Supplemental Material

Supplemental Experimental Procedures

Antibodies

Anti-EB1 (mouse) and CLIP-170 (rabbit) antibodies were generous gifts from Franck Perez (Institut Curie, Paris). MARK1 (rabbit, 3319) antibody was from Cell Signaling. Monoclonal anti-HA was from Covance, CKAP5 (rabbit) from QED Bioscience, MAP4 (rabbit, A301-488A) and GEF-H1 (rabbit, A301-928A) from Bethyl Laboratories.

FACS analysis

Cultured A549 cells were treated with control reagents or USP21 siRNA at 45nM for 120 hours before harvesting. Cells were washed with ice cold PBS and fixed by adding drop-wise into 70% ethanol. After 12 hours incubation at 4ºC, cells were washed once with cold PBS and resuspended in PBS/0.1%TX100 supplemented with 20µg/ml propidium iodide and 100µg/ml RNase (both from Sigma). Samples were incubated at 37ºC for 15 minutes and then stored at 4ºC protected from light until analysis by flow cytometry using a FACScan cytometer (Becton Dickinson).

Real time PCR

Cultured A549 cells were incubated with USP21 siRNA (oligos 5, 6, 8, and pool) for 24 hours before RNA was extracted with the Qiagen RNAeasy kit according to manufacturer's instructions. cDNA was generated using a QuantiTect® reverse transcription kit (Qiagen). RT-PCR reactions were carried out with the DyNAmo HS SYBR Green qPCR kit (Finnzymes) on a BioRad iQ5 system. USP21 mRNA levels were normalised to actin. Primer sequences used are as follow, USP21 (forward 5'-GAGCTCACTGAAGCCTTTGC, reverse 5'-CCATGAGGAGCTTCAGGAAC) and actin (forward 5'-GATCATTGCTCCTCCTGAGC, reverse 5'-CGTCATACTCCTGCTTGCTG).

MTS assays

500,000 A549 cells were seeded in a 6cm dish and treated with non-targeting control oligos (NT1) or USP21 siRNA (oligos 5, 6, and 8) for 48 hours. Cells were collected and counted; no difference in cell number was observed. 4000 cells per condition were re-seeded into a 96 well plate in 7 replicates and incubated with NT1 or USP21 siRNA for a further 72 hours, (120 hours total). The amounts of viable cells resulted per condition was compared using CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega), in which a MTS tetrazolium compound is bio-reduced by metabolically active cells into a colored formazan product, and measured by spectrometry.

Mass spectrometry

Following immunoprecipitation from NP40 cell lysates, samples were reduced with 10mM DTT (98°C, 5 minutes) and alkylated with 50mM 2-Chloroacetamide (room temperature, 30 minutes) before subjected SDS PAGE analysis (4-12% NuPAGE (Invitrogen)). Protein gels were stained with Novex Colloidal Blue staining kit (Invitrogen). Each lane was cut into 12 slices for in gel digestion with trypsin. Tryptic peptides were separated on a 75μmx25cm C18 column with a 39 minutes acetonitrile gradient using a nanoAquity UPLC system (Waters) connected to a Thermo LTQ orbitrap XL mass spectrometer. Spectra were acquired with a top 5 CID method and analyzed with MaxQuant 1.0.13.13 (Cox et al, 2008).

FAMILY	GENE NAME	COMMON ALIAS	GENE ID	LOCALIZATION	FIG.
UCH	UCHL1		7345	N=C	S1C
UCH	UCHL3		7347	N=C	S1C
UCH	UCHL5		51377	N>C	S1C
UCH	BAP1		8314	N>>C	S1C
USP	USP1		7398	Nuc	S1A
USP	USP2		9099	Cyt aggr.	-
USP	USP4		7375	N=C	S1C
USP	USP5		8078	N>C, (N<C)	S1C
USP	USP6		9098	Plasma Membrane	1
USP	USP7		7874	Nuc	S1A
USP	USP8	UBPY	9101	Cyt & endosomes	1
USP	USP9X		8239	N=C, (&PM)	S1C
USP	USP10		9100	Cyt	S1B
USP	USP11		8237	Nuc	S1A
USP	USP12		219333	N<C	S1C
USP	USP13		8975	N>C, &PM	S1C
USP	USP14		9097	N<C	S1C
USP	USP15		9958	N<C	S1C
USP	USP16		10600	Cyt	S1B
USP	USP18		11274	Cyt	S1B
USP	USP19		10869	ER	1
USP	USP20		10868	ER?	1
USP	USP21		27005	MTs & Centrosome	1
USP	USP25		29761	Cyt & Cyt aggr.	-
USP	USP26		83844	Nuc	S1A
USP	USP28		57646	Cyt aggr.	-
USP	USP29		57663	Nuc	S1A
USP	USP30		84749	Mitochondria	1
USP	USP36		57602	Nucleoli (excl)	S1A
USP	USP38		84640	N>C, & filipodia?	S1C
USP	USP39	SAD1	10713	Nuc (incl. nucleoli)	1
USP	USP42		84132	Nuc	S1A
USP	USP44		84101	Nuc	S1A
USP	USP46		64854	N<C	S1C
USP	USP49		25862	Nuc	S1A
USP	USP50		373509	N<C	S1C
USP	PAN2	USP52	9924	Cyt	S1B
USP	USP53		54532	PM (restricted), Cyt	1
USP	USP54		159195	Cyt aggr.	-
USP	USPL1		10208	Nuc	S1A
USP	CYLD		1540	N<C, & PM	S1C
OTU	OTUB1		55611	N<C	S1C
OTU	OTUB2		78990	N=C	S1C

