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Supplemental Information

Phosphorylation-Dependent PIH1D1 Interactions Define Substrate Specificity of the R2TP Cochaperone Complex

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Supplemental Experimental Procedures

Protein expression and purification

Proteins for structural and ITC analysis were expressed in BL21 (DE3) cells with a N-terminal hexahistidine tag. Cells were re-suspended in lysis buffer containing 50 mM Tris pH 8.0, 500 mM NaCl, 20 mM imidazole pH 8.0, 0.5 mM TCEP pH 8.0. 1.25 U ml^{-1} benzonase (Novagen, Merck) and complete EDTA free protease inhibitor cocktail (Roche). Lysis supernatant was added to 2 ml of Ni-NTA beads (GE healthcare) and beads were washed with 10 column volumes of wash buffer [50 mM Tris pH 8.0, 500 mM NaCl, 20 mM imidazole pH 8.0, 0.5 mM TCEP] followed by elution with 12 column volumes of elution buffer [50 mM Tris pH 8.0, 500 mM NaCl, 200 mM imidazole pH 8.0, 0.5 mM TCEP]. Appropriate fractions were pooled, concentrated and further purified by size exclusion chromatography on a Superdex 200 26/60 column (GE Healthcare) equilibrated in 20 mM Tris pH 8.0, 150 mM NaCl, 0.5 mM TCEP. Protein identity and integrity was confirmed by electrospray mass spectrometry. GST-fusion protein production, LB medium was inoculated 1:50 with an overnight culture of *E. coli* transformed with GST-PIH1D1 wt or K64A and grown to OD 0.6-1.0. The expression was with 0.1 mM IPTG for 2 hours at 37^{o}C. Cells were pelleted by centrifugation at 3,000 x g for 15 mins at 4^{o}C. The pellets were resuspended in 30ml of cold PBS (140 mM NaCl, 2.7 M KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.3) supplemented with protease inhibitor cocktail (Roche), and lysed by sonication. The samples were centrifuged for 2 x 10min at 30 000 x g and incubated with 1.33ml of glutathione Sepharose 4 Fast Flow beads (GE Healthcare), washed 3 x with PBS prior adding to the samples. The beads were washed 3 x with 10 ml of PBS and eluted with 3 x 1 ml aliquots of glutathionine buffer (50 mM Tris pH 8.0, 10 mM reduced glutathionine) and dialysed over night against Tris buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% v/v glycerol).

Mass spectrometry analysis and protein identification

SYPRO ruby-stained polyacrylamide gel slices (1-2 mm) were excised using a scalpel and processed for mass spectrometry using the Janus automated liquid handling system (PerkinElmer, U.K.). Briefly, the excised protein gel pieces were placed in individual wells of a 96-well microtitre plate and destained with 50 % vol/vol acetonitrile and 50 mM ammonium bicarbonate, reduced with 10 mM DTT, and alkylated with 55 mM iodoacetamide. After alkylation, proteins in gel pieces were digestedwith 6 ng/µl trypsin overnight at 37 °C. The resulting peptides were extracted in 1% v/v formic acid, 2 % v/v acetonitrile. The digests

were analyzed by nano-scale capillary LC-ESI MS/MS using a Waters nanoACQUITY UPLC to deliver a flow of 300 nl/min. A Waters µ-Precolumn, C18 Symmetry 5 mm, 180 mm x 20 mm (Waters, U.K.) guard column trapped the peptides prior to separation on a C18 BEH130 1.7 mm, 75 µm x 250 mm nanoAcquity UPLC column. Peptides were flushed from the guard column onto the analytical column at 300 nl/min and eluted with a gradient of acetonitrile. The column outlet was directly coupled to a Triversa nanomate microfluidic chip interface (Advion, U.K.). Mass spectrometric information was obtained using an orthogonal acceleration Quadrupole-Time of Flight mass spectrometer (SYNAPT HDMS, Waters, U.K.). Data dependent analysis was carried out where automatic MS/MS was acquired on the 8 most intense, multiply charged precursor ions in the m/z range 400–1500. MS/MS data were acquired over the m/z range 50–1995. LC/MS/MS data were processed using Mascot Distiller (Matrix Science, U.K) to generate mgf files. The processed data was then searched against a concatenated, non-redundant protein database (UniProt KB release 15.5) using the Mascot search engine programme V2.3 (Matrix Science, U.K.). Oxidation (Met), phosphorylation (STY), Gln->pyro-Glu (N-term Q) and carbamidomethylation (Cys) were included as variable modifications and a maximum of one missed tryptic cleavage was allowed. Precursor and fragment ion tolerances were set to 20 ppm and 0.05 Da respectively.

