Systematic survey of deubiquitinase localisation identifies USP21 as a regulator of centrosome and microtubule associated functions.

Sylvie Urbé§*, Han Liu§ #, Sebastian D. Hayes§†, Claire Heride§, Daniel J. Rigden¶, Michael J. Clague§

§ Cellular and Molecular Physiology, Institute of Translational Medicine, University of Liverpool, Crown Street, Liverpool L69 3BX, UK, ¶ Institute of Integrative Biology, University of Liverpool, Crown Street, Liverpool L69 7ZB, UK.† Current Address: Department of Pathology, Harvard Medical School, Boston, MA 02115, USA.

* corresponding author, email: urbe@liv.ac.uk
# these authors contributed equally to this study

Key words: USP21, ubiquitin, deubiquitination, microtubules, centrosome, DUB, localisation

Running head: USP21 regulates microtubule dynamics

Abstract

Ubiquitination is a reversible modification that influences a broad range of physiological processes. There are approximately 90 DUBs encoded in the human genome, of which 79 are predicted to have catalytic activity. We have tagged 66 DUBs with GFP and systematically surveyed their sub-cellular distribution, identifying enzymes specific to the nucleus, plasma membrane, secretory and endocytic pathways. USP21 is unique in showing clear association with both centrosomes and microtubules. Using an in vitro assay, we show that microtubule binding is direct and identify a novel microtubule binding motif, encompassed within amino acids 59-75 of the N-terminus of USP21. Our functional studies indicate a key role for USP21 in the governance of microtubule and centrosome associated physiological processes: Depletion of USP21 in A549 cells compromises the re-establishment of a radial array of microtubules during recovery from cold-induced depolymerisation and also reduces the probability of primary cilium formation, whilst USP21 knockdown in PC12 cells inhibits NGF-induced neurite outgrowth.

Supplemental Material can be found at: http://www.molbiolcell.org/content/suppl/2012/01/31/mbc.E11-08-0668v1.DC1.html
Introduction

Ubiquitination is a reversible post-translational modification involved in most complex cellular processes, including cell division, DNA repair, membrane trafficking and cell signalling. Deubiquitination is mediated by specific deubiquitinases (DUBs), of which there are around 79 active enzymes that can be predicted from the human genome sequence (Komander et al., 2009; Reyes-Turcu et al., 2009). They can be sub-divided into 5 families, Ubiquitin C-terminal Hydrolases (UCH), Ubiquitin Specific proteases (USP), Ovarian Tumour (OTU), Josephins (Machado-Joseph disease; MJD) and JAB1/MPN/Mov34 metalloenzymes (JAMM/MPN+). The first four families are cysteine proteases, whilst the JAMM family belongs to the zinc metalloproteases. In general, DUBs may act to rescue proteins from ubiquitin-dependent degradation processes or to reverse ubiquitin signals.

We have surveyed the subcellular localisation of > 60 GFP-tagged DUBs in HeLa cells and subsequently focused our attention on the unique example of clear centrosome and microtubule association, namely USP21. This sparsely studied member of the USP family has been suggested to influence cell growth and to display dual specificity for both ubiquitinated and neddylated conjugates (Gong et al., 2000). However, a recent study has clearly shown promiscuous activity towards ubiquitin chain types and a high degree of discrimination against Nedd8 conjugated substrates (Ye et al., 2011). A microarray study observed USP21 levels to change during liver regeneration and proposed the relevant target to be mono-ubiquitinated histone H2A (Nakagawa et al., 2008). Recently, USP21 has also been proposed to be a negative regulator of NFκB signalling (Xu et al., 2010).

Reversible ubiquitination is fundamental to mitosis and cytokinesis, but other links between the ubiquitin/proteasome system and microtubule cell biology are emerging. Ubiquitination sites for α- and β-tubulin have been identified using mass spectrometry (Meierhofer et al., 2008). The DUB CYLD can associate with microtubules through a CAP-Gly domain, regulate their dynamics and control entry into mitosis (Stegmeier et al., 2007; Gao et al., 2008; Wickstrom et al., 2010), whilst a ubiquitin C-terminal hydrolase, UCHL1, has also been found associated with microtubules in certain cell types (Bheda et al., 2010). The E3
ligase BRCA1 is required for γ-tubulin localisation to the centrosome (Sankaran et al., 2007). Following proteasome inhibition, various proteins accrue at the pericentriole and the cells become impaired in microtubule aster formation (Didier et al., 2008). In post-mitotic neurons, a centrosomal Cdc20-APC ubiquitin signalling pathway drives dendrite formation (Kim et al., 2009).

