Cellular functions of the DUBs

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Summary

Ubiquitylation is a reversible post-translational modification that has emerged as a key regulator of most complex cellular processes. It may rival phosphorylation in scope and exceed it in complexity. The dynamic nature of ubiquitylation events is important for governing protein stability, maintaining ubiquitin homeostasis and controlling ubiquitin-dependent signalling pathways. The human genome encodes ~80 active deubiquitylating enzymes (DUBs, also referred to as deubiquitinases), which exhibit distinct specificity profiles towards the various ubiquitin chain topologies. As a result of their ability to reverse ubiquitylation, these enzymes control a broad range of key cellular processes. In this Commentary we discuss the cellular functions of DUBs, such as their role in governing membrane traffic and protein quality control. We highlight two key signalling pathways – the Wnt and transforming growth factor β (TGF-β) pathways, for which dynamic ubiquitylation has emerged as a key regulator. We also discuss the roles of DUBs in the nucleus, where they govern transcriptional activity and DNA repair pathways.

This article is part of a Minifocus on Ubiquitin. For further reading, please see related articles: 'Ubiquitin and SUMO in DNA repair at a glance' by Helle D. Ulrich (*J. Cell Sci.* **125**, 249-254). 'Emerging regulatory mechanisms in ubiquitin-dependent cell cycle control' by Annamaria Mocciaro and Michael Rape (*J. Cell Sci.* **125**, 255-263). 'The role of ubiquitylation in receptor endocytosis and endosomal sorting' by Kaisa Haglund and Ivan Dikic (*J. Cell Sci.* **125**, 265-275). 'HECT and RING finger families of E3 ubiquitin ligases at a glance' by Meredith B. Metzger et al. (*J. Cell Sci.* **125**, 531-537). 'Non-canonical ubiquitin-based signals for proteasomal degradation' by Yelena Kravtsova-Ivantsiv and Aaron Ciechanover (*J. Cell Sci.* **125**, 539-548). 'No one can whistle a symphony alone – how different ubiquitin linkages cooperate to orchestrate NF-κB activity' by Anna C. Schmukle and Henning Walczak (*J. Cell Sci.* **125**, 549-559).

Introduction

Ubiquitin is a 76 amino acid polypeptide that was originally characterised as a covalently attached signal for ATP-dependent proteasomal degradation of substrate proteins (Hershko and Ciechanover, 1998). It has since been appreciated that ubiquitin also has a role in both the lysosomal and autophagic degradation pathways (Clague and Urbe, 2010). Furthermore, the reversible nature of ubiquitylation (Fig. 1) and the evolution of more than 20 distinct classes of protein domains that can interact with ubiquitin (Dikic et al., 2009) extend its influence beyond the degradative pathways to many dynamic cellular processes, such as the transduction of cellular signals and gene transcription (Hunter, 2007).

The C-terminus of ubiquitin is ligated to lysine residues in substrate proteins by the concerted action of an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligase. This modification (monoubiquitylation) can then be extended by ligation of further ubiquitin molecules to any of the seven lysine residues that are present in the ubiquitin molecule, thereby producing ubiquitin chains of various topologies (Xu et al., 2009). Specific functions associated with distinct chain types are only just emerging, although Lys63 chains appear to have key roles in lysosomal sorting and several signalling pathways, without involving the proteasome (Chiu et al., 2009; Lauwers et al., 2009). To maintain ubiquitin homeostasis, ubiquitin must be recycled once a substrate has been committed to the degradative pathway. Hence, both the proteasome and lysosomal sorting machinery have deubiquitylating enzymes (DUBs) associated with them (Clague and Urbe, 2006; Finley, 2009). Free ubiquitin is generated by DUBs through processing the proteins encoded by four ubiquitin genes (UBC, UBB, UBA52 and UBA80) that express either linear polyubiquitin chains or ubiquitin fused to one of two ribosomal proteins (L40 and S27a) (Komander et al., 2009a; Ozkaynak et al., 1987).

The classical role of ubiquitin is to serve as a tag for protein destruction (Hershko and Ciechanover, 1998). It follows that deubiquitylation can promote protein stability (Fig. 1) and a diverse array of DUBs ensure some selectivity to this process. The human genome encodes ~90 DUBs, which can be grouped into five distinct families. Of these proteins, 79 are predicted to be active. The largest of the five families is the ubiquitin-specific protease (USP) family (~55 members). The USPs as well as the ubiquitin C-terminal hydrolases (UCHs), ovarian tumour proteases (OTUs) and members of the Josephin family are cysteine proteases. The fifth DUB family comprises a group of Zn^{2+} metalloproteases that are referred to as the JAB1/MPN/MOV34 metalloenzymes (JAMMs, also known as MPN^{+}). The domain architectures and structural features of this superfamily have been reviewed extensively elsewhere (Komander et al., 2009a; Nijman et al., 2005b; Reyes-Turcu et al., 2009). In this article we will, therefore, focus on recent advances in the understanding of the broad range of cellular functions that are associated with specific DUBs.

The substrate specificity of DUBs is determined by sub-cellular localisation, specific binding interactions and the preference of the catalytic domain for particular types of ubiquitin chain linkages. The principle of DUB chain linkage specificity was first established for the endosomal JAMM family member associated molecule with the SH3 domain of STAM (AMSH, officially known as STAMBP) and, later, for the closely related AMSH-like protein (AMSH-LP, officially known as STAMBPL1), both of which are stringently selective for Lys63-linked ubiquitin chains (McCullough et al., 2004; McCullough et al., 2006; Nakamura et al., 2006; Sato et al., 2008). More recent studies have characterised a broad variety of DUBs and have pointed towards a spectrum of linkage preferences and promiscuities. Surprising levels of discrimination for different ubiquitin chains are observed amongst the DUBs, even within the same family (Bremm et al., 2010; Komander et al., 2009b; Virdee et al., 2010). There is also a notable tendency for