Cell culture, siRNA and drug treatment

HEK293T (Cancer Research UK Cell Services), HEK293 Flp-In (Invitrogen), U2OS (Cancer Research UK Cell Services) and RPE cells were maintained as adherent monolayer in DMEM media containing 10% FBS at 37°C in a humidified atmosphere of 5% carbon dioxide. Stable HEK293 Flp-In cell lines were created by transfection of empty pDEST-Flag/FRT/TO and pDEST-Flag/FRT/TO - PIH1D1 wt, K64A or K57A according the Flp-In cell lines manual and selected in media containing 150mg/ml hygromycin B (Invitrogen). Stable RPE cells were created by retroviral infection of empty pDEST LXSN-Flag and pDEST LXSN Flag-PIH1D1 wt or K64A and selection with neomycin (final concentration 500 µg/ml). Single colonies were picked and checked for PIH1D1 expression. Transient transfection was done using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions.

siRNA targeting PIH1D1 number one was a mixture of 2 Stealth siRNAs for PIH1D1, purchased from Invitrogen HSS123721 [CCUUCCACCGGAAGAGAAAGCAAUU] and HSS12372 [GACGUAGCUGUCAACAGCGACUUCU]. siRNA targeting PIH1D1 number two is a mixture of 2 siRNAs targeting GGAGAGGCGGCTAAGGAAA and GCTAAGGAAAGGTGCCACA sequences of untranslated region of PIH1D1 cDNA. Cells were transfected with 30nM siRNA using Lipofectamine 2000. The transfection was repeated

after 48 hours and cells were collected 72 hours after the first transfection. For replication stress induction, the cells were treated with 5mM hydroxyurea (HU) (Sigma) for 2 hours before harvesting.

Plasmids

pDEST-GST PIH1D1 wt was previously described (*4*). All mutations were introduced using the QuickChange II site-directed mutagenesis kit (Stratagene). For mammalian cell expression the PIH1D1 constructs were cloned by Gateway LR reaction to pDEST-FTF/FRT/TO. cDNAs for SNRP116, UBR5, ECD and RPAP3 were purchased from Origene and cloned to pDONR221 (Invitrogen), from which they were cloned by Gateway LR reaction to pDEST-FTF/FRT/TO plasmid. pDEST LXSN-Flag was created from pLXSN by insertion of Gateway cassette and Flag sequence into the multiple cloning site.

Protein extracts, Immunoprecipation, λ phosphatase treatment and peptide pull-down

For whole cell extracts, the cells were solubilized on ice in lysis buffer (50 mM Tris-HCL pH 8, 150 mM NaCl, 1 % Triton X-100, 1 mM EDTA, 2.5 mM EGTA, 10% v/v glycerol) supplemented with protease inhibitor cocktail (Roche). Cleared lysates were produced by centrifugation of the resulting samples at 16,000 x g for 15 mins at 4°C. 1mg of lysate was incubated with 25 μl of anti-Flag M2 agarose beads (Sigma) for 2 hours at 4^{o}C. Beads were then pelleted and washed three times in 20x bed volume of the lysis buffer. Bound protein was eluted by boiling in 2x Laemmli SDS sample buffer (100 mM Tris pH 6,8, 200 mM DTT, 4% SDS, 0,2% bromophenol blue, 20% v/v glycerol). For mass spectrometry analysis the bound protein was eluted with Flag peptide according to Sigma instructions and the resulting eluates were passed through a BioRad column to remove traces of contaminating beads.
Prior to lambda phosphatase treatment and/or incubation with GST-tagged PIH1D1 proteins, beads with bound Flag-tagged protein were successively washed in lysis buffer and wash buffer (50 mM Tris-HCl pH 8.0, 1 M NaCl, 1% Triton X-100, 1 mM DTT, 1 mM EDTA). Treatment with λ phosphatase (NEB) was performed using 4000U of λ phosphatase per 25μl of beads with bound protein. The reaction was incubated 30min at 30^{o}C. For pull-down assay 50μl of the M2 beads with bound Flag-tagged proteins and 30μg of purified GST proteins were used per reaction. The beads and proteins were diluted in 500μl of peptide pull-down buffer (50 mM Tris pH7.5, 150 mM NaCl, 1% Triton X-100, 1 mM DTT, 1 mM EDTA, 10% v/v

glycerol) and incubated for 2hrs at 4^{o}C. Proteins were eluted from the M2-Flag agarose beads by boiling in 2x LSB buffer. Peptide pull down was previously described (*4*).