In this study, we show that USP21 associates with both centrosomes and microtubules. We map the sequence determinants responsible for such localisation, demonstrate direct microtubule-binding properties and identify several microtubule associated proteins as USP21 binding partners. We go on to conduct functional studies, in which we implicate USP21 in microtubule network regeneration following cold depolymerisation, in primary cilia formation and in neurite outgrowth from rat pheochromocytoma PC12 cells.

Results

**Analysis of subcellular localisation of DUBs**

We have undertaken a systematic analysis of the subcellular localization of GFP-tagged DUBs in HeLa cells (Figure 1 and Supplemental Figure S1 and Table S1). A significant number accumulate in the nucleus (12 exclusively nuclear, 9 predominantly nuclear), including some, which are enriched in identifiable sub-nuclear structures such as the nucleolus (USP36 (Endo et al., 2009), Figure 1, Supplemental Table 1, Supplemental Figure S1). Many are cytosolic under our conditions of over-expression, but clear association with identifiable structures can be seen in several cases, including endosomes (AMSH/STAMBP, AMSH-LP/STAMBPL, UBPY/USP8) (McCullough et al., 2004; Nakamura et al., 2006; Row et al., 2006), mitochondria (USP30) (Nakamura and Hirose, 2008), plasma membrane (USP6) (Martinu et al., 2004) and endoplasmic reticulum (USP19) (Hassink et al., 2009), which we have verified through co-localisation with other markers. By its very nature, our screening procedure will be prone to the occasional example where the appended GFP tag interferes with the correct localisation. Indeed, our N-terminally GFP-tagged CYLD localised to the plasma membrane and cytosol, but did not show noticeable microtubule staining, in contrast to the clear microtubule localisation of CYLD tagged with GFP at the C-terminus and of endogenous protein reported by others (Supplemental Figure S1, (Stegmeier et al., 2007; Gao et al., 2008; Wickstrom et al., 2010)).
**USP21 associates with the microtubular cytoskeletal network**

Out of 66 GFP-tagged DUBs, only GFP-USP21 (or Myc-tagged USP21, see Supplemental Figure S2), displayed a distribution that was suggestive of microtubular localisation (Figure 2, 2A-C). In interphase cells, expressing moderate levels of GFP-USP21, fluorescent microtubules were mostly located in close proximity to the nucleus (Figure 2C and D-F and Supplemental Figure S2 D-F). Microtubule-association of GFP-USP21 was also prominent in late telophase and in cells undergoing cytokinesis; in these cells USP21 appears to redistribute to the polar and midbody microtubule network (Figure 2A, B, G-I). At low expression levels, GFP-USP21 and Myc-USP21 are predominantly cytosolic together with a clear accumulation at the centrosome where they co-localise both with Pericentrin and γ-Tubulin, suggesting that the microtubule organising centre/centrosome provides a high-affinity recruitment site for USP21 (Figure 3).

High levels of GFP-USP21 promote microtubule bundling in a manner commonly observed for microtubule-associated proteins (MAPs) (Figure 4A-C) (Olson et al., 1995). Intriguingly, a catalytically inactive mutant of USP21 (C221S, Supplemental Figure S3) is not effectively recruited to microtubules in HeLa cells but retains the primary localisation to the mid-body (Figure 4D-F) as well as to centrosomes (Figure 4G-I). We did however observe some microtubule localisation for this inactive mutant in the osteosarcoma cell line U2OS, albeit at high expression levels only (Figure 4J-L).

**Mapping the requirements for USP21 association with centrosomes and microtubules**

Whilst the C-terminus of USP21 contains its catalytic domain, the amino-terminus (1-212) is intrinsically disordered and contains multiple proline-rich sequences that are commonly involved in protein-protein interactions. We therefore constructed a set of N-terminal and C-terminal deletion mutants for analysis of USP21 associations in HeLa and U2OS cells (Figure 5 and Supplemental Figure S4). Deletion of the first 184 amino acids (USP21Δ1-184) abolishes microtubule and centrosomal localisation. This construct partially localises to the nucleus with enrichment in nucleoli, consistent with the loss of a CRM1-dependent nuclear export sequence (NES; aa134-147). USP21Δ1-121 can still localise to centrosomes, but does not extend along microtubules or bundle them (Figure 5 and Supplemental Figure S4). USP21Δ1-47 behaves as full length with respect to microtubule bundling, centrosome and microtubule
association. Our C-terminal deletion constructs appeared largely cytosolic in HeLa cells, but retained microtubule localisation in U2OS cells to different degrees (Supplemental Figure S5). Removal of the catalytic domain confirmed that the first 210 amino acids are sufficient to target GFP to microtubules. Further truncation to aa 174 did not affect microtubule association, whilst truncation to the first 120 amino acids markedly reduced microtubule localisation.

