SUPPLEMENTAL INFORMATION

SUPPLEMENTAL METHODS

Protein Expression and Purification

The human PP5-Cdc37 chimera was cloned in two steps. A linker (sequence $(GS)_3ASR$) was added 3' to the PP5 catalytic domain, comprising residues 175-499, using PCR, and subsequently used as a template for the further addition of residues 5-20 of Cdc37, including the S13→E mutation, 3' to the linker. The chimera gene was subcloned as a BamHI-XhoI fragment into a modified pET-44 vector (Novagen), with an N-terminal, Tobacco Etch Virus (TEV) cleavable His_6-NusA tag. The PP5-Cdc37 chimera was purified using a DEAE-sepharose anion exchange column (GE Healthcare) followed by cleavage of the tag with TEV protease. The protein was reloaded onto the anion exchange column to separate the cleaved protein from the tag, and further purified using a Superdex 200 HR16/60 gel filtration column (GE Healthcare). Full-length human Cdc37 was cloned into the pET-28a vector (Novagen) with a C-terminal His_6 tag, and purified using Talon metal affinity resin (Clontech) followed by PreScission protease cleavage, glutathione sepharose resin (GE Healthcare) and finally Superdex 200 HR16/60 gel filtration (GE Healthcare). Phospho-Cdc37-Ser13 was generated by co-expression of Cdc37 with GST-tagged *Z. mays* CK2α in pGEX 6P-1 and purified as described for WT Cdc37. Phosphorylation was confirmed by mass spectrometry and Western blot using anti-phospho-Ser13 Cdc37 (Sigma). Human PP5 (residues 16-499) was purified as previously described (1). Full-length wild-type Hsp90 with an N-terminal His_6 tag in a pRset-A vector was purified as previously described(2). All constructs were expressed in *E.coli* BL21-star (DE3) cells (Invitrogen).

PP5 and Cdc37 mutants were created using the QuikChange®II site-directed mutagenesis method (Stratagene) and confirmed by DNA sequencing. Mutant proteins were expressed and purified using the same method as wild-type proteins.

Crystallisation, Data Collection and Structure Determination

The PP5-Cdc37 chimera crystals were grown by hanging drop vapour diffusion at 4 °C by mixing the protein at 12 mg/ml, in a buffer containing 100 mM NaCl, 20 mM Tris pH 8 and 2% glycerol, with an equal volume of well buffer containing 18% PEG 10K, 8% ethylene glycol, 0.1 M Hepes pH 7.5 and 20 mM hexamine cobalt chloride. Crystals were cryoprotected in well buffer supplemented with 15% ethylene glycol. Data were collected at the Diamond light source, beamline I24, wavelength 0.968600 Å. Data were processed using XDS (3) and Scala (4). The chimera structure was solved by molecular replacement using Phaser (5) with the PP5 catalytic domain (PDB code 1S95) as a search model. Clear difference density was observed for the phospho-mimetic Glu13 of Cdc37. Additional rounds of refinement using Phenix Refine (6) allowed the remainder of the peptide to be built manually into difference density using Coot (7). There is clear electron density for the main-chain of residues 175-490 of PP5 and residues 7-15 of the Cdc37 peptide. The αJ-helix of PP5 (residues 491-499), the linker and the N- and C-termini of Cdc37 (residues 5-6 and 16-20) are not seen in the maps. 95.3% of residues lie in the most favoured region of the Ramachandran plot (only 2 residues, PP5-Phe272 in the core of the protein, and Cdc37-His9 are outliers; density for both of these residues is clearly defined).

PP5 Phosphatase Activity Assays

The phosphatase activity of PP5 wild-type and mutants was assayed by monitoring hydrolysis of the artificial substrate *para*-Nitrophenyl Phosphate (*p*NPP). Samples were assayed in 384-well plates in triplicate. Each well contained 0.015 μM of PP5 and 1.5 μM of Hsp90 in a

buffer containing 0.5 mM $MnCl_2$, 50 mM NaCl and 100 mM Tris, pH 8.0. The reaction was started by addition of *p*NPP and the change in absorbance at 405 nm was measured over 10 minutes at 30 °C. Data were fit to the Michaelis-Menten equation using non-linear least squares regression in MATLAB.