The peptide pull down was carried out using following biotinylated peptides (LRI protein chemistry facility):
pECD: Bio-eahx-NYFDKILGPRPNESDpSDDLDDEDFECLDSDDDL
ECD: Bio-eahx-NYFDKILGPRPNESDSDDLDDEDFECLDSDDDL
pEIF5B:Bio-eahx-DKKPSKEMSSDSEYDpSDDDRKEERAYDKAKRRI
pUBR5:Bio-eahx-RHGSSRSVVDMDLDDpTDDGDDNAPLFYQPGKRG
pPTGES3: Bio-eahx-VDLPEVDGADDDSQDpSDDEKMPDLE
pTRIP12:Bio-eahx-LVVRGYGRVREDDEDpSDDDGSDEEIDESLAAQF
pSFRS18:Bio-eahx-GDSEDERSDRGSESSDpTDDEELRHRIRQKQEAF
pTEL2: Bio-GGGIVDGGVPQAQLAGSDSDLDpSDDEFVPYDMSGDREL
TEL2: Bio-GGGIVDGGVPQAQLAGSDSDLDSDDEFVPYDMSGDREL

'Pep-spot' substitution array

Peptide arrays were synthesized on an Intavis Multipep Peptide Synthesiser (Intavis Bioanalytical Instruments AG, Cologne, Germany). The peptides were synthesized using 9-fluorenylmethyloxycarbonyl for temporary α-amino group protection. Protecting groups used are Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl) for arginine, OtBu (t-butyl ester) for glutamic acid and aspartic acid, Trt (trt) for asparagine, glutamine, histidine, and cysteine tBu (t-Butyl) for serine, threonine and tyrosine and Boc (t-butoxycarbonyl) for lysine and tryptophan. Phosphorylated serine was incorporated as required. Loading of the membrane was reduced to 10% of standard by the incorporation of 1:9 Fmoc-beta-Ala-OH:Ac-Beta-Ala-OH as the first cycle for all peptides. Standard loading is 400 nmol/cm2. For a 6 mm diameter spot this equates to 113 nmol peptide per spot. Following coupling of the beta–Ala mix, loading was 10 nmol peptide per spot. Each amino acid was coupled by activating its carboxylic acid group with diisopropylcarbodiimide (DIC) in the presence of hydroxybenzatriazole (HOBT). Individual aliquots of amino acids were spotted on to a cellulose membrane derivatised to contain 8 to 10 ethylene glycol spacers between the cellulose and an amino group. Synthesis was accomplished by cycles of coupling of amino acids, washing and subsequent removal of the temporary α-amino protecting group by piperidine followed by more washing. Once the required number of cycles of coupling and deprotection and washing had been completed, the membranes were treated with a solution

of 20 mls containing 95% trifluoroacetic acid, 3 % tri-isopropylsilane and 2 % water for four hours. Following this treatment membranes were washed 4 times with dichloromethane, 4 times with ethanol, and twice with water to remove side chain protecting groups and TFA salts and once again with ethanol for easier drying.

The peptide array for PIH1 consensus binding-site screen was made from 16mer peptides derived from the TEL2 protein. The original sequence AGSDSDLD(pS)DDEFVPY was mutated at each position to every other amino acid. The peptide array was activated by 20 min incubation in 50 % MetOH, washed with water, blocked with 5 % milk for 30 min at room temperature and incubated with 6 ml of 5 mM 6xHis PIH1D1 N-terminal fragment 1-180, diluted in IP buffer (50 mM Tris-HCL pH 8, 150mM NaCl, 1 % w/v Triton X-100, 1 mM EDTA, 2.5 mM EGTA, 10 % v/v glycerol), at 4 ^{o}C over night. Following 3 washes with IP buffer the membrane was incubated with PIH1D1 antibody for 1 hr at room temperature. Following 3 washes with IP buffer the membrane was incubated with secondary antibody conjugated with HRP and incubated for 1hr at room temperature. Following 3 washes with IP buffer the membrane was incubated with ECL and processed in similar way as western blot.