OTU	OTUD3		23252	N<C	S1C
OTU	OTUD4		54726	Cyt	S1B
OTU	OTUD6A		139562	Cyt & NE	S1B
OTU	OTUD6B		51633	Cyt	S1B
OTU	OTUD7B	CEZANNE	56957	N=C, & PM	1
OTU	ZRANB1	TRABID	54764	N>C, & PM	S1C
OTU	VCPIP1	VCPIP	80124	N=C, & Filipodia	1
OTU	YOD1		55432	N>C, & PM	S1C
OTU	TNFAIP3	A20	7128	Cyt aggr.	-
MJD	JOSD1		9929	N<C, & PM	1
MJD	JOSD2		126119	N<C, & PM	S1C
MJD	ATXN3		4287	N=C, &PM	S1C
MJD	ATXN3L		92552	Cyt & PM	1
JAMM	BRCC3		79184	N=C	S1C
JAMM	COPS5	CSN5	10987	N<C, &PM	S1C
JAMM	COPS6	CSN6	10980	N>C, N=C	S1C
JAMM	EIF3H	EIF3S3	8667	N>C & N<C	S1C
JAMM	EIF3F	EIF3S5	8665	Cyt	S1B
JAMM	PSMD7		5713	N<C, &PM	S1C
JAMM	STAMBP	AMSH	10617	N=C, & endosomes	S1C
JAMM	STAMBPL1	AMSH-LP	57559	N=C, & endosomes	S1C
JAMM	MPND		84954	N<C	S1C
JAMM	MYSM1		114803	Nuc	S1A

Supplemental Table 1. Localization data of 66 EGFP-tagged DUBs expressed in HeLa cells. Nuc: exclusively nuclear; Cyt: cytoplasmic and excluded from the nucleus; N=C: equally distributed in nucleus and cytoplasm; N>C: predominantly nuclear; N<C: predominantly cytoplasmic; PM: plasma membrane; ER: endoplasmic reticulum; MTs: Microtubules; NE: nuclear envelope; Cyt aggr. (cytosolic aggregates).

Phosphorylation site	Probability	
	HeLa	HEK293T
S93	1	1
S111	n.d.	0.894
S113	0.579	0.915
S115	0.825	0.973

Supplemental Table 2. Phosphorylation sites identified in USP21 from label free mass spectrometry. HeLa or HEK293T cells were transfected with GFP-USP21 (WT) or C221S expressing constructs for 24 hours. GFP tagged USP21 proteins were immunoprecipitated with anti-GFP antibody coupled beads and analyzed by tandem mass spectrometry (Orbitrap XL)using collision induced dissociation. Phosphorylation sites were identified with MaxQuant 1.0.13.13 with probability cutoff of 0.75 (S113 from HeLa is shown because high probability from HEK293T). n.d., not detected.

Gene names	Protein names	TSC (HeLa)		TSC (HEK293T)	
		WT	Mut	WT	Mut
MARK2	MAP/microtubule affinity-regulating kinase 2	9	7	5	13
MAP4	Microtubule-associated protein 4	2	2	5	3
GEF-H1	Rho guanine nucleotide exchange factor 2	4	0	3	0
CKAP5	Cytoskeleton-associated protein 5	0	0	8	2
MARK3	MAP/microtubule affinity-regulating kinase 3	0	0	1	3

Supplemental Table 3: Co-immunoprecipitation of cytoskeleton related proteins. HeLa or HEK293T cells were transfected with GFP, GFP-USP21 (WT), or GFP-USP21 C221S (Mut) expressing constructs. GFP or GFP tagged proteins were immunoprecipitated with anti-GFP antibody coupled beads and analyzed by mass spectrometry. All listed proteins were seen in two experiments absent from control experiments and identified by at least two peptides. TSC, total spectral counts.