Cdc37 Dephosphorylation Assays

To monitor dephosphorylation of Cdc37-Ser13 by PP5, 5 μM of purified phospho-Ser13 Cdc37 (wild-type or mutant) was mixed with 2.5 μM of Hsp90 in a buffer containing 100 mM NaCl, 50 mM Tris pH 7.5, 2 mM DTT, 1 mM $MnCl_2$. The reaction was started by adding 0.25 μM PP5 (wild-type or mutant) and the samples were incubated at 30^{o}C. Samples were taken every 45 minutes for SDS-PAGE analysis. The phosphorylation state of Cdc37 Ser13 was probed by Western blot using a phospho-Ser13 specific antibody (Sigma). Figures are representative of results from a minimum of two independent assays.

Conservation Analysis

Consurf (8) was used to map the relative conservation of residues between PP5 homologues and within the PPP family. The input alignments were generated in ClustalW2 (9). For PP5 subfamily analysis greater than 70 PP5 homologues were identified using Blast (10). For the PPP family all common homologues were identified for each family member (typically 10 homologues per family member isoform, except for PP7 for which only *A. thaliana* was included).

Plasmids for cellular assays

N-terminally FLAG or c-myc tagged human PP5 and N-terminally FLAG tagged human Cdc37 in mammalian expression plasmid pCDNA3 and their mutants were derived using a

QuikChange®II site-directed mutagenesis kit (Stratagene) with primers listed in Table S3. Mutations were checked by DNA sequencing.

Immunoprecipitation and immunoblotting

HEK293 cells were transiently transfected with each construct using TransIT®-2020 (mirus) transfection reagent and incubated at 37ºC for 24 hours. Cells were washed with cold 1X PBS (Dulbecco's Phosphate Buffered Saline, 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. Lysates were incubated with anti-FLAG (Sigma) or anti-myc antibody conjugated beads (ThermoScientific) for 2 hours at 4°C with gentle rotation. After incubation, beads were washed 4 times with cold lysis buffer, eluted with 5X Laemmli buffer and boiled for 5 minutes. Western blotting was carried as previously described (11). Coimmunoprecipitated proteins were detected by immunoblotting with indicated antibodies recognizing FLAG (Sigma-Aldrich), Raf-1 and CDK4 (Santa Cruz), Cdc37, Ulk1, myc-tag, GR, phospho-GR (S211), CKIIα and PP5 (Cell Signalling Technology), Hsp90 (Enzo), or PP5 and phospho-Cdc37 (S13) (Abcam). The data are representative of three biological replicates.

Drug Binding Assay

Total cell lysates prepared as described above were incubated with 50 nM or 100 nM biotinylated ganetespib (Synta Pharmaceuticals) at 4°C for 1 hour followed by streptavidin agarose beads (ThermoScientific) for an additional 2 hours with gentle rotation. Bound Hsp90 and PP5-FLAG were detected by immunoblotting as described above. The data are representative of three biological replicates.

PP5 knock-down by siRNA

Short interfering RNA (siRNA) scramble control and PP5 targeting duplexes were purchased from OriGene (SKU: SR303702) and suspended in provided re-suspension buffer. In a 6-well plate, HEK293 cells were transiently transfected with the siRNA using TransIT®-2020 (mirus), with either 30 nM of control siRNA (SKU: SR30004), or 10 nM of each PP5 siRNA duplex mixed (SKU: SR303702A, SR303702B, and SR303702C). Transfections were incubated at 37ºC for 72 hours. Then cells were harvested and protein extracted as described above, or cells scraped for RNA isolation (see below). The data presented are representative of three biological replicates.