Antibodies

The anti-phospho TEL2 antibody was reported previously (*4*). TEL2 (HCLK2) antibody has been described previously (*5, 6*). Antibodies against ATM (ab91, rabbit polyclonal), PIH1D1 (ab57512, mouse monoclonal), HSP90 (ab13492, mouse monoclonal), GST (ab6613, goat polyclonal), RUVBL1 (ab51500, mouse monoclonal and ab75826, rabbit polyclonal) and RUVBL2 (ab36569, rabbit polyclonal), UBR5 (EDD, ab4376, goat polyclonal), actin (ab8226, mouse monoclonal), ECD (hSGT1, ab99293, rabbit polyclonal) and SNRP116 (EFTUD2, ab72456) were purchased from Abcam. Antibody against RPAP3 (WH0079657M1, mouse monoclonal anti-FLJ21908), and antiFlag M2-peroxidase conjugate (A 8592, mouse monoclonal) were purchased from Sigma. RPB1 antibody (N20, sc-899) and p53 (FL-393, sc-6243) were purchased from Santa Cruz Biotechnology, p53 pS15 (#9284, rabbit polyclonal) and p53 (#9282, rabbit polyclonal) were purchased from Cell Signaling Technology and from BD Biosciences, respectively.

Supplemental Tables

Table S1, related to Figure 2. Crystallographic statistics

	PIH1D1-Tel2 (Se SAD)	PIH1D1
Data Collection		
PDB ID	4PSI	4PSF
Space group	$P2_12_12_1$	$P4_12_12$
Cell dimensions (Å)		
a, b, c (Å)	49.6, 81.8, 84.0	60.6, 60.6, 170.0
	Peak	
Wavelength (Å)	0.9790	0.92
Resolution (Å)	30.0 – 2.45	35 – 1.58
R_{merge} (%)*	12.2 (93.0)	6.8 (39.7)
R_{pim} (%)	5.4 (45.7)	2.1 (11.9)
$< I/\sigma(I) >$	10.3 (2.3)	19.0 (5.8)
Completeness (%)	99.9 (99.2)	100.0 (100.0)
Redundancy	11.1	11.4
Refinement		
Resolution (Å)	30.0 – 2.45	35.0 – 1.58
No. reflections	12991	44637
R_{work}/R_{free} (%)	24.0/26.8	17.5/19.2
No. atoms (non-H)		
Protein	1848	2107
Peptide	161	-
Water	33	287
R.m.s deviations		
Bond lengths (Å)	0.003	0.005
Bond angles (°)	0.80	0.96
Ramachandran plot (%)		
Favoured	96	97
Allowed	4	3
Disallowed	0	0

*Values in parentheses are for highest-resolution shell

Table S2, related to experimental procedures. ITC analysis

			K_d	ΔH^o	$-T\Delta S^o$	N
Protein	**Peptide**	**Sequence**	(μM)	(kcal mol^{-1})	(kcal mol^{-1})	
PIH1D1 (1-180)	TEL2	482-YAGSDSDL<u>DpSDD</u>EFVPY	3.1±0.6*	-4.9	2.3	0.8
PIH1D1 (1-180)	TEL2	482-YAGSDpSDL<u>DSDD</u>EFVPY	30.1±2.4*	-1.1	2.5	0.9
PIH1D1 (1-180)	TEL2 pT491	482-YAGSDSDL<u>DpTDD</u>EFVPY	15.0±1.5*	-1.3	5.2	0.7
PIH1D1 (1-180)	TEL2	482-YAGSDpSDL<u>DpSDD</u>EFVPY	1.5±0.2*	-5.4	2.5	0.8
PIH1D1 (40-180)	TEL2	482-YAGSDpSDL<u>DpSDD</u>EFVPY	1.4±0.2*	-5.2	2.7	1.0
PIH1D1 (51-180)	TEL2	482-YAGSDpSDL<u>DpSDD</u>EFVPY	1.5±0.2*	-6.5	1.4	0.8
PIH1D1 (51-180)	TEL2	488-DL<u>DpSDD</u>EY	10.3±2.3*	-5.2	1.5	0.7
PIH1D1 (51-180) K57A	TEL2	488-DL<u>DpSDD</u>EY	NI			
PIH1D1 (51-180) K64A	TEL2	488-DL<u>DpSDD</u>EY	NI			
PIH1D1 (51-180) R168A	TEL2	488-DL<u>DpSDD</u>EY	NI			
PIH1D1 (51-180) R163A	TEL2	488-DL<u>DpSDD</u>EY	111.9±9.4	-7.0	-1.7	0.6
PIH1D1 (51-180) K113A	TEL2	488-DL<u>DpSDD</u>EY	90.0±8.5	-6.2	-0.8	0.7
PIH1D1 (51-180) K166A	TEL2	488-DL<u>DpSDD</u>EY	58.1±4.9	-6.0	-0.3	0.6
PIH1D1 (51-180)	TEL2 S491E	488-DL<u>DEDD</u>EY	NI			
PIH1D1 (51-180)	TEL2 D490A	488-DL<u>ApSDD</u>EY	NI			
PIH1D1 (51-180)	TEL2 D492A	488-DL<u>DpSAD</u>EY	NI			
PIH1D1 (51-180)	TEL2 D493A	488-DL<u>DpSDA</u>EY	NI			
Kintoun (73-197)	TEL2	488-DL<u>DpSDD</u>EY	78.7±16.9	-6.2	-0.7	0.6
PIH1D1 (1-180)	ECD	499-RPNES<u>DpSDD</u>LDDY	16.1±0.7	-4.5	1.9	0.7