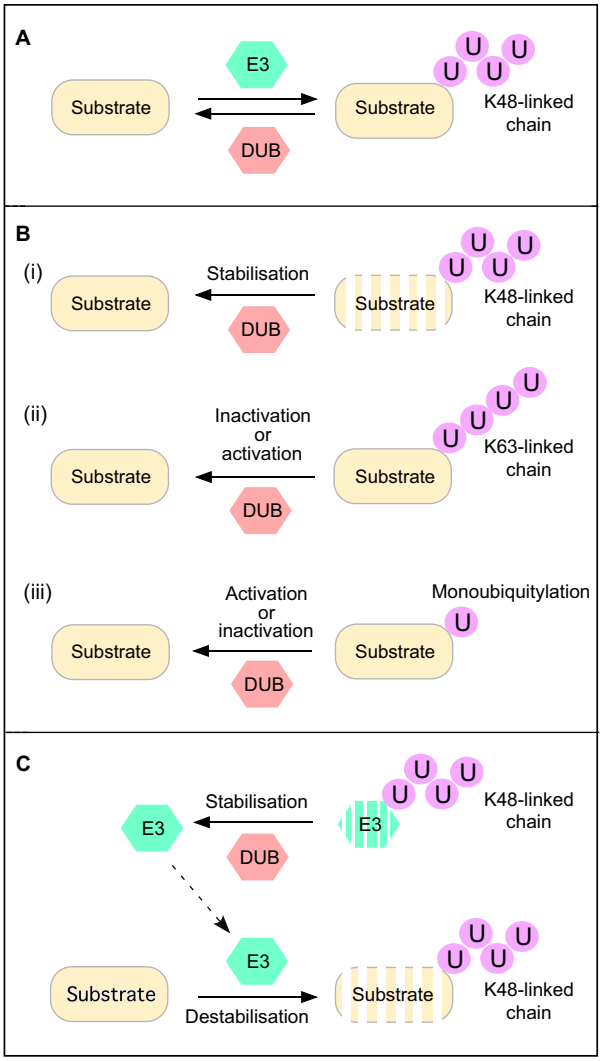

Fig. 1. DUBs regulate the stability or activity of proteins. (**A**) DUBs oppose the action of E3 ubiquitin ligases. (**B**) Depending on the type of ubiquitin chain that is attached to the substrate, DUBs can stabilise, or inactivate or activate their target protein. Examples from the text include the following DUB (substrate) configurations: (i) AMSH (EGFR), USP34 (AXIN), (ii) CYLD (DVL), (iii) USP9X (SMAD4). (**C**) Many E3 ligases undergo autoubiquitylation and can be stabilised by DUBs. In this scenario, DUBs may indirectly destabilise the protein that is targeted by the E3 ligase.

DUBs to interact with E3 ubiquitin ligases, which themselves have a propensity to autoubiquitylate. Hence, one fundamental function of DUBs might be to control the stability of E3 ligases (of which more than 600 are found in mammals) (Komander et al., 2009a). In this case the ultimate effect of the DUB will be to destabilise the substrates of the cognate E3 ligase (Fig. 1C).

The cellular functions that have been ascribed to DUBs are growing rapidly. Many are based on small interfering RNA (siRNA) screens, which do not directly inform on whether enzymatic activity is actually required unless they are accompanied by rescue experiments that compare active and inactive forms of the enzyme. In fact, some DUBs may have scaffolding functions independent of their catalytic activity. In this Commentary, we will focus on three broad areas that recently saw substantial progress. First, we will discuss the role that DUBs have in membrane trafficking events and protein quality control. Second, we will focus on their role in cell signalling: the reversibility of ubiquitylation, together with the myriad of ubiquitin-binding domains, allows for the establishment of dynamic networks of protein interaction and the relay of signals in a manner akin to phosphorylation (Dikic et al., 2009). The nuclear factor kappa B (NF-κB) pathway was the first pathway for which an elaborate dependence on reversible ubiquitylation was unravelled and this has recently been reviewed elsewhere (Chiu et al., 2009; Harhaj and Dixit, 2011). Here, we will focus on Wnt and on transforming growth factor beta (TGF-β) signalling, for both of which the ubiquitin system is emerging as a master regulator. Finally, the important role of deubiquitylation in the regulation of nuclear events, including transcription and DNA-damage repair, will be reviewed.

Membrane trafficking and control of protein quality

Membrane trafficking is crucial to the organisation of the cell, and ubiquitylation can be used as a means to regulate the trafficking itinerary of cargo molecules. Functional studies have largely focused on DUB activity that is associated with the endocytic pathway because of the well-established role for ubiquitylation in dictating the lysosomal degradation of various cell-surface receptors (Clague and Urbe, 2006; Hicke and Dunn, 2003). Ubiquitylated receptors are selected for lysosomal sorting through engagement with the endosomal sorting complex required for transport (ESCRT) machinery, which promotes the formation of multivesicular bodies through budding of small, cargo-laden vesicles into the lumen of the sorting endosome (Williams and Urbe, 2007). The first point of contact is proposed to be the interaction of ubiquitylated receptors with the ESCRT-0 complex, which comprises hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) and signal transducing adaptor molecule (STAM), both of which contain ubiquitin interaction motifs (UIMs) (Henne et al., 2011). This is followed by an incompletely understood transfer of the ubiquitylated receptor to the downstream multimeric ESCRT-I and ESCRT-II complexes. ESCRT-III components and the AAA-ATPase VPS4 conclude the process by generating internal vesicles (Alonso and Teis, 2011; Williams and Urbe, 2007; Wollert et al., 2009).