RNA extraction, cDNA and qRT-PCR quantification

Cells from one 6-well plate of control siRNA and PP5 siRNA knock-down in HEK293 cells were scraped and washed in cold 1X PBS (Dulbecco's Phosphate Buffered Saline, without calcium or magnesium) (Sigma). RNA was isolated using the RNeasy® Plus Mini Kit (QIAGEN) according to manufacturer's protocol. cDNA was synthesized using the iScript™ cDNA synthesis kit (BioRad) according to manufacturer's protocol. qRT-PCR was carried out using PP5 primers forward: 5'-AAGACTCAGGCCAATGACTAC-3' and reverse: 5'-CGCGTAGCCATAGCACTCAG-3' (PrimerBank ID: 324021714c1), GAPDH primers forward: 5'- GGAAGGTGAAGGTCGGAGTCA-3' and reverse: 5'-GCAACAATATCCACTTTACCAGAGTTAA-3'. PCR product was detected using iTaq™ universal SYBR® green supermix (BioRad) and CFX96 thermocycler (BioRad). The data presented are representative of three biological replicates.

Statistical Analysis

Statistics for RNA relative quantitation are presented as mean ± SE. Data were analyzed with an unpaired t-test. Asterisks in the figure represent significance, *** indicates p-value < 0.0005.

SUPPLEMENTAL FIGURE LEGENDS

Supplementary Figure 1: Construct design and structural analysis for the PP5-Cdc37 chimera; related to Figure 1

(A) Schematic representation of human Cdc37, PP5 and the chimeric construct used to determine the crystal structure.

(B) Structural alignment of the Cdc37-bound PP5 with toxin inhibitor-bound PP1 structures. The PP1 backbone is omitted for clarity since the phosphatase catalytic domains are highly structurally homologous. The cartoon representation of PP5 is coloured as Figure 1B. The Cdc37 substrate, okadaic acid (PDB 1JK7), microcystin (PDB 1FJM), Caliculin (PDB 1IT6), Nodularin-R (PDB 3E7A) and Tautomycin (PDB 3E7B) are shown as grey, cyan, green, magenta, yellow and pink sticks respectively.

(C) Surface representation of the PP5 catalytic domain. Cdc37 substrate (sticks) binds in the hydrophobic groove and extends towards the C-terminal groove. The hydrophobic, acidic and C-terminal grooves are highlighted.

(D) Least squares fit of the phosphate-bound catalytic domain (PDB 1S95), in grey, with the chimera structure, in blue. The αJ-helix in 1S95 is highlighted in magenta. The residues that show the greatest change in position are highlighted in stick format. The N- and C-termini are labeled.

Supplementary Figure 2: Sequence and structural alignment of phosphatase families; related to Figure 2

Sequence alignment of the catalytic domain of (A) PP5 homologues and (B) PPP family members. Residues that contribute H-bonds or van der Waals contacts to Cdc37 via their side-chain are coloured green and via their main-chain are coloured cyan. Residues involved in interactions at the catalytic site are coloured yellow. Arg400 and Arg275, coloured orange, both coordinate the substrate and play a role in the catalytic centre. The αJ-helix is coloured magenta. Alignments were generated using ClustalW2 (9) and illustrated with ESPRipt3.0 (12).

Sequences used for the PPP family alignment in (B) are all human and α-isoform, with the exception of PP7 from Arabidopsis. The accession numbers are: P62136, PP1A; P67775,PP2Aα; Q08209, PP2Bα; P60510, PP4; P53041, PP5; O00743, PP6; Q9FN02, PP7.

(C) Least squares fit of the PP1 (light blue; PDB 4MOV), PP2A (light green; PDB 2IE4) and PP2B (pink; PDB 4F0Z) structures to PP5 (grey). Only residues that line the substrate-binding cleft are shown. Hydrogen-bonds made between PP5 and Cdc37 (coloured rainbow) are shown. Structures with the highest resolution from human PPP superfamily members were used in this analysis. Cdc37 substrate is shown as sticks and coloured as Figure 1 and labeled from -4 through +2 to identify location relative to S13E (position 0).