Numbers preceding the peptide sequences correspond to the position of the first amino acid in the protein sequence. The DpSDD motif is underscored and positions substituted for other amino acids are in bold with pS indicating phosphoserine. A C-terminal tyrosine residue was included for concentration determination. Experiments were performed in triplicate (indicated by an asterisk), with data shown as mean of K_d ± standard deviation. For the weaker binding titrations, errors of the fit are shown. No interaction (NI) indicates weak and/or constant signal under the condition used (~80μM protein and ~800μM peptide).

Table S3, related to Figure 3. Mass spectrometry analysis of PIH1D1 phospho-specific interacting partners

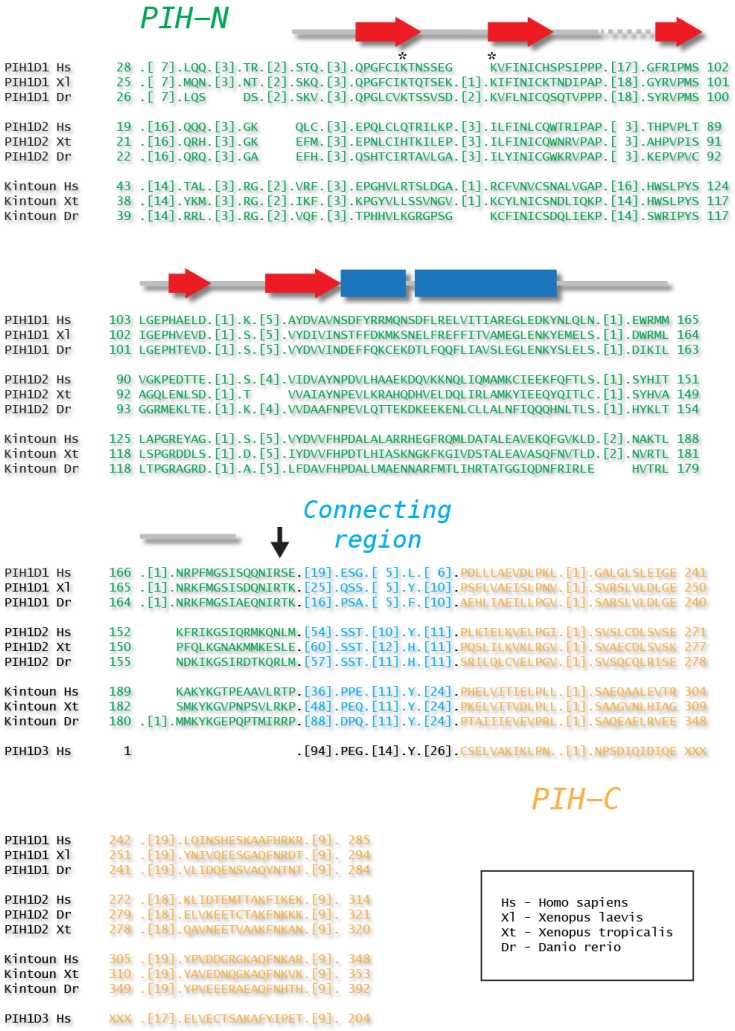
Chromatin extracts of stable transfected 293 Flp-In cells with Flag control, Flag-tagged PIH1D1 wt and Flag-tagged PIH1D1 K64A mutant expressing cells were used for anti-FLAG immunoprecipitation followed by mass spectrometry. Numbers of unique peptides identified by mass spectrometry are indicated in the table.