DUBs balance receptor degradation and recycling

In yeast and mammalian cells DUBs show a widespread cellular distribution with some specificity for particular organelles (Fig. 2) (Kouranti et al., 2010; Sowa et al., 2009). In human cells, such organelle-specific localisation is most stringent for the two DUBs with trans-membrane domains, USP19 and USP30, which localise to the endoplasmic reticulum and the outer membrane of mitochondria, respectively (Hassink et al., 2009; Nakamura and Hirose, 2008). The clearest examples of DUB sub-cellular localisation to endosomal compartments are AMSH and USP8 (also known as UBPY), which both localise to sorting endosomes predominantly through interactions with components of the ESCRT machinery. They share a binding site on the Src homology 3 (SH3) domain of the ESCRT-0 component STAM (reviewed in Clague and Urbe, 2006) but each also possess a microtubule-interacting and -trafficking (MIT) domain that mediates interactions with the ESCRT-III charged multivesicular body proteins (CHMPs) (Hurley and Yang, 2008). Both AMSH and USP8 bind to CHMP1A and CHMP1B, but only AMSH is able

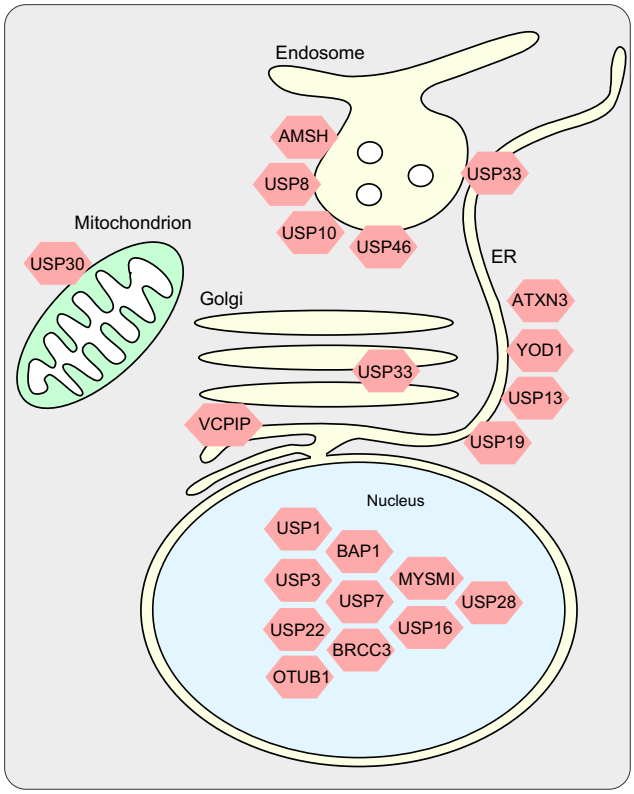

Fig. 2. Localisation and organelle-specific functions of DUBs. Multiple DUBs have been associated with endosomal trafficking of a variety of receptors and channels. USP33, an ER and Golgi associated DUB, has been implicated in the recycling of β-adrenergic receptor and might be able to deubiquitylate endosomal cargo at ER–endosome junctions. Other ER DUBs shown are candidates for the regulation of the ERAD pathway through association with p97. USP30 localises to the outer mitochondrial membrane. Nuclear DUBs with specific functions described in the text are indicated.

to interact with CHMP3 (Agromayor and Martin-Serrano, 2006; McCullough et al., 2006; Row et al., 2007). ESCRT-III CHMP components have also been implicated in cytokinesis and viral budding (Carlton and Martin-Serrano, 2007; McDonald and Martin-Serrano, 2009; Morita et al., 2007). Accordingly, roles for AMSH and USP8 in these processes have also been indicated (Mukai et al., 2008).

The predominant form of receptor ubiquitylation that promotes sorting along the lysosomal pathway appears to be in form of short Lys63-linked chains (Duncan et al., 2006; Galan and Haguenauer-Tsapis, 1997; Huang et al., 2006a; Lauwers et al., 2009). Depletion of the Lys63-specific DUB AMSH by using RNA interference (RNAi) accelerates epidermal growth factor receptor (EGFR) trafficking to lysosomes (Bowers et al., 2006; McCullough et al., 2004). This observation led to the simple working model, wherein the efficiency of ubiquitin-dependent lysosomal sorting (which occurs at the expense of receptor recycling) is governed by the balance of E3 ligases [e.g. Cbl in the case of EGFR (Thien and Langdon, 2005)] and DUBs (e.g. AMSH) (Clague and Urbe, 2006). This principle of negative regulation of lysosomal sorting can be extended to other ubiquitylated receptors and to other DUB family members. For example, in the *Caenorhabditis elegans* ventral nerve chord, Usp46 negatively regulates the degradation of glutamate receptors (Kowalski et al., 2011). Similarly, USP10 depletion in mammalian cells, promotes degradation of cystic fibrosis transmembrane conductance regulator (CFTR) (Bomberger et al., 2010).

Additional roles for DUBs on the endocytic pathway

The simple negative regulatory role of DUBs with respect to receptor ubiquitylation (described above) cannot account for all aspects of their endosomal function. The AMSH MIT domain has an exceptionally high-affinity binding site for CHMP3, which does not overlap with that for CHMP1 (Solomons et al., 2011). Studies have pointed to a coupling between CHMP3 and AMSH functions, suggesting a role for this DUB late in the endosomal pathway. Furthermore, both AMSH and USP8 seem to have positive rather than negative roles in the downregulation of protease-activated receptor 2 (Hasdemir et al., 2009). One possibility is that AMSH or USP8 can serve to release ubiquitylated cargo from ESCRT-0 to allow transfer to ESCRT-I and -II (Hurley, 2011). It is known that AMSH can simultaneously bind the ESCRT-0 protein STAM and the ESCRT-III protein CHMP3A in vitro (McCullough et al., 2006) but it is presently unclear whether this ternary complex is required for AMSH function or whether the association with different sub-complexes of the ESCRT-machinery reflects distinct sequential functions of this DUB.

USP8 exhibits pleiotropic effects, which could partially explain some of the confusion in the complex literature surrounding this protein. Two reports suggest that depletion of USP8 by using RNAi leads to accumulation of an ubiquitylated form of EGFR, and blocks the degradation of both the EGFR (Bowers et al., 2006; Row et al., 2006) and the MET receptor (Row et al., 2006). This is accompanied by clustering of multivesicular bodies and the depletion of ESCRT-0 components, which USP8 otherwise protects from proteasomal degradation. Both EGFR degradation and ESCRT-0 stability can be rescued by ectopic expression of GFP-tagged USP8, but not by a catalytically inactive form or a MIT-domain deletion construct (Row et al., 2007). Mice in which USP8 has been conditionally knocked-out also show reduced levels of ESCRT-0 components but, in contrast to transient depletion in tissue culture cells, EGFR levels are markedly reduced (Niendorf et al., 2007). The principle that DUBs can regulate the sorting machinery rather than the receptor per se – which may reflect an aspect of USP8 function – is further illustrated by the vasopressin-dependent expression of USP10, which increases the amount of amiloride-sensitive epithelial Na^+ channels (ENaC, officially known as SCNN1) in the plasma membrane. However, whereas USP8 controls the stability of ESCRT-0 components, USP10 stabilises sorting nexin 3 (SNX3), a positive regulator of endosomal recycling (Boulkroun et al., 2008; Strochlic et al., 2008).