We next tested for direct binding to microtubules using an *in vitro* assay, which depends upon redistribution of purified protein in the presence of microtubules to a pellet fraction following high-speed ultracentrifugation (Figure 5E). Full length protein was not amenable to this analysis owing to its own tendency to pellet independently of microtubules under the conditions of this assay. We therefore took a fragment based approach. Both GST-USP21 (1-120) and GST-USP21 (1-87) showed substantial microtubule association. Serendipitously, our GST-USP21 (1-120) preparation contains a lower molecular weight degradation product, fractionally smaller than the first 87 amino acids, which has lost the ability to bind microtubules. Analysis of this sequence revealed high homology with motifs previously identified in Tau and Adenomatous Polyposis Coli (APC) proteins. The Tau motif is also involved in microtubule binding (Goode *et al*., 1997), whilst the APC motif is part of a larger region known to bind microtubules (Deka *et al*., 1998). Mutation of matching lysine residues within this motif abrogates microtubule binding of the 1-87 N-terminal fragment *in vitro* (Figure 5F and G). Interestingly this motif is adjacent to the major sites of phosphorylation we have been able to detect within USP21 using mass spectrometry (S93, S111, S113 and S115, Supplemental Table 2). Although this sequence is essential for *in vitro* binding of aa 1-87, expression of the full length protein bearing these mutations in HeLa and U2OS cells, nevertheless retained a microtubular disposition indistinguishable from wild-type USP21, suggesting that multiple binding modes must exist, reflecting a common feature of microtubule associated proteins (Supplemental Figure S5). Mutation of the microtubule binding motif in the context of the (1-210) N-terminal fragment reduces microtubule association, whilst mutation of the shorter (1-120) fragment, largely abolished the residual level of microtubule association observed with this construct (Supplemental Figure S5).
Candidate interacting partners

In a recent comprehensive proteomic survey of DUB interaction partners, Sowa and collaborators found that all four microtubule-affinity regulating kinases (MARK) co-immunoprecipitate with HA-tagged USP21, stably expressed in HEK293 cells (Sowa et al., 2009). We confirmed this interaction by Western blotting following co-immunoprecipitation of GFP-USP21 and HA-tagged MARKs 1 and 2 and mapped this interaction to the same region that was required for microtubule localisation in intact cells (Supplemental Figure S6). Our own mass spectroscopic analysis of GFP-USP21 interactors in HeLa and HEK293T cells also suggested association with endogenous MARK2 and MARK3 as well as MAP4, GEF-H1 (ARHGEF2), CKAP5, which are all annotated as microtubule associated (Supplemental Table S3). Despite multiple attempts to generate anti-peptide and anti-fusion protein antibodies against USP21 in three different species, we failed to obtain a high affinity antibody capable of detecting endogenous levels of USP21 by immunofluorescence. We were however able to determine the efficacy of three out of four siRNA oligonucleotides targeting USP21 by RT-PCR and by western blotting, using our affinity-purified anti-USP21 antibody on concentrated RIPA buffer cell lysates (Supplemental Figure S7A, B). However, significant knock-down of USP21 did not result in changes to the protein expression levels of any of these USP21 interacting proteins, nor did we observe an upshift in protein mass that might be indicative of enhanced ubiquitination (Supplemental Figure S7 C and D).

USP21 regulates microtubule regrowth and primary cilium formation in A549 cells

Knock-down of USP21 had no effect on the levels of γ-tubulin, a centrosomal component known to influence microtubule aster formation, nor on a range of proteins known to be associated with interphase microtubule dynamics (Didier et al., 2008) (Supplemental Figure S7C). We also assessed a potential role for USP21 in mitosis, but did not find any significant alteration in the distribution of cells within the cell cycle (as judged by FACS analysis) in accordance with published data (Garnett et al., 2009), although a modest effect on cell growth/viability could be detected after prolonged knock-down (Supplemental Figure S8A, B).

A549 lung adenocarcinoma cells treated with siRNA oligos targeting USP21 display an extensive microtubule network comparable to control cells. Given its preferential centrosomal localisation at low expression levels, we
wondered whether USP21 may play a role in nucleating new microtubule growth radiating from the centrosome. We used a well-established assay measuring re-growth of microtubules after cold-induced depolymerisation to assess the effect of depleting USP21 by siRNA (Figure 6). Within 2.5 minutes, control cells re-establish a radial array of new microtubules extending to the periphery of the cell. Following USP21 knock-down with either of two independent oligonucleotides this process is markedly impaired. In those cells, which do establish a microtubule network, it is usually less ordered, only rarely displaying a clear radial pattern emanating from the centrosome and extending all the way to the cell periphery.