Name	*Number of unique peptides*		
	Flag	PIH1D1 wt	PIH1D1 K64A
PIH1D1	1	11	10
RPAP3	0	17	17
RUVBL2	0	15	13
RUVBL1	0	7	9
URI/RMP	0	6	5
TEL2	0	2	0
RPB1	0	11	1
GRDN	0	19	5
RPB3	0	2	0
GRL1A	0	4	0
RPAP2	0	8	2
HSP90B	3	7	1
Coilin	0	6	0
SNRP116	0	4	1
B7Z6H4	0	6	1
MPP9	0	10	4
PHF8	0	6	0
PSMD4	0	3	0
RL7	0	3	0
UBR5	0	7	0
LRPPR	0	12	2

Supplemental Figures

Figure S1, related to Figure 1. PIH1D1 architecture and sequence conservation.
(A) Sequence comparison of human, amphibian and fish orthologues for PIH1D1, PIH1D2 and Kintoun (NCBI CDD server). The secondary structural elements observed in the crystal structure are shown. Helix α1 occurs in a poorly conserved loop between β2 and β3 and is not shown. The connecting region of variable length and sequence is highlighted in blue and the PIH-C domain that is also present in PIH1D3 is shown in gold. Asterisks denote the positions of K57 and K64 in the human protein. The vertical arrow show the site of C-terminal tryptic cleavage observed in limited proteolysis experiments. (B) The two phospho-interacting lysine residues observed in the human PIH1D1 structure (K57 and K64) are conserved from yeast to humans. (C) Binding isotherms for ITC titration of PIH1D1 1-180 against TEL2 phosphopeptides containing single phosphosites (pS487 or pS491) or both.

A



B

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                                        57                  64
Homo sapiens                45 STQIQPQPGFCIKTNSS--------EG--KVFINICH 73
Bos Taurus                  45 STQIQPQPGFCIKTNSA--------EG--KVFINICH 73
Mus musculus                45 STQIQPKPGFCVKTNSS--------EG--KVFINICH 73
Danio rerio                 46 SKVIRPQPGLCVKTSSV--------SDKKKVFLNICQ 74
Xenopus laevis              42 SKQIRPQPGFCIKTQT---------SEKAKIFINICK 69
Caenorhabditis elegans      13 TWLIKPLPGYVCKWKDVKING-IVNDEYRKCFVNVCH 48
Saccharomyces cerevisiae    28 VSKIEPIADFVIKTKLLSANGPEKLQDGRKVFINVCH 65
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C

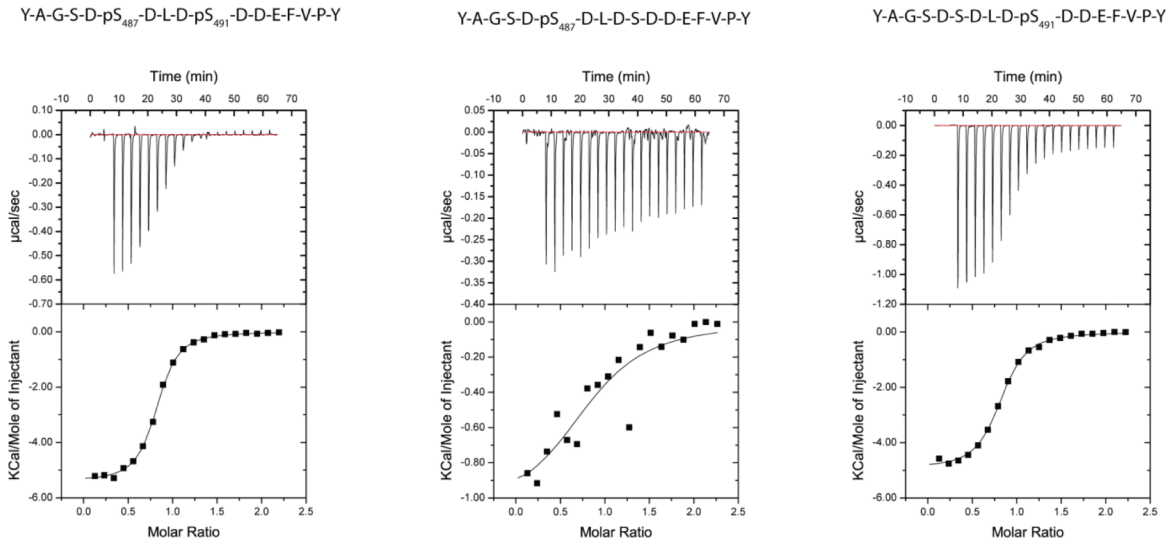


Figure S2, related to Figure 2B. TEL2 binds to a region of positive electrostatic potential.

Orthogonal views of the electrostatic potential surface are shown contoured from -10 kT (red) to +10 kT (blue).