(D) Hydrophobicity of the PP5 surface from red (most hydrophobic) to white (least hydrophobic). The grooves in which the side-chains of Glu11 and Asp14 are bound display intermediate properties. Cdc37 is coloured and labeled as in (C).

Supplementary Figure 3: Related to Figure 3

(A) Dephosphorylation of phospho-Cdc37-Ser13 in the context of purified WT and indicated mutants of Cdc37 and PP5, in the absence of Hsp90. Activity was assessed using a phospho-specific antibody over time (minutes).

(B) Rate of dephosphorylation of the model substrate *p*NPP by PP5 mutants. W386F, orange; WT, red; N308Q, green; N308D, cyan; M309C, blue; Y313F, magenta; without Hsp90, black).

(C) HEK293 cells were transiently transfected with empty plasmid (C), wild-type Cdc37-FLAG (WT), or indicated mutants. PP5 was immunoprecipitated (IP) using beads conjugated with anti-PP5 antibody and co-IP of Hsp90 and Cdc37-FLAG were examined by immunoblotting,

(D) Sequence alignment of the extreme N-terminus of Cdc37. Residues that contribute H-bonds to PP5 via their side-chain are coloured green, via their mainchain are coloured cyan and via both are coloured magenta. For the remaining residues, those that are completely conserved are shown as white text in red, whereas residues that are strongly conserved are coloured red text in white. Residues of the CK2 consensus motif SXXE/D are indicated with a triangle. Alignments were generated using ClustalW2 (9) and illustrated with ESPRipt3.0 (12).

Supplementary Figure 4: Related to Figure 4

HEK293 cells were transiently transfected with control or PP5 siRNA. RNA was isolated from cells, reverse transcribed and DNA quantitated. Quantification is reported as relative expression of mean ± standard error, *** indicates p-value < 0.0005.

SUPPLEMENTAL TABLES

Supplementary Table 1: Data Collection and Refinement Statistics for the PP5-Cdc37 chimera structure

	PP5-Cdc37 chimera
Data Collection	
Space group	P212121
Cell dimensions	
a, b, c (Å)	42.31, 65.16, 132.12
α, β, γ (°)	90, 90, 90
Resolution (Å)	46.39 – 2.27 (2.34 – 2.27)*
R_{merge}	0.164 (0.657)
I/σI	9.1 (2.9)
Completeness (%)	95.3 (89.5)
Redundancy	5.3 (4.3)
Refinement	
Resolution (Å)	2.3
No. reflections	16806
R_{work}/R_{free}	0.168/0.235
No. atoms	
Protein	2626
Ligand/ion	14/2
Water	105
B-factors	
Protein	18.1
Ligand/ion	29.1/8.7
Water	19.0
R.m.s. deviations	
Bond lengths (Å)	0.017
Bond angles (°)	1.63

* The values for the highest resolution shell are shown in parenthesis

Supplementary Table 2: Residue Variety (%) By Amino-Acid Position* Generated Using CONSURF for Residues Lining the Substrate-Binding Cleft (see Methods)

	PP5 Amino-Acid	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr	Max % identity/similarity
PP5 family†	R275														100						R 100.000
	N308			7.246						1.449		62.319				4.348	18.841	4.348		1.449	N 62.319
	M309										100										M 100.000
	I312								49.275	1.449	46.377							2.899			I/M/V 98.511
	Y313																			100	Y 100.000
	P375									1.449			98.551								P 98.551
	P376			1.449									98.551								P 98.551
	W386						1.449												98.551		W 98.551
	R400														100						R 100.000
	E428				100																E 100.000
	V429		1.471							1.471								97.059			V 97.059
	Y451																			100	Y 100.000
PPP superfamily‡	R275														100						R 100.000
	N308							20.69				5.747		39.08		31.034	1.149	1.149		1.149	Q 39.080
	M309		1.149						67.816	20.69	10.345										I/L/M 98.851
	I312								34.483		5.747							39.08		20.69	I/M/V 79.31
	Y313					20.69														79.31	Y 79.310
	P375								14.943				29.885					55.172			I/V 70.115
	P376												100								P 100.000
	W386																		100		W 100.000
	R400														100						R 100.000
	E428				32.184									67.816							Q/E 100.00
	V429	20.69					1.149			40.23								37.931			L/V 78.161
	Y451																			100	Y 100.000