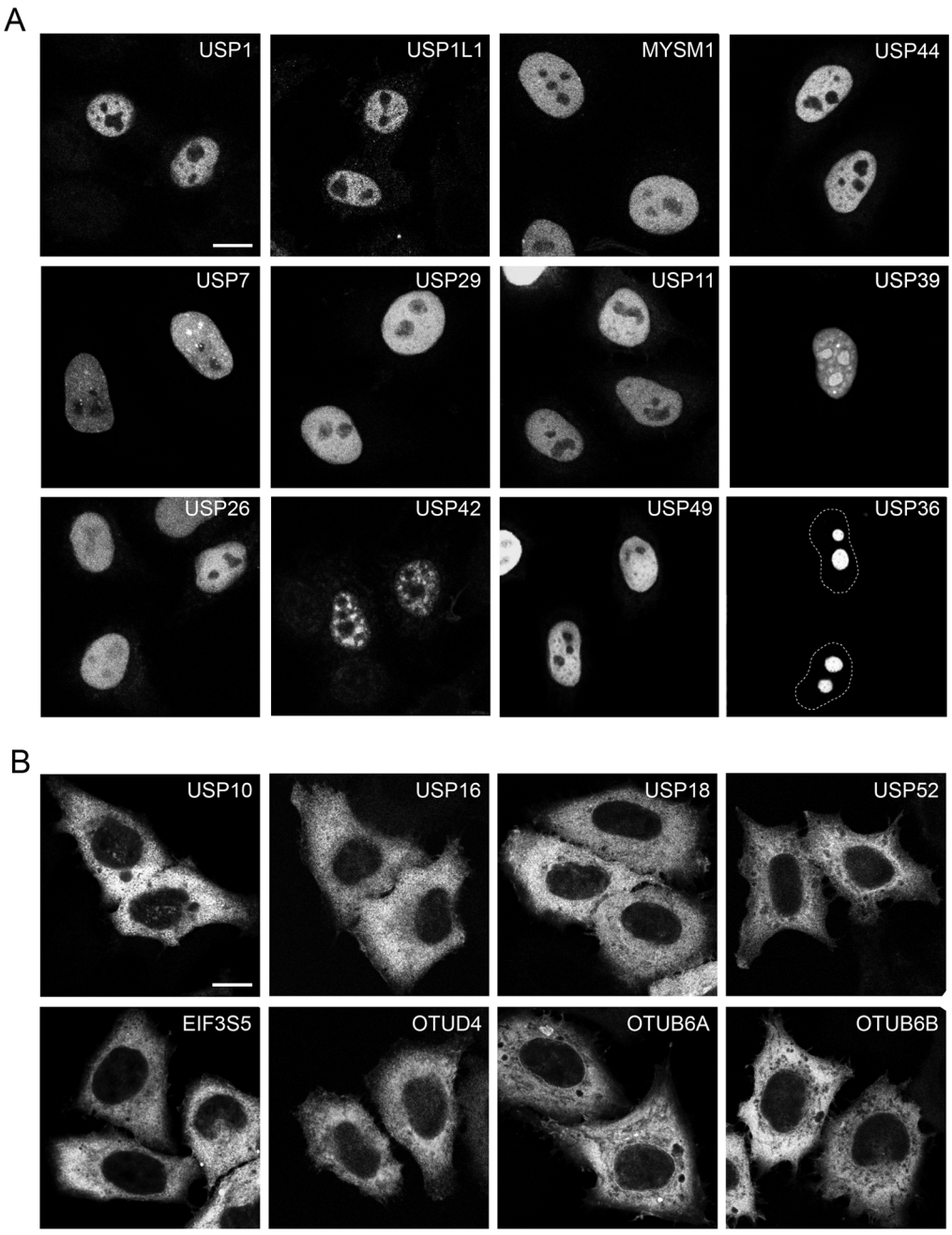
