
IC261- 2.0μM
Caki-1
786-O
A-498
2.39%
1.88%
94.30%
1.48%
1.50%
2.21%
93.50%
2.77%
2.90%
1.32%
92.5%
3.30%
3.01%
6.47%
87.90%
2.59%
4.64%
5.55%
84.40%
5.46%
5.04%
5.25%
75.1 %
11.6%
4.99%
2.08%
92.60%
0.30%
8.82%
3.51%
86.20%
1.52%
5.59%
5.10%
73.7%
15.6%
PI
Annexin V - FITC


B
Caki-1
A-498
786-O
IC261 (0μM)
IC261(2μM)
100μm

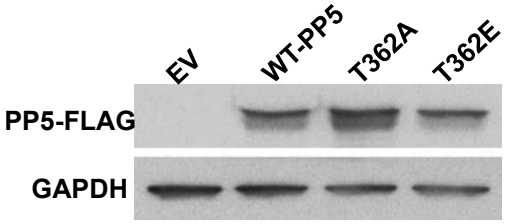

C
EV
WT-PP5
T362A
T362E
PP5-FLAG
GAPDH

Figure S6. CK1δ inhibition induces apoptosis and reduced proliferation in VHL-null ccRCC cells. (Related to Figure 6).

A) AV/PI graphs of Caki-1, A498 and 786-O cells untreated (0μM), or treated with 0.5μM and 2μM IC261 for 16hr for 2 hr. The top left quadrants represent dead cells stained only with PI. The bottom right quadrants represent apoptotic cells stained only with AV. The top right quadrants represent cells stained with both PI and AV (secondary necrosis and late apoptosis). Percentage of each stained cell population is indicated. Dot plots shown are representative of one of three independent experiments.
B) Soft agar colony formation assay for A498, 786-O and Caki-1 cells treated with indicated amounts of IC261.
C) 786-O cells transiently expressing wild-type PP5-FLAG, T362A-PP5-FLAG and T362E-PP5-FLAG and then treated with indicated amounts of IC261. These cells were used for the MTT assay. Levels of PP5-FLAG protein expression was assessed by immunoblotting. GAPDH was used as a loading control.