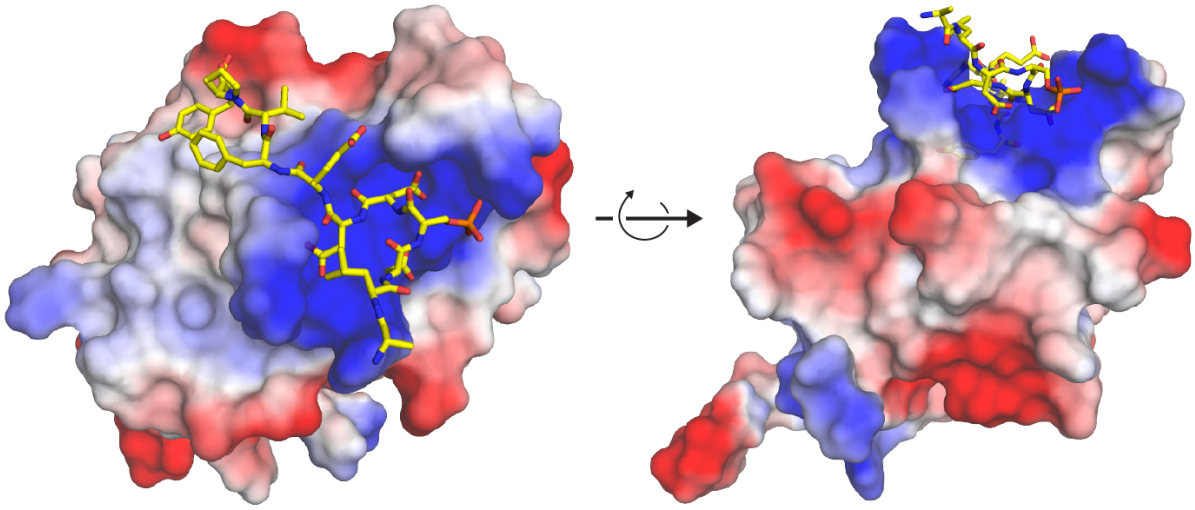


Figure S3, related to Results and Discussion. ITC analysis of Kintoun/pTEL2 interactions.

A recombinant Kintoun fragment incorporating the predicted PIH-N domain (residues 73-197) shows significant binding to the phospho-TEL2 8'mer wild-type peptide (open squares). Binding isotherm of PIH1D1 to the same peptide is overlayed (closed squares).