* Numbering as for PP5 catalytic domain

† A total of 69 homologues were identified using BLAST with identity greater than 56% throughout both the TPR and catalytic domains

‡ A total of 87 homologues were identified using UniProt (>10 homologues for each of the PPP family members). The exception is PP7, for which only Arabidopsis was included

Supplementary Table 3: Primer sequences. For the PP5-Cdc37 chimera construct (Primers 1-3), PP5 sequence is in bold, the linker sequence is highlighted in green, and Cdc37 sequence is italic. Restriction sites are underlined. For immunoprecipitation experiments (Primers 4-22), mutated sequences are highlighted in red. Epitope sequences are highlighted in blue.

Primer		**Sequence**
1	Chimera-BamHI-F	CGGGGATCC**TACAGCGGACCC**
2	Linker-Nhe1-R	CCG**GCTAGCGCTACCGCTGCCGCTACCCATCATTCCTAGCTG**
3	Chimera-XhoI-R	CCGCTCGAGTTA*GTGCGTCTCGTCTTCATCATCCTCCACCTCAATGTG GTCCCACACGCT*CCG**GCTAGCGCTACCGC**
4	Cdc37-FLAG-Hind3F	TATGCGAAAGCTTATG**GATTACAAAGACGATGACGATAAG**GTG GACTACAGCGTGTGG
5	Cdc37-FLAG-EcoIR	CTAATGCGAATTCTCACACACTGACATCCTTCTCATC
6	Cdc37-H9A-F	GACTACAGCGTGTGGGACGCAATTGAGGTGTCTGATGATGAAGAC
7	Cdc37-H9A-R	GTCTTCATCATCAGACACCTCAATTGCGTCCCACACGCTGTAGTC
8	Cdc37-E11A-F	AGCGTGTGGGACCACATTGCCGTGTCTGATGATGAAGAC
9	Cdc37-E11A-R	GTCTTCATCATCAGACACGGCAATGTGGTCCCACACGCT
10	Cdc37-D14N-F	GACCACATTGAGGTGTCTAACGATGAAGACGAGACGCACC
11	Cdc37-D14N-R	GGTGCGTCTCGTCTTCATCGTTAGACACCTCAATGTGGTC
12	PP5-MYC-BamHI-F	GGTACCTTGGATCCATG**GAACAAAAGTTAATCTCCGAAGAAGAT TTA**GGTGCGGAGGGCGAGAGGACTGAGTG
13	PP5-FLAG-Kpn1	TATGCGGTACC**ATGGATTACAAGGATGACGATGACAAGGGA**GC GGAGGGCGAGAGGACTGAGTGTG
14	PP5-Xho-R	GGATCGTCTCGAGTCACATCATTCCTAGCTGCAG
15	PP5-N308D-F	GGCAACCACGAGACAGACGACATGAACCAGATCTACGGTT
16	PP5-N308D-R	AACCGTAGATCTGGTTCATGTCGTCTGTCTCGTGGTTGCC
17	PP5-M309C-F	CGAGGCAACCACGAGACAGACAACTGTAACCAGATCTACGGTTTC GAGGGTG
18	PP5-M309C-R	CACCCTCGAAACCGTAGATCTGGTTACAGTTGTCTGTCTCGTGGTT GCCTCG
19	PP5-W386F-F	CCCATGTGTGACCTGCTCTTTTCAGATCCACAGCCACAG
20	PP5-W386F-R	CTGTGGCTGTGGATCTGAAAAGAGCAGGTCACACATGGG
21	PP5-Y313F-F	GACAACATGAACCAGATCTTCGGTTTCGAGGGTGAGGTGA
22	PP5-Y313F-R	TCACCTCACCCTCGAAACCGAAGATCTGGTTCATGTTGTC

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