The effect of USP8 on receptor trafficking has been most controversial, but this might be a function of the degree of depletion in individual studies. Mizuno et al. originally found that depletion of USP8 enhances EGFR degradation (Mizuno et al., 2005) but subsequently – in line with observations by others – showed that a more complete depletion blocks degradation and induces endosomal clustering (Bowers et al., 2006; Mizuno et al., 2006; Row et al., 2006). In a similar fashion, the intermediate-conductance Ca^{2+}-activated K^+ channel (KCa3.1) responds to USP8 depletion by exhibiting enhanced ubiquitylation but reduced degradation (Balut et al., 2011). By contrast, Nash and colleagues have recently resurrected the observation that depletion of USP8 enhances EGFR degradation and propose this to occur in a manner that is dependent

on Hrs (Berlin et al., 2010b). However, they also find that lysosomal degradation of the activated chemokine receptor 4 (CXCR4) is blocked under similar conditions (Berlin et al., 2010a).

In addition to maintaining the balance between receptor degradation and recycling, endosomal DUBs, such as USP8, may also be involved in recycling ubiquitin from receptors once they have been committed to the lysosomal pathway. This ensures the maintenance of free ubiquitin homeostasis and is analogous to the role previously proposed for the *S. cerevisiae* DUB Doa4 – the yeast orthologue of USP8 (Amerik et al., 2000; Dupre and Haguenauer-Tsapis, 2001).

Another DUB that is associated with endocytic trafficking is USP33. Berthouze and colleagues have reported that both USP33 and its close paralogue USP20 constitutively associate with the β-adrenergic receptor. The prompt dissociation of these proteins in response to agonist allows for efficient receptor ubiquitylation and routing to lysosomes. Prolonged stimulation promotes the reassociation of USPs, which deubiquitylate and, thus, stabilise the receptor (Berthouze et al., 2009). In addition, USP33 has been proposed to bind directly to and deubiquitylate the G-protein-coupled receptor (GPCR), and the endocytic adaptor and signalling scaffold protein β-arrestin, leading to enhanced recycling and stability of the GPCR–β-arrestin signalling complex (Shenoy et al., 2009). However, in HeLa and A549 cells, both ectopically expressed GFP–USP33 and endogenous enzyme are largely confined to the secretory pathway, ER and Golgi compartments together with unidentified punctate structures that are negative for established endosomal markers (Thorne et al., 2011). This distribution is consistent with a proposed role for USP33 in the regulation of the stability of the ER-localised type-2 iodothyronine deiodinase (D2) that generates 3,5,3′-triiodothyronine (T3), which is essential for brain development (Curcio-Morelli et al., 2003). To reconcile the data on USP33 localisation with the effects on endocytic trafficking of the β-adrenergic receptor, we suggest an in-*trans* interaction between the ER-localised enzyme and the receptor – much as the ER localised phosphatase PTP1B has been shown to regulate EGFR on endosomes (Eden et al., 2010).

DUBs and the secretory pathway

The most prominent role for ubiquitylation in the secretory pathway that has been established so far is in the quality control endoplasmic-reticulum-associated degradation (ERAD) pathway. The ER-membrane-anchored DUB, USP19 is a target of the unfolded protein response (UPR) and is able to rescue the ERAD substrates CFTRΔF508 and T-cell receptor alpha (TCRα) from proteasomal degradation (Hassink et al., 2009). The AAA-ATPase p97 (officially known as VCP) in complex with a dimeric co-factor (UFD1L–NPLOC4) recognises ubiquitylated ERAD substrates, and participates in ratcheting them from the ER membrane and presenting them to the proteasome. p97 interacts with several DUBs, including YOD1, VCPIP1 (also known as VCIP135), ataxin 3 and USP13 (Sowa et al., 2009; Wang et al., 2006; Wang et al., 2004). Depletion of USP13 or expression of catalytically inactive ataxin 3 results in the accumulation of model ERAD substrates (Sowa et al., 2009; Wang et al., 2006). Expression of a catalytically inactive form of YOD1 also inhibits the dislocation of model ERAD substrates from the ER (Ernst et al., 2009), but this can be overcome by targeting a DUB from the Epstein-Barr virus to p97 through the appendage of an ubiquitin regulatory X (UBX) domain (Ernst et al., 2011). Although this artificial, generic p97-associated DUB can fulfil the requirement for dislocation, the degree of redundancy between p97-associated endogenous DUBs in this pathway remains an open question. It has been proposed that deubiquitylation is required for completion of p97-dependent dislocation of ERAD substrates, in which case another round of ubiquitylation would be needed to target dislocated proteins to the proteasome. In *C. elegans*, p97 and ataxin 3 homologues fulfil redundant functions in determining lifespan through an influence on the insulin–IGF1 signalling pathway independent of ER homeostasis (Kuhlbrodt et al., 2011). Interestingly, another cellular function of p97 – the reassembly of the Golgi complex following mitosis – also requires an associated DUB activity that corresponds to VCPIP1 (Wang et al., 2004).

Cellular signalling

In addition to controlling receptor availability, reversible ubiquitylation is an important downstream element of signalling cascades, such as the NF-κB and the receptor tyrosine kinase pathways (Buus et al., 2009; Harhaj and Dixit, 2011). Here, we will focus on the role of DUBs in the regulation of the Wnt and TGF-β pathways, for which substantial recent progress has been made (Fig. 3).