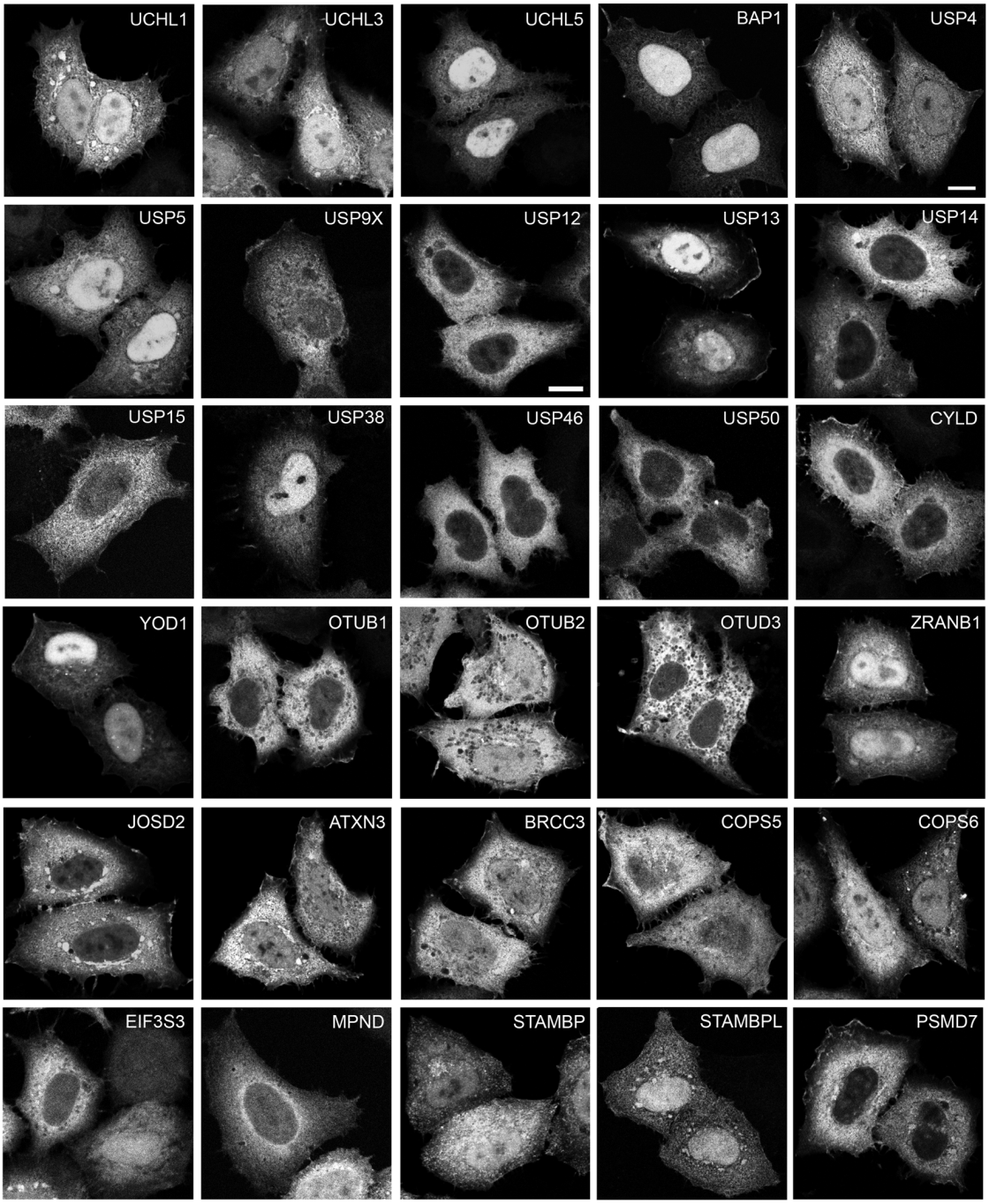