One particular aspect of centrosome function in resting cells is the establishment and maintenance of the primary cilium, a sensory organelle emanating from a modified centriole, the basal body, and consisting of a discrete array of particularly stable microtubules, that is a key site of multiple signal transduction cascades (Goetz and Anderson, 2010). Based on our observation that USP21 is required for effective microtubule regrowth from centrosomes, we wondered whether its depletion may also negatively affect the generation of a primary cilium upon serum starvation. Indeed, we observed a significant reduction in the number of A549 cells developing a primary cilium upon serum starvation when USP21 was depleted with two independent siRNA oligos (Figure 7). This reduction was comparable (~60% in our hands) to that observed with a known regulator of ciliogenesis, VHL, (Thoma et al., 2007). We also confirmed these results in hTERT-RPE1 cells, in which a higher % of cells respond to serum starvation by forming a primary cilium: 69% in cells treated with non-targeting control oligo, compared to 47% in cells treated with siUSP21 Oligo 1, 39% with Oligo 2 and 43% with Oligo4. We did not observe any significant changes in primary cilium length in USP21-depleted cell populations.

Requirement of USP21 for neurite out-growth in PC12 cells

We turned to another physiological process intimately connected with microtubule reorganisation, that of neurite outgrowth. Rat pheochromocytoma PC12 cells respond to application of NGF by the outgrowth of branching neurites, which become visible after two to three days (Black et al., 1986). We transfected cells with HUSH plasmids expressing shRNA targeting USP21 together with RFP translated from the same transcript using an IRES (internal ribosomal entry site). After 3 days in the presence of NGF, we measured neurite length relative to the
cell body (Figure 8). We observed a significant decrease for all four shRNA targeting plasmids in the number of cells with neurites, defined as extensions larger than the maximum chord length across the cell body.

**Discussion**

Systematic analysis of interaction partners (Sowa et al., 2009) or subcellular localisation of DUBs can provide clues to cellular processes, within which they may function. In this study, we provide the most comprehensive analysis of mammalian DUB localisation hitherto undertaken, providing a unique resource for future studies of this important class of enzymes. Our data is complementary to a recent survey of DUB localisation within fission yeast (Kouranti et al., 2010). However, in that organism there are only 20 family members and assignment of mammalian orthologues is not straightforward.

We focused our further attention on USP21, which presented in our screen of GFP-tagged DUBs as the only microtubule and centrosome associated protein. Further analysis revealed that the centrosome represents the site of highest concentration at low expression levels, suggesting that this may be the primary recruitment or highest affinity binding site for USP21. Whilst CYLD and UCHL1 have also been shown to associate with microtubules (Bheda et al., 2010)(Stegmeier et al., 2007; Gao et al., 2008), this is a unique example of a centrosome associated DUB. As we have been unable to generate an antibody that is capable of detecting endogenous USP21 by immunofluorescence, our localisation data are limited to tagged proteins. Whilst these are strongly supported by our in vitro binding and interaction data, as well as by functional evidence discussed below, the localisation of endogenous USP21 will require further validation in future studies as and when new tools become available. We were able to show that binding to microtubules is direct and specified in part by a region of the N-terminal domain distinct from the sequence necessary for centrosome binding. Interestingly, the USP21 sequence suggested to encompass a microtubule binding site contains a KKLeLgR motif juxtaposed to a proline rich region, similar to a motif required for microtubule binding of Tau (KKVaVvR) (Goode et al., 1997).

Our identification of USP21 as a DUB associated with centrosomes and microtubules, and functioning in several physiological processes depending on microtubule reorganisation, ties in well with the mass spectrometric DUB
interaction survey data provided by Sowa et al., which indicated an association with all four members of the microtubule affinity regulating kinase family (MARK 1-4) (Sowa et al., 2009). The DUB USP9X has previously been shown to regulate MARK4 activity through its ubiquitination status (Al-Hakim et al., 2008). We have here confirmed interactions with MARK1 and MARK2, however we have been unable to provide any parallel observation in this case using co-expressed MARKs, nor did we observe any changes in steady state levels of endogenous MARK1 and MARK3 proteins. We also report three novel interactions with microtubule associated proteins: MAP4, GEF-H1 (ARHGEF2) and CKAP5. Whilst these do not appear to be direct targets of USP21, as their stability and ubiquitination status are not altered upon manipulation of USP21 expression, they lend further credence to the association of USP21 with microtubule-dependent processes.

We observed no major influence on cell growth, or in distribution of cell population between cell-cycle phases. We therefore pursued a functional role of USP21 in regulating microtubule dynamics of interphase cells. Our data suggest a physiological role for USP21 in the establishment and organisation of a new microtubule network following cold-induced depolymerisation. A similar observation has been reported in HeLa cells following knock-down of CYLD (Gao et al., 2008). Dominant negative effects, we observe upon overexpression of USP21 (not shown), have so far thwarted our attempts to demonstrate a direct requirement for catalytic activity in this assay. USP21 depletion in both resting A549 and hTERT-RPE1 cells also reduces the probability of serum starvation induced primary cilium formation, further corroborating a requirement for USP21 in centriole (basal body) dependent microtubule outgrowth in interphase cells. Major microtubule reorganisation is also an essential component of NGF-dependent neurite extension, but is not required for the transcription-dependent “priming” of this remodelling event (Greene et al., 1982). We propose a role for USP21 in this physiologically important process that is concordant with its governance of microtubule dynamics. Taken together these data indicate a complex and fundamental role for reversible ubiquitination events in the choreography of microtubule regrowth.

Future work will need to address the full range of ubiquitination events necessary for centrosome and microtubule function in non-mitotic cells, as well as the salient targets of USP21, which have so far remained elusive. We
anticipate that as a unique centrosomal DUB and a rare microtubule associated enzyme, USP21 will be one of the major DUBs controlling these processes.

Materials and Methods

Cell culture and transfection

A549, HeLa, U2OS and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% non-essential amino acids. HeLa cells stably expressing either mCherry-α-tubulin or GFP-EB1 were kindly provided by Anna Akhmanova (Erasmus University, Rotterdam). Telomerase-immortalized retinal pigmented epithelial (hTERT-RPE1) cells were provided by Francis Barr and cultured in DMEM/F12 supplemented with 10% FBS and 1% non-essential amino acids. PC12 cells were provided by Giampietro Schiavo (CRUK London Research Institute) and grown in DMEM, 7.5% FBS, 7.5% filtered horse serum, 4mM Glutamine, 1% Pen/Strep. HeLa, U2OS and HEK293T cells were transfected with plasmids using Genejuice (Novagen) and fixed or lysed after 24 hrs. PC12 cells were transfected with HuSH-29 constructs using Lipofectamine LTX reagent at a 3:1 ratio and Plus reagent (Invitrogen). For siRNA experiments, A549, HeLa and hTERT-RPE1 cells were treated with non-targeting (NT1) or target specific siRNA oligos (Dharmacon On Target Plus oligos, Thermo Fisher Scientific) at 40-50nM final concentration using Oligofectamine transfection reagent (Invitrogen) twice over a period of 120 hrs.

Plasmids, antibodies and other reagents

The human USP21 ORF (NM_012475.4) was amplified from human brain cDNA library (Clontech) using a two step PCR reaction with KOD Hot Start DNA Polymerase (Novagen) and fully sequence verified; primers available upon request. The ORF was inserted into pDONR223 and then shuttled into Gateway converted pEGFP-C, pCMV-Myc and pGEX6P expression vectors. A catalytically inactive mutant was constructed by QuickChange mutagenesis introducing a T to A mutation at position 661 resulting in Cys 221 to Ser (CS) conversion. Lys 64 and Lys 65 were converted to Ala using Quickchange mutagenesis (KKAA). All mutant ORFs were sequenced and subcloned into relevant vectors. Full-length and C- as well as N-terminal truncation mutants of USP21 (1-87, 1-120, 1-174, 1-210, ∆1-47, ∆1-121, ∆1-184) were amplified,
inserted into pCR4-TOPO (Invitrogen) and sub-cloned into pGEX6P1 and/or pEGFP-C1 vectors. HA-MARKs 1 and 2 constructs were kindly provided by Dario Alessi (University of Dundee). Rabbit polyclonal anti-Pericentrin (ab4448), β-tubulin (ab6046), and γ-tubulin (ab11317) antibodies were purchased from Abcam. Mouse anti-myc (4A6) was obtained from Millipore. Rabbit anti-actin (A2066), Myc (C3956) and -ubiquitin (U5379), mouse anti-α-tubulin (T5168 and DMA1), γ-tubulin (T6557), mouse acetylated α-tubulin as well as Poly-L-Lysine were obtained from Sigma. Secondary donkey anti-mouse and anti-rabbit IRDye (680 and 800nm) antibodies were purchased from LI-COR and Alexa Fluor 488 and 594-labelled secondary antibodies were from Invitrogen (Molecular Probes). Sheep anti-GFP was a gift from Ian Prior (University of Liverpool). rHuNGF was purchased from First Link, UK. On Target Plus USP21 siRNA oligos were purchased from Dharmacon, the target sequences are as follows: #1 (Cat. No. J-006071-05, CCACCCACUUUGAGACGUA), #2 (Cat. No. J-006071-06, GAUCCAAGCUACCAUUUGC), #3 (Cat. No. J-006071-07, CGAGAGCCACCTGTTAATA), #4 (Cat. No. J-006071-08, CUUCGGGACUUCUGUCUGA). HuSH-29 constructs expressing 4 individual shRNAs targeting rat USP21 together with RFP were purchased from Origene.

**GFP-DUBs**

GFP-USP8 and GFP-AMSH have been described elsewhere (McCullough *et al.*, 2004; Row *et al.*, 2006). GFP-A20 and GFP-USP9X were kind gifts of Paul Evans, Imperial college, UK and Dario Alessi, University of Dundee, UK, respectively. A subset of DUBs were obtained in a Gateway compatible entry vector from the human ORFeome collection v1.1, courtesy of Mark Vidal (Harvard Medical School, Boston, USA). The rest of the DUB collection was assembled by PCR-amplifying the ORFs of the reference sequences either from a) human cDNA libraries (liver, brain or testis, Clontech), b) from human IMAGE clones (Mammalian gene collection), or c) from constructs kindly donated by Rohan T Baker (USP4), René Bernard (CYLD), Boudewijn MT Bugering (USP7), Erich E. Wanker (USP13), Sirano Dhe-Paganon (USP50), and Paul Evans (Cezanne, ZRANB1). ORFs were cloned into the Gateway™ based entry vector pDONR223, and subsequently shuttled by LR reaction into a Gateway converted pEGFP-C vector. USP30 was cloned by restriction enzyme and ligation based cloning into pEGFP-N3, resulting in a C-terminal GFP-tag. MYSM1 and AMSH-LP were cloned by restriction enzyme and ligation based cloning into
pEGFP-C1. All ORFs were fully sequence verified and all complete sequences are available on request. A complete description of this library will be published elsewhere.

**Protein purification and DUB assays**

Rosetta cells (Invitrogen) were transformed with pGEX6p1-USP21 constructs encoding various GST-tagged USP21 derived proteins and purified with Glutathione Sepharose 4B (GE healthcare) according to the manufacturer’s instructions. For DUB assays, purified GST-USP21 or GST-USP21(C221S) (both at 100nM) were incubated with K48 or K63 linked tetra-ubiquitin chains (Boston Biochem, Boston, MA) (both at 0.4µM) in DUB assay buffer (50mM Tris pH7.5, 25mM KCl, 5mM MgCl₂, and 1mM DTT) at 37°C for 2 hours. Samples were subjected to NuPAGE analysis (Invitrogen) followed by immunoblotting with anti-ubiquitin antibodies.

**Microtubule spin-down assay**

Microtubule Binding Protein Spin-down Assay Kit (BK029) was purchased from Cytoskeleton Inc. (Denver, CO). GST, GST-USP21(1-87), GST-USP21(1-87)KKAA or GST-USP21(1-120) were incubated ± in vitro assembled microtubules at 21°C for 30 minutes. The reaction mixture was centrifuged at 100,000g for 40 minutes at 25°C. Supernatant and pellet fractions were collected and analysed by SDS PAGE and staining with Coomassie blue.

**Cell lysis and immunoblotting**

Cultured cells were lysed with either Nonidet P-40 (NP40) buffer (0.5% NP-40, 25mM Tris pH7.5, 100mM NaCl, and 50mM NaF), RIPA buffer (1% NP-40, 10mM Tris pH7.5, 150mM NaCl, 1% sodium deoxycholate, and 0.1% SDS) supplemented with proteinase and phosphatase inhibitors, or “hot lysis buffer” (1% SDS, 50mM NaF, and 1mM EDTA at 110°C). In some cases, N-ethylmaleimide (NEM) was added to a final concentration of 10mM to inactivate cysteine proteinases. Proteins were resolved by SDS PAGE and transferred to nitrocellulose. Visualisation of Western blots was achieved using an Odyssey infrared scanner (LI-COR Biosciences, Lincoln, NE).
**Immunofluorescence microscopy**

Cells were processed for immunofluorescence by fixation either with 3% paraformaldehyde in PBS, followed by permeabilization with 0.2% Triton in PBS (localisation of GFP-tagged DUBs), or with -20°C Methanol (microtubule staining). Cells were stained as indicated with primary antibodies for 30-60 minutes at room temperature in 3% BSA followed by Alexa-Fluor 488 or 594-labelled secondary donkey anti-mouse and -rabbit antibodies. Confocal images were captured with a Leica confocal SP2 AOBs (HCX PL APO CS 63x 1.40 oil objective) for all co-localisation experiments and the localisation of the GFP-DUB collection. Microtubule regrowth experiments were visualised using a Leica DML or a Nikon Ti-Eclipse microscope.

**Microtubule regrowth assay**

A549 cells seeded on coverslips were incubated for 90 min on ice to depolymerise microtubules in ice-cold PBS++ (PBS supplemented with 1mM CaCl₂, 1 mM MgCl₂), then the buffer was exchanged for pre-warmed PBS++ (37°C) and held at this temperature for 2.5 minutes before fixation with -20°C Methanol (5 minutes). Cells were then stained with anti-α-tubulin and anti-Pericentrin antibodies followed by secondary labelled antibodies.

**Primary cillum assay**

A549 cells (3.5x10⁵ cells per 6 cm dish) were treated with non-targeting or USP21-targeting siRNA oligos (40 nM) twice over 120 hours. 24 hrs after the second hit, cells were seeded at 1x10⁵ cells per well onto 22x22 mm coverslips, and serum starved for a further 48 hrs to induce primary cillum formation. hTERT-RPE1 cells (5x10⁵ cells per 6 cm dish with 22x22mm coverslip) were treated with siRNA (100 nM) 12h after seeding, and serum starved for 48h. Cells were then washed with PBS++, and incubated in PBS++ on ice for 60 (hTERT-RPE1) or 90 (A549) min to promote microtubule depolymerization. Cells were fixed with methanol (A549) or 4% paraformaldehyde followed by Triton permeabilization (hTERT-RPE1), and stained with antibodies against Pericentrin and acetylated Tubulin to identify primary cilia. Nuclei were counterstained with 4,6 diamidino-2-phenylindole (DAPI) and slides were mounted with p-phenylenediamine (PPD). Wide field microscopy was performed with a Nikon Ti-Eclipse microscope (CFI Plan Apochromat VC 100X Oil objective, N2 N.A. 1.4). Per condition, 800 and 100 cells were counted for A549 and hTERT-RPE1 cells.
respectively. The percentage of cells developing a primary cilium was approximately 20% in A549 and 70% in hTERT-RPE1 cells.

Neurite outgrowth assay

PC12 cells were seeded at 100,000 cells per well of a 6 well plate and transfected with 1 µg rHuSH-29 plasmid DNA expressing either non-targeting (NC15; control) or rat USP21-targeting shRNA (77, 78, 79, 80) together with Red Fluorescent Protein (RFP). Two days after transfection, cells were reseeded onto Poly-L-Lysine coated coverslips situated in a 6 well plate at 40,000 cells per well. Upon attachment, cells were incubated in full growth medium supplemented with 100 ng/ml NGF. Cells were fixed after 3 days of NGF treatment with 3% PFA in PBS and counter-stained with Alexa-Fluor 488-labelled Phalloidin (Invitrogen). Coverslips were mounted using Moviol infused with DAPI, and cells visualized using an epifluorescence microscope and a 50x objective. Between 130 and 350 RFP expressing cells were assessed for each condition and scored as differentiated if one or more neurites had reached a length larger than the maximum chord length of the cell body.

Acknowledgements

This work was supported by a CRUK Senior Fellowship award to SU and a project grant from the Wellcome Trust. SDH was supported by a Wellcome Trust studentship. We thank the many colleagues listed in the Materials and Methods section for reagents, Dario Alessi, Franck Perez and Giampietro Schiavo for helpful discussions, Zehra Akerman, Sara Cadeco and Rebecca Eccles for technical assistance and Monika Chojnowska, Ewan Macdonald, Carla Bento and Matthew Edmonds for help in producing some constructs and preliminary experiments.

References


Figure Legends

**Figure 1.** Subcellular localisation of EGFP-tagged DUBs.

A. Selection of DUBs showing distinctive subcellular distributions. All panels correspond to confocal sections and are shown at identical magnification. Scale bar = 10µm. 

B. Venn diagram representing the relative distribution of EGFP-tagged DUBs between Nuclear and Cytoplasmic compartments. Those DUBs also localizing to clearly identifiable organelles or structures are indicated with a number corresponding to the following locations; 1: Plasma Membrane, 2: Endosome, 3: ER, 4: Mitochondria, 5: Microtubules and Centrosome, 6: Nucleoli. 

Note that Plasma Membrane localisation may be confined to particular structures, e.g. filipodia or lamellipodia. The full data-set corresponding to fluorescence images of 61 EGFP-DUB family members is provided in Supplemental Figure S1 and Supplemental Table 1.
Figure 2. GFP-USP21 associates with microtubular structures: HeLa cells were transfected for 24 hours with GFP-USP21. (A-C) Different aspects of GFP-USP21 microtubular staining. (D-F) HeLa cells stably expressing Cherry-α-tubulin transiently transfected with GFP-USP21. (G-I) Co-localisation with α-tubulin in a HeLa cell undergoing cytokinesis. Scale bar = 10µm.
Figure 3. USP21 localises to centrosomes at low expression levels.
HeLa cells were transfected with GFP-USP21(A-F) or myc-USP21 (G-I) and counter stained with Pericentrin (A-C, G-I) or γ-tubulin antibodies (D-E). Insets show threefold enlargement of the pericentriolar region (indicated by arrows). Scale bar = 10µm.
Figure 4. Catalytic activity favours microtubule association but is not required for centrosome or midbody colocalisation. HeLa cells were transfected for 24 hours with GFP-USP21 wild-type or catalytically inactive mutant (CS) tagged with GFP or myc. (A-C) Co-localisation with microtubule bundles in cells expressing high levels of GFP-USP21. (D-F) GFP-USP21-CS localises to the mid-body but is absent from cytosolic microtubules. (G-I) myc-USP21-CS colocalisation with Pericentrin. (J-L) High level expression of GFP-USP21-CS in U2OS cells promotes its localisation to microtubules. Scale bar = 10µm.
Figure 5. Mapping the requirements for USP21 localisation and binding to microtubules.

(A-D) HeLa cells were transfected for 24 hours with full-length GFP-USP21 wild-type or the indicated N-terminal truncation mutant. Scale bar = 10µm. Note, this localisation was also confirmed in U2OS cells. (E) In vitro spin-down assay for direct binding to purified microtubules (MT). Binding of both USP21 N-terminal fragments (1-87 and 1-120) to microtubules is evidenced by redistribution from...
supernatant (S) to pellet fraction (P). Asterisk indicates a shorter truncation, which has lost microtubule binding. (F) Alignment of USP21 residues 59-75 with motifs in Tau and Adenomatous Polyposis Coli proteins: mutation of matching lysine residues diminishes \textit{in vitro} binding to microtubules. (G) Quantitation of data presented in (F), bars indicate range of two distinct experiments. (H) Summary of constructs used and properties reported in this Figure and Supplemental Figure S4 and S5.
Figure 6. USP21 depletion impairs the re-establishment of a microtubule network after cold-depolymerization.

A549 lung cells were transfected with a non-targeting siRNA oligonucleotide (siControl), or one of two individual siRNA oligonucleotides (siUSP21#1, siUSP21#2) specifically targeting USP21 for 120 hours prior to experimentation. Following cold-induced microtubule depolymerisation, cells were incubated for 2.5 minutes at 37°C followed by methanol fixation and staining with α-tubulin antibodies. Cells were classified into 3 categories of microtubule regrowth status, (a) green: advanced (full) regrowth; (b) orange: partially defective regrowth; (c) red: severely defective (minimal) regrowth. Shown are the results of a representative experiment in which 287 (siControl), 323 (siUSP21#1) and 385 (siUSP21#2) cells were counted respectively.
Figure 7. USP21 is required for primary cilium formation
A549 were transfected with siRNA specific to USP21 (OL1, OL4) or non targeting siRNA (NT1) and serum starved for 48 hrs to induce primary cilium formation.
(A) A maximal projection of NT1 and USP21-targeting siRNA (OL1) transfected cells are shown after staining of acetylated α-tubulin as a marker for primary cilia (Ac Tub, green) and Pericentrin (PCNT, red). DNA was stained with DAPI (blue).
scale bar: 10 µm. (B) The percentage of cells with a primary cilium (approximately 800 cells counted per condition) is significantly reduced for cells transfected with siRNA specific to USP21 (error bars = sd, n=3 independent experiments).
**Figure 8.** USP21 depletion interferes with NGF-induced neurite outgrowth in PC12 cells.

PC12 cells were transfected with pRFP-C-RS vector expressing either non-targeting shRNA (NC15, Control) or HuSH-29 shRNA targeting rat USP21 (shUSP21#1-4). Transfected cells co-express RFP. Neurite outgrowth was induced by application of 100 ng/ml NGF 48 hours after transfection and three days later cells were fixed and counterstained with Alexa Fluor 488-labelled Phalloidin (Invitrogen) to visualise non-transfected cells. Cells expressing RFP were scored as differentiated if one or more neurites had reached a length larger than the diameter of the cell body (30-48% of control cells, compared to 4-15% in USP21-depleted cells). The data are derived from three independent experiments, in each of which, between 130 and 350 cells were scored for each condition. Error bars = +/-SD.