DUBs and the Wnt signalling pathway

The canonical Wnt signalling pathway has a key role in development and tissue homeostasis and its deregulation is associated with multiple diseases. The key mediator of this pathway is β-catenin, which activates transcription of a palette of genes by associating with transcription factors (TFs) of the TCF/LEF family. In the absence of a Wnt signal, β-catenin is constitutively targeted for proteasomal degradation through association with a destruction complex that comprises the scaffold proteins adenomatous polypopis coli (APC) and AXIN, as well as glycogen synthase kinase 3β (GSK3β) and casein kinase 1 (CK1). The simultaneous binding of Wnt to Frizzled (FZD) and its co-receptors, low-density lipoprotein receptor-related proteins 5 or 6 (LRP5 or LRP6, respectively), leads to their association with the cytoplasmic effector DVL and the recruitment of AXIN and GSK3β. This causes the disassembly of the destruction complex, allowing β-catenin to accumulate and activate transcription (Fig. 3A) (Tauriello and Maurice, 2010).

The levels of FZD receptor expressed on the cell surface determine the cellular responsiveness to Wnt signalling. In *Drosophila melanogaster*, the endosomal DUB Ubpy was found to be a positive regulator of Wnt signalling (Mukai et al., 2010). Elegant studies suggest this occurs through influencing constitutive reversible monoubiquitylation and endosomal cycling of FZD. Both Ubpy and its mammalian homologue USP8 can deubiquitylate endosomal FZD receptors, thereby favouring recycling, rather than sorting to the lysosomal degradation pathway (Mukai et al., 2010).

A search for binding partners of components of the AXIN complex has also highlighted several DUBs. USP34 was identified as a binding partner for AXIN by mass spectrometry and has been shown to influence Wnt signalling by controlling AXIN stability (Lui et al., 2011). Another DUB, USP15 has been implicated in the stabilisation of the second scaffold protein APC. In the absence of USP15, APC is degraded by the proteasome. Intriguingly, USP15 is also indirectly involved in the ubiquitylation of β-catenin as an obligate cofactor of the COP9 signalosome, which assists in the formation of the destruction complex (Huang et al., 2009). The

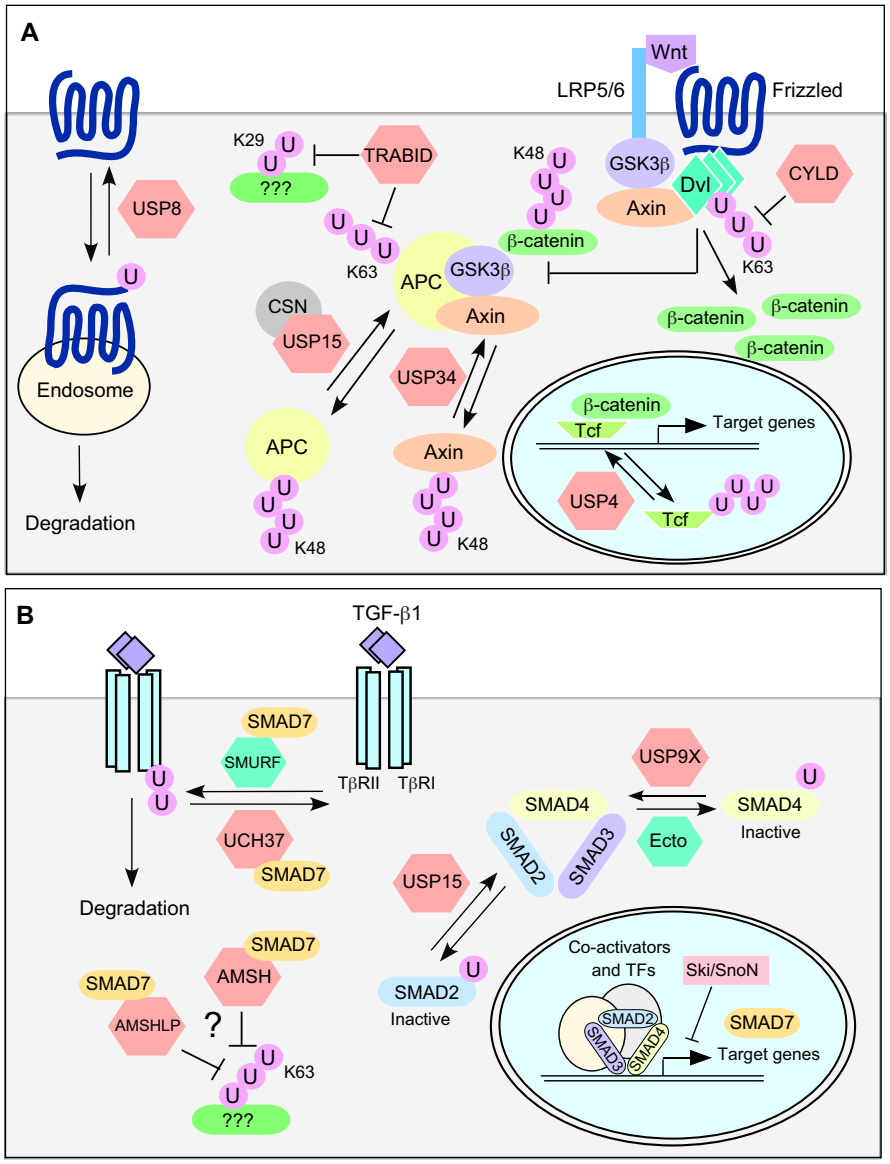

Fig. 3. Wnt and TGF-β signalling pathways regulated by DUBs. (**A**,**B**) The roles of DUBs in Wnt signalling (A) and TGF-β signalling (B); details are given in the main text. Red hexagons, DUBs; green hexagons, E3 ligases. SKI and SnoN (also known as SKIL) are transcriptional cofactors that inhibit TGF-β signalling.

OTU family member TRABID (officially known as ZRANB1) was identified as an APC interacting partner using a yeast two-hybrid assay with the Armadillo repeat domain of APC as bait (Tran et al., 2008). Knockdown of TRABID leads to the accumulation of a hyper-ubiquitylated form of APC but does not markedly affect its stability. Wnt target gene transcription is inhibited by TRABID depletion, but epistasis experiments suggest that the effect lies below the level of β-catenin accumulation, suggesting a role for this DUB in TCF-mediated transcription (Tran et al., 2008). Whether the relevant physiological substrate is APC or not is presently unclear, although APC has been shown to directly repress TCF target gene transcription under conditions of sustained Wnt signalling (Sierra et al., 2006). Tran and colleagues speculated that the hyper-ubiquitylated APC is hyper-repressive (Tran et al., 2008), and found TRABID to be highly specific for Lys63-linked ubiquitin chains over Lys48 chains. However, with the increased appreciation of different chain types, Virdee and colleagues have been able to show that TRABID possesses a 40-fold greater specificity for cleaving the Lys29 linkage over the Lys63 type (Virdee et al., 2010). The relevance of this relative selectivity and the physiological role of Lys29 linkages in this signalling cascade are currently unknown.