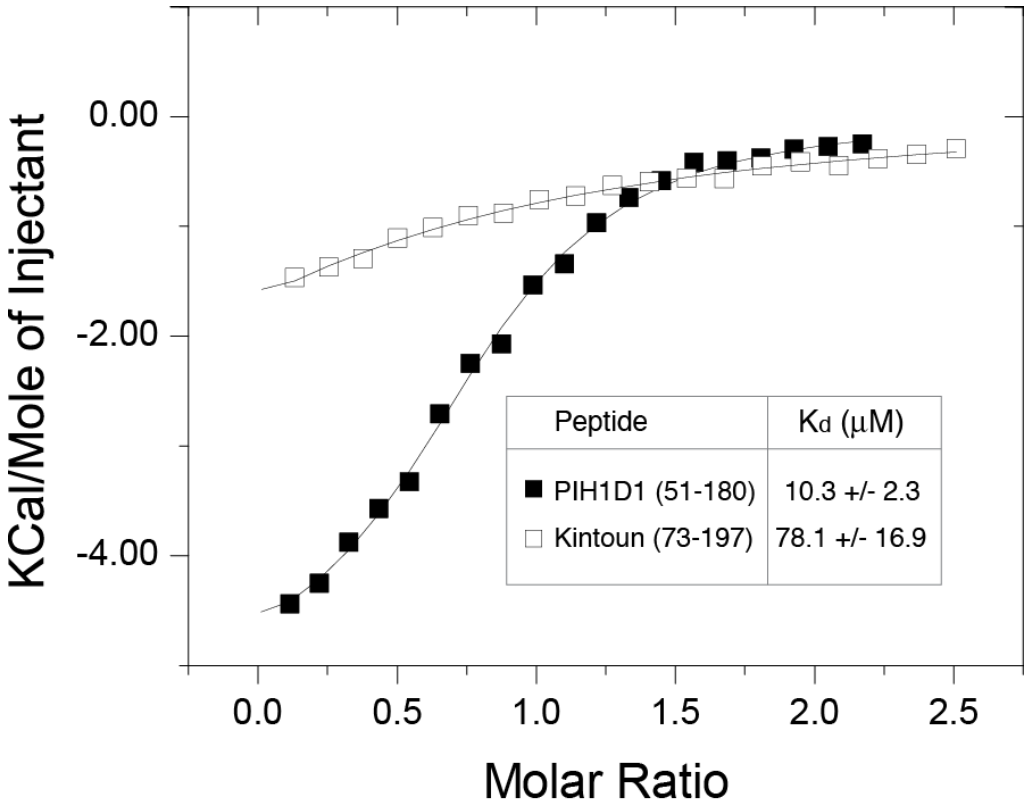
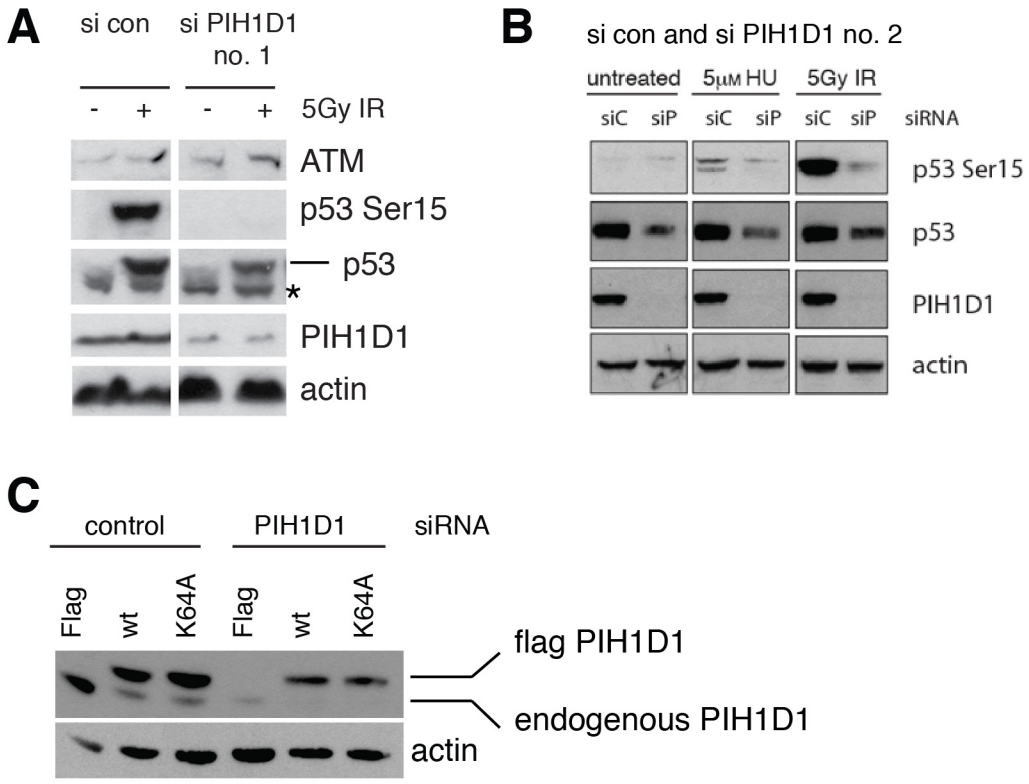


Figure S4, related to Figure 4. Depletion of PIH1D1 leads to decreased levels of p53 protein and phosphorylation levels after DNA damage

(A) RPE cells were treated with control siRNA (si con) or siRNA number 1 targetting 5' UTR region of PIH1D1 mRNA. Subsequently the cells were treated with 5 Gy of γ irradiation. Samples were collected 1 hour after irradiation. The asterisk indicates a non-specific band.

(B) U2OS cells were treated with control siRNA (siC) or siRNA number 2 targeting PIH1D1 (siP). Subsequently the cells were treated with 5mM hydroxyurea (HU) or 5Gy of IR. Samples were collected 2 hours after treatment.

(C) RPE cells were stably infected with Flag-tagged PIH1D1 wt, PIH1D1 K64A or empty retroviral vector expressing Flag. Single clones expressing Flag- PIH1D1 at levels similar to endogenous PIH1D1 were picked. The cells were treated with control siRNA (control) or siRNA targeting 5'UTR of PIH1D1 cDNA.



Supporting literature for Experimental procedures

4. Z. Horejsi *et al.*, CK2 phospho-dependent binding of R2TP complex to TEL2 is essential for mTOR and SMG1 stability. *Molecular cell* **39**, 839 (Sep 24, 2010).
5. A. Ciccia *et al.*, Identification of FAAP24, a Fanconi anemia core complex protein that interacts with FANCM. *Mol Cell* **25**, 331 (Feb 9, 2007).
6. S. J. Collis *et al.*, HCLK2 is essential for the mammalian S-phase checkpoint and impacts on Chk1 stability. *Nat Cell Biol* **9**, 391 (Apr, 2007).