

# Riding the DUBway: regulation of protein trafficking by deubiquitylating enzymes

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Ubiquitylation is a key regulator of protein trafficking, and much about the functions of ubiquitin ligases, which add ubiquitin to substrates in this regulation, has recently come to light. However, a clear understanding of ubiquitin-dependent protein localization cannot be achieved without knowledge of the role of deubiquitylating enzymes (DUBs). DUBs, by definition, function downstream in ubiquitin pathways and, as such, have the potential to be the final editors of protein ubiquitylation status, thus determining substrate fate. This paper assimilates the current evidence concerning the substrates and activities of DUBs that regulate protein trafficking.

## Introduction

Ubiquitylation is a key posttranslational regulator of protein activity, stability, and/or localization. That the addition of this highly conserved 76-amino acid moiety can result in such a range of outcomes is, in part, a result of the variety of ways ubiquitin can be covalently attached to the substrate protein. Ubiquitylation is the formation of an isopeptide bond between a substrate lysine and the COOH group of the COOH-terminal glycine of ubiquitin. The addition of a single ubiquitin is termed monoubiquitylation or, if several substrate lysines are modified in this manner, multiubiquitylation. However, because ubiquitin itself has seven lysines, which can also be subjected to isopeptide formation, a polyubiquitin chain can form. Polyubiquitylation can have several forms, depending on which lysine is used. All lysines (K6, 11, 27, 29, 33, 48, and 63) can be subjected to chain formation, but the most common linkages are K48 and K63. K48 polyubiquitylation is a very efficient tag for marking proteins for degradation at the proteasome, whereas K63 chains are involved in nonproteasomal functions, including protein trafficking and DNA repair (for review see Pickart and Eddins, 2004). Mono- and multiubiquitylation are major regulators of protein trafficking in both the exocytic and endocytic pathways.

Ubiquitin can affect protein trafficking in two ways: either by direct attachment to the cargo protein (cis-regulation) or modification of the protein-trafficking machinery (trans-regulation; Hicke and Dunn, 2003). Ubiquitylation of cargo proteins at the plasma membrane can be sufficient to induce endocytosis, and it also acts as a sorting signal at the TGN and the multivesicular body (MVB; Hicke and Dunn, 2003). Furthermore, ubiquitin may participate in vesicle targeting, as several proteins involved in vesicle docking and fusion bind ubiquitin (Donaldson et al., 2003). At the plasma membrane, the ubiquitin signal is recognized by epsin and Eps15, and, at the MVB, it is recognized by a complex containing hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and signal-transducing adaptor molecule (STAM). Subsequent interactions with protein complexes ESCRT (endosomal sorting complex required for transport) I, II, and III result in the targeting of ubiquitylated cargo into internal vesicles to be degraded at the vacuole/lysosome (Katzmann et al., 2002; Raiborg et al., 2003). Many of the protein-trafficking machinery components are themselves ubiquitylated without being subject to proteasomal degradation, raising