A short hairpin RNA (shRNA) screen for DUBs involved in Wnt signalling identified USP4 to be a unique DUB that is required for hyper-activation of this pathway in SW480 cells (Zhao et al., 2009). In these cells, Wnt signalling is constitutively active owing to an inactivating mutation in APC. Evidence was provided for an interaction of USP4 with a post-translationally modified form of TCF4 – the major binding partner of β-catenin – which led the authors to propose that USP4 regulates the turnover of a specific pool of TCF4.

A conceptually similar screen using the same shRNA library (Brummelkamp et al., 2003) identified the product of the cylindromatosis tumour suppressor gene (*CYLD*) as an outlying DUB that serves as a negative regulator of Wnt signalling (Tauriello et al., 2010). In previous studies, CYLD has also been identified as a negative regulator of NF-kB signalling (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003), but Tauriello and co-workers published results indicating that the effect on Wnt signalling is independent of NF-

kB activation. Human skin appendage tumours associated with mutations in CYLD display hyperactive Wnt signalling (Tauriello et al., 2010). CYLD might, therefore, coordinately regulate both pathways to promote tumour formation in patients who suffer from cylindromatosis.

Epistasis experiments suggest a role for CYLD upstream of β-catenin stabilisation, and biochemical experiments identified the accumulation of Lys63-chain ubiquitin on the DIX domain of DVL following CYLD depletion. In vitro, CYLD has been shown to process unanchored Lys63 chains but not those attached to two of its 'established' substrates TRAF6 and NEMO (officially known as IKBKG) (Xia et al., 2009). Similarly, Tauriello and colleagues were unable to observe direct deubiquitylation of DVL by CYLD in vitro, suggesting the requirement of a co-factor or a more indirect effect on DVL ubiquitin status (Tauriello et al., 2010). Lys63-linked ubiquitylation of DVL in CYLD-depleted cells, leads to enhanced signalling rather than decreased DVL stability. This distinguishes it from Lys48-linked DVL polyubiquitylation, which is mediated by the KLHL12 enzyme and negatively regulates Wnt signalling by promoting DVL proteasomal degradation (Angers et al., 2006). Under starvation conditions the Von Hippel-Lindau tumour suppressor (VHL) E3 ligase complex can, additionally, promote DVL2 ubiquitylation and degradation through the autophagy pathway (Gao et al., 2010). DUBs that rescue DVL proteins from either degradation pathway may have important roles in the regulation of Wnt signalling but remain to be identified.

DUBs and the TGF-β signalling pathway

The TGF-β signalling pathway is elicited by a variety of cytokines and regulates a diverse set of biological functions that are highly context dependent (Heldin et al., 2009; Schmierer and Hill, 2007). The activated TGF-β receptor phosphorylates receptor-regulated SMADs (R-SMADs), which then form an active nuclear transcriptional complex by association with SMAD4 (Fig. 3B). Bone morphogenic protein (BMP) stimulation of type I TGF-β receptors activates R-SMADs 1, 5 and 8 (SMAD1, SMAD5 and SMAD 8, respectively), whereas TGF-β stimulation of a different subtype of the same class of receptors leads to phosphorylation of SMAD2 and SMAD3. In the absence of stimulus, SMAD3 but not SMAD2 undergoes constitutive proteasomal turnover under the control of the AXIN–GSK3β axis and, thereby, provides an example of crosstalk between the TGF-β and Wnt signalling systems (Guo et al., 2008). The inhibitory SMADs (I-SMADs) SMAD6 and SMAD7 oppose signalling by interacting with type I receptors and preventing R-SMAD phosphorylation, and by competing with SMAD4 for binding to activated R-SMADs. I-SMADs also recruit multiple HECT E3 ubiquitin ligases, including the SMAD-specific E3 ubiquitin protein ligases 1 and 2 (SMURF1 and 2, respectively), which promote polyubiquitylation as well as the degradation of receptors, R-SMADs and I-SMADs themselves (Inoue and Imamura, 2008).

An siRNA screen of 75 DUBs for two characteristic responses of TGF-β activation – SMAD3 phosphorylation and induction of $p21^{Waf1}$ – identified a number of DUBs that might be involved in TGF-β signalling. Of these, USP9X showed the most penetrant requirement for eliciting TGF-β responses in follow-up studies (Dupont et al., 2009). In the absence of USP9X, the activity of the RING E3 ligase ectodermin (also known as TRIM33) results in the accumulation of SMAD4 in a monoubiquitylated form. This interferes with R-SMAD binding and, consequently, inhibits SMAD4 signalling. The current working model proposes that SMAD4 undergoes cycles of ubiquitylation and deubiquitylation in the nucleus and cytosol, respectively. More recently, a luciferase-reporter-assay-based siRNA screen, identified USP15 as a DUB that is required for the response to both TGF-β and BMP. In analogy to the way in which USP9X acts on SMAD4, USP15 was shown to remove inactivating monoubiquitin from DNA-binding domains of R-SMADs, thereby enabling their association with target promoters (Inui et al., 2011).

The endosomal DUBs AMSH and AMSH-LP were found to be regulators of BMP signalling and TGF-β signalling, respectively, before they were recognised as members of the DUB super-family. AMSH interacts specifically with the I-SMADs, SMAD6 and SMAD7, and AMSH overexpression prolongs BMP signalling, apparently through sequestration of SMAD6 (Itoh et al., 2001). Similarly, AMSH-LP interacts with SMAD2 and SMAD7, and its overexpression suppresses the inhibitory action of SMAD7 on TGF-β signalling (Ibarrola et al., 2004). It would be surprising if more detailed studies on the function of endogenous AMSH-family proteins within these pathways did not implicate a role for their DUB activity in these effects.