Figure S1. Subcellular localisation of EGFP-tagged DUBs. Supplementing Figure 1. (A) Nuclear DUBs (B) Cytosolic DUBs (C) Nuclear/cytosolic DUBs.

C



Urbé, Figure S1C

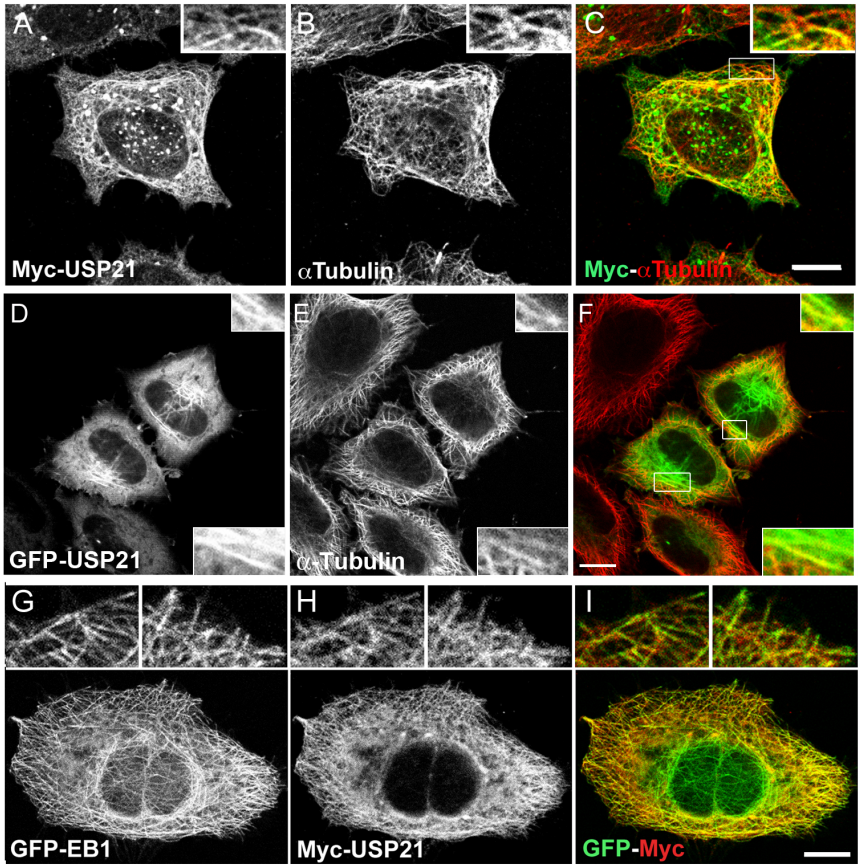


Figure S2. USP21 co-localisation with α-tubulin.
(A-C) Hela cells expressing Myc-USP21 co-stained with antibodies recognising endogenous α-tubulin and rabbit anti-Myc. (D-F) HeLa cells expressing GFP-USP21 stained with α-tubulin. (G-I) HeLa cells stably expressing GFP-EB1 transfected with Myc-USP21 and stained with a mouse anti-Myc antibody. Scale bar = 10μm.

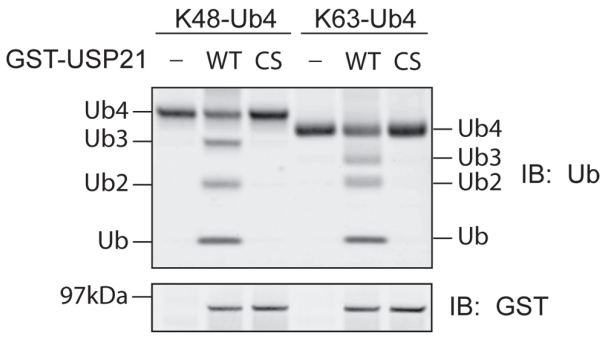


Figure S3. Catalytic activity of USP21.
In vitro cleavage of K63 and K48 tetraubiquitin (Ub4) chains by full length wild type GST-tagged USP21 (WT) but not by C221S (CS) mutant enzyme.