The proteasome-associated DUB, UCH37 (UCHL5) also binds to SMAD7 and, similarly to AMSH-LP, its overexpression enhances TGF-β signalling. The type-I TGF-β receptor is ubiquitylated and destabilised by the E3 ligase SMURF2, which is recruited through SMAD7; however, this ubiquitylation is greatly diminished when UCH37 is coexpressed. Thus, the relative levels of SMURF2 and UCH37 can determine receptor expression levels and cellular responsiveness to TGF-β stimulation (Wicks et al., 2005). Functional studies have indicated that this can determine the pattern of individual gene expression responses and is particularly relevant to the early phase of TGF-β signalling that encompasses enhanced cellular motility (Cutts et al., 2011). In zebrafish, a systematic morpholino knockdown of 85 potential DUBs has identified crucial roles for Otud4, Usp5, Usp15 and Usp25 in dorsoventral patterning through regulating the BMP pathway (Tse et al., 2009).

Nuclear functions of DUBs

Signalling pathways, like the ones described above, culminate in changes to gene transcription through specific transcription factors, which can be regulated by reversible ubiquitylation with respect to stability, activity and sub-cellular localisation. In addition, DUBs can directly influence the structure of chromatin and co-ordinate DNA repair pathways.

Regulation of transcription and RNA processing

USP7 provides an example of a DUB that influences several factors associated with transcription. By counteracting polyubiquitylation, USP7 stabilises many nuclear substrates, such as the transcription factor p53 (Li et al., 2002) or the DNA methyl transferase DNMT1 (Du et al., 2010; Felle et al., 2011). USP7 can also increase the binding affinity of p53 for its target genes through a mechanism that is independent of its deubiquitylase activity (Sarkari et al., 2010). Lastly, USP7 counteracts oxidative stress-dependent monoubiquitylation of the transcription factor FOXO4, thereby opposing its nuclear accumulation and transcriptional activity (van der Horst et al., 2006). Studies of the DUB interactome have also revealed a preponderance of interactions between DUBs and RNA-associated proteins (Sowa et al., 2009), and a role for USP4 in mRNA splicing has recently been described (Song et al., 2010).

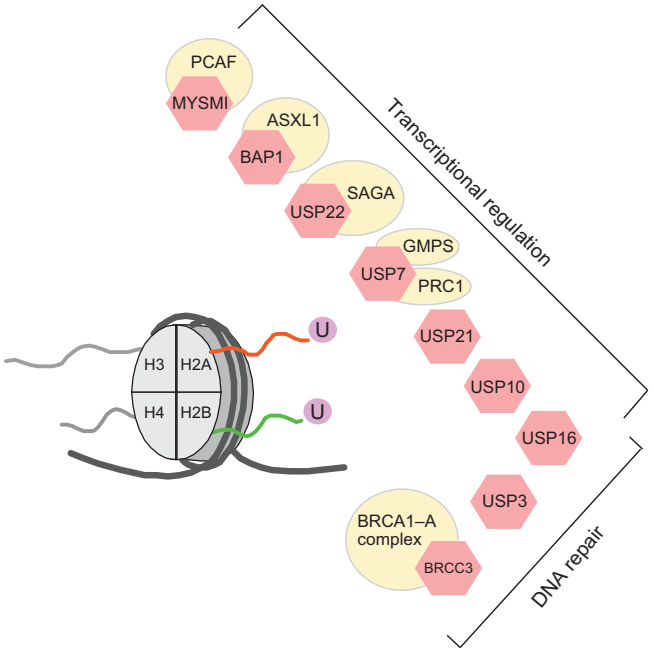

Fig. 4. Diversity of histone DUBs. Many DUBs have been shown to remove monoubiquitin from either histone H2A or histone H2B, thereby providing a mechanism for the regulation of transcription and DNA repair. USP10 deubiquitylates H2AZ, and BRCC3 removes Lys63-linked Ub chains from histone H2A. DUBs are commonly embedded in large protein complexes that promote deubiquitylase activity.

DUBs as histone modifiers

The cellular genome is packaged into chromatin, which must undergo remodelling to accommodate transcription, DNA replication and cell division. Nucleosomes are the basic unit of chromatin comprising 147 bp of DNA wound around eight core histones, whose protruding tails are targets for multiple post-translational modifications that contribute to chromatin remodelling. A number of histone DUBs that exhibit a spectrum of specificity for H2A or H2B have recently been reported (Fig. 4) (Atanassov et al., 2011). Many of these enzymes, including MYSM1 (Zhu et al., 2007), USP16 (also known as Ubp-M) (Joo et al., 2007), USP21 (Nakagawa et al., 2008) and *Drosophila* Calypso (BAP1 in mammals) (Scheuermann et al., 2010) act on monoubiquitylated H2A (H2AUb), whereas the Lys63-specific DUB BRCC3 shows specificity for diubiquitylated H2A (Feng et al., 2010) and USP10 specifically acts on the H2AZ variant (Draker et al., 2011). Other DUBs that can deubiquitylate both H2A and H2B include USP3 (Nicassio et al., 2007), *Xenopus laevis* USP12 and USP46 (Joo et al., 2011), and USP22 (Ubp8 in yeast) (Zhang et al., 2008a; Zhang et al., 2008b). In yeast and flies, H2B is the histone that is predominantly ubiquitylated, and this is processed by Ubp10 and Ubp8 in yeast, and USP7 in flies and mammalian cells (Frappier and Verrijzer, 2011; Gardner et al., 2005; Sarkari et al., 2010; van der Knaap et al., 2005). Intriguingly, in mammalian cells USP7, together with USP11, is also part of the protein regulator of cytokinesis 1 (PRC1) E3 ligase complex, where their DUB activity stabilises PRC1 components, thereby promoting H2A ubiquitylation (Maertens et al., 2010).

The cellular requirement for this plethora of histone DUBs is gradually being unravelled, revealing crosstalk between deubiquitylation and other histone modifications, and participation across a variety of nuclear processes. Individual histone DUBs can influence the transcription of a sub-set of genes. For example, MYSM1 and USP22 participate in activation of the androgen receptor gene (Zhao et al., 2008; Zhu et al., 2007), yet USP16 and calypso exert opposing effects on *HOX* gene expression (Joo et al., 2007; Scheuermann et al., 2010). Association of DUBs with specific transcriptional cofactors may allow their recruitment to discrete subsets of genes or enable crosstalk between deubiquitylation and alternative histone modifications. H2A deubiquitylation by USP3 or USP16 promotes phosphorylation of H3S10 that, in turn, is required for G2–M cell-cycle progression (Joo et al., 2007; Nicassio et al., 2007). The coordination of deubiquitylation with acetylation is highlighted by the finding that USP22 (Ubp8) is a functional component of the Spt–Ada–Gcn5–acetyltransferase (SAGA) complex and MYSM1 also interacts with the histone acetyltransferase p300/CBP associated factor (PCAF). Embedding in a complex is often required for full DUB activity; the SAGA 'DUB module' components ATXN7, ATXN7L3 and ENY2 allosterically activate USP22 (Lang et al., 2011), and the nuclear activity of BRCC3 is expressed within two multicomponent complexes (BRISC and BRCA1 A) (Cooper et al., 2009; Shao et al., 2009; Wang and Elledge, 2007).

DUBs in DNA-repair pathways

DNA lesions embracing double-strand breaks (DSB), single-strand breaks (SSB) and inter-strand crosslinks (ICL) are rectified by tightly controlled pathways that include homologous recombination (HR), non-homologous end joining (NHEJ), base excision repair (BER) and trans-lesion synthesis (TLS) (Ulrich and Walden, 2010). Double-stranded DNA breaks are particularly hazardous to cells and are rapidly marked by escalating phosphorylation-dependent protein recruitment around the breaks. Lys63-linked ubiquitin accumulates at these foci through the concerted action of HERC2, RNF8, RNF168 and Ubc13 (officially known as UBE2N) (Bekker-Jensen and Mailand, 2011; Ulrich and Walden, 2010). It is required for recruitment of proteins, such as BRCA1 and TP53BP1, that activate the DNA-damage checkpoint and trigger the DSB-repair pathway. Several DUBs, including USP3 and BRCC3, have been implicated in removal of these Lys63-chains (Nicassio et al., 2007; Shao et al., 2009). The DUB OTUB1 antagonises this ubiquitylation in a non-catalytic fashion: interaction of its UIM domain with the E2 ubiquitin-conjugating enzyme UBC13 inhibits the catalytic activity of the latter, thereby blocking the function of the E3-ligase RNF168 (Nakada et al., 2010).

DNA base lesions also generate genomic instability and activate the BER pathway, in which two DUBs have so far been implicated. DNA polymerase beta (POLB) is part of a complex with XRCC1, which patches DNA after the corrupted base is removed. The cytoplasmic reserve of newly synthesised POLB is sequentially mono- and then polyubiquitylated by the E3s Mule (officially known as HUWE1) and CHIP (officially known as STUB1), which regulate its nuclear availability. DNA damage inhibits Mule and allows USP47 to deubiquitylate POLB (Parsons et al., 2011). USP7 promotes chromatin remodelling around lesions induced by hydrogen peroxide and, although the substrate remains obscure, has been suggested to act by stabilising MDM2, which in turn ubiquitylates H2B and, thereby, opens up the DNA for repair (Khoronenkova et al., 2011).

Fork-blocking lesions are often the result of chemically induced inter-strand crosslinks, and the activity of proteins such as PCNA (proliferating cell nuclear antigen) and FANCD2 (Fanconi anaemia,

complementation group D2), which are involved in crosslink repair, is dependent on their ubiquitylation status. USP1, in cooperation with its allosteric activator UAF1, can remove ubiquitin from each of these substrates (Cohn et al., 2007; Huang et al., 2006b; Lee et al., 2010b; Nijman et al., 2005a). Fanconi anaemia patients have recessive deletions of a dozen genes, including the one encoding FANCD2, and exhibit hypersensitivity to DNA crosslinking agents. Monoubiquitylated FANCD2 binds BRCA1 in chromatin foci to participate in DNA repair and is later recycled by the activity of USP1 (Nijman et al., 2005a). Transcription of USP1 is repressed by P21 on exposure to DNA-damaging agents to permit DNA-damage-induced accumulation of monoubiquitylated FANCD2 (Rego et al., 2011). Stalling of the replication fork can result in mono- or Lys63 poly-ubiquitylated forms of PCNA, which then promote the alternative pathways of TLS or template switching, respectively. USP1 has a complex role in this balance, but is important to remove monoubiquitin from PCNA and limit error-prone TLS (reviewed in Fox et al., 2011). Most recently USP1 was shown to also participate in DSB repair by promoting HR over the more error-prone NHEJ (Murai et al., 2011).

The cellular responses to DNA damage are coordinated by the ATM–CHEK2 and ATR–CHEK1 kinase cascades, which monitor the cell cycle checkpoints and are activated by DSBs or SSBs. Several DUBs participate at this interface between cell cycle progression and DNA repair. USP1 fine-tunes initiation and termination of the damage response by inhibiting the damage-specific DNA-binding protein 1 (DDB1)-dependent degradation of phosphorylated CHEK1 (Guervilly et al., 2011). The ATM- and ATR-checkpoint kinases include USP15, USP19, USP28 and USP34 amongst their portfolio of substrates (Matsuoka et al., 2007; Mu et al., 2007). Of these, USP28 is known to stabilise multiple proteins, including CHEK2, in response to DSBs (Zhang et al., 2006). Ultimately, deubiquitylation of H2A and H2B by USP3 promotes dephosphorylation of the variant histone H2AX and concomitant checkpoint recovery (Nicassio et al., 2007).

Concluding remarks

In recent years, our appreciation of the dynamic aspect of ubiquitin modifications has increased dramatically, which has led to the DUBs assuming equal prominence to the ubiquitin ligases. In this Commentary, we have highlighted their central roles in the endocytic and ERAD pathways, their emergence as key regulators of major signal transduction pathways as well as nuclear processes. In reality, virtually no complex cellular process will be untouched by their activity. DUBs are, therefore, now emerging as attractive drug targets, particularly for cancer and neurological diseases. First-generation inhibitors that show specificity have recently been reported (Daviet and Colland, 2008; Lee et al., 2010a; Liu et al., 2011), which will heighten interest in this rapidly developing field.

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