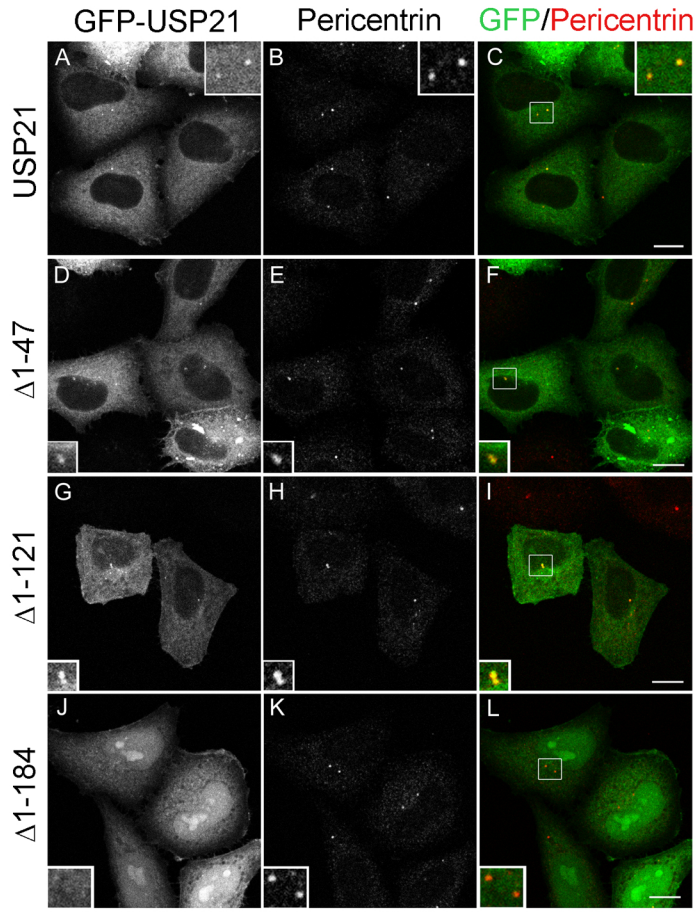


Figure S4. Deletion of the first 121 amino acids of USP21 does not interfere with centrosomal localisation.
Centrosomal localisation in HeLa cells is retained upon deletion of the first 121 amino acids (D-I), but this property is lost upon further truncation (Δ1-184) (J-L). Scale bar = 10μm.

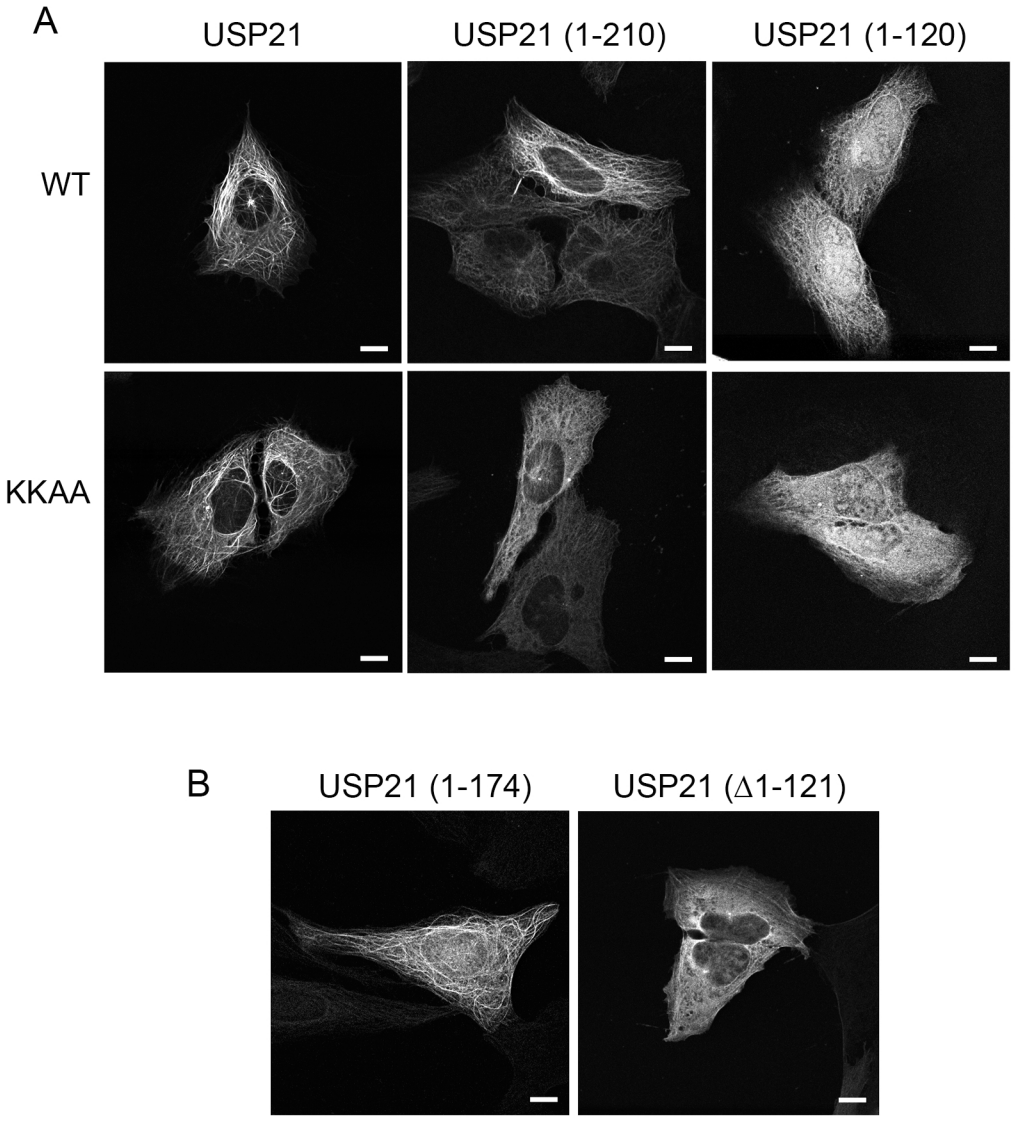


Figure S5. Mutation of putative microtubule binding motif weakens the association of the amino-terminus of USP21 to microtubules.
A) U2OS cells were transfected with GFP-USP21, GFP-USP21 (1-120), and (1-210) (WT) as well as their respective microtubule binding motif mutant counterparts (KKAA). The 1-120 fragment is only weakly associated with microtubules. Mutation of the microtubule binding motif reduces microtubule localisation of USP21 (1-210) and USP21 (1-120). B) U2OS cells were transfected with GFP-USP21 (1-174) and Δ1-121, and stained with anti-tubulin antibodies. The 1-174 fragment localises to microtubules, whilst the Δ1-121 fragment does not.

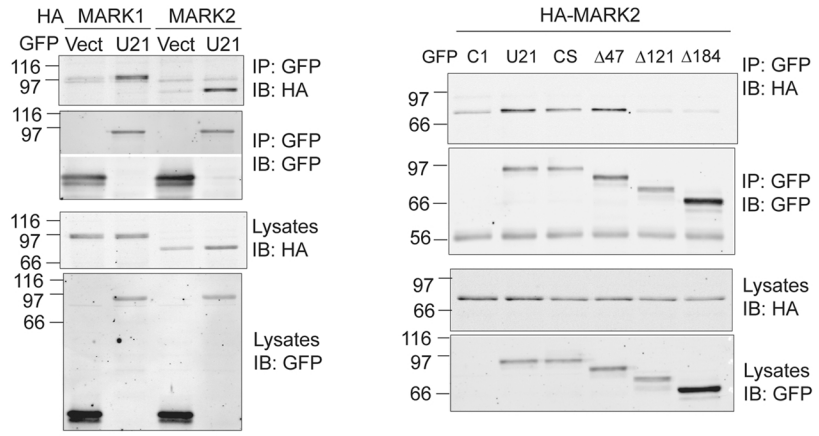


Figure S6. Mapping the requirements for USP21 interaction with MARK2. (A) HEK293T cells were co-transfected either with GFP-vector control or GFP-USP21 (U21) and HA-MARK1 or HA-MARK2 as indicated. Top panel shows specific co-immunoprecipitation of HA-MARKs. (B) Interaction of HA-MARK2 with full length USP21 independent of catalytic activity and with the N-terminal truncation mutant Δ1-47, but not with further truncations.

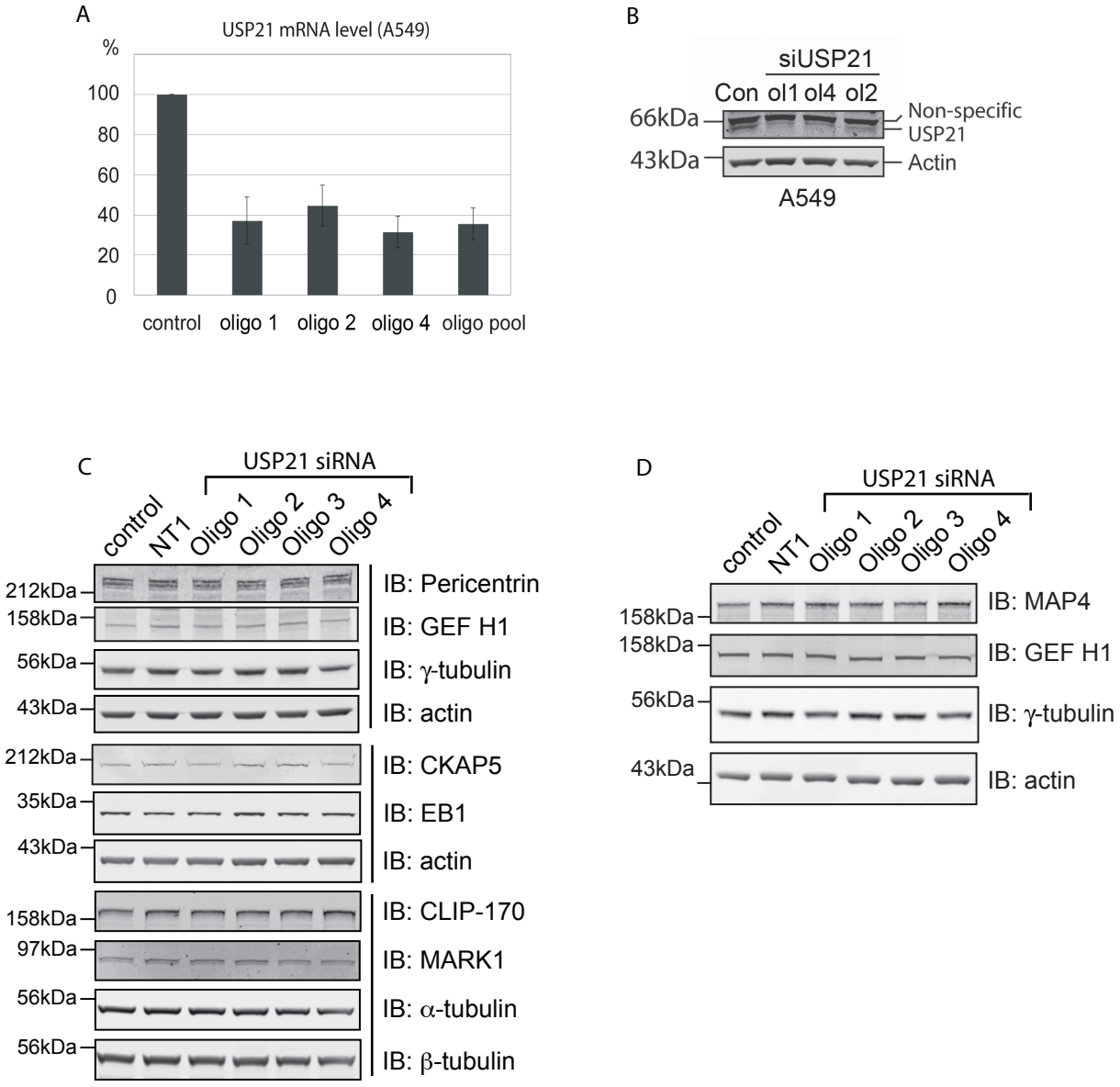
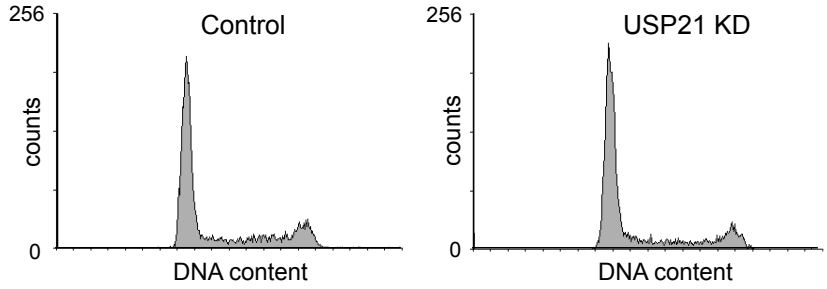


Figure S7. Characterisation of siRNA oligonucleotides.
(A) RT-PCR, A549 lung adenocarcinoma cells 24 hours post-transfection with indicated oligonucleotides (B) Western blot analysis of endogenous USP21 treated with USP21-targeting siRNA oligos for 120 hrs. (C) Levels of centrosomal proteins pericentrin and γ--tubulin, the +end microtubule binding proteins EB1 and CLIP-170, α- and β-tubulin and MARK1, all proteins associated with microtubule function, as well as USP21 interacting proteins CKAP5 and GEF-H1 are unaffected by siRNA-mediated USP21 depletion, following "hot lysis" of A549 cells. (D) USP21 interacting proteins MAP4 and GEF-H1 are unaffected by siRNA-mediated USP21 depletion, following RIPA lysis of A549 cells.

A



Cells	%G1	%S	%G2/M
Control	63.7	22.5	13.8
USP21 KD	66.7	20.8	12.5

B

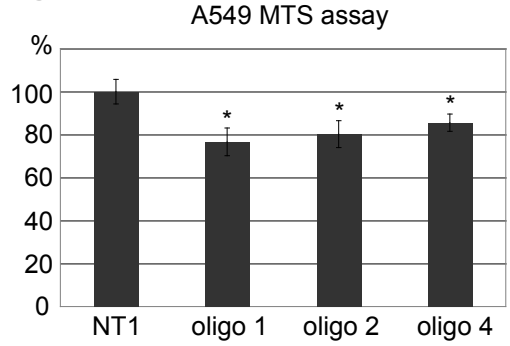


Figure S8. Effects of siRNA knock-down.
(A-D) A549 cells were incubated for 120 hours with siRNA oligos specific to USP21 (A) Cell-cycle parameters as judged by FACS analysis were not significantly affected (pooled oligos). Control corresponds to no oligo. (B) Cell viability as judged by MTS assay shows a modest reduction across 3 individual oligos versus a non-targeting oligo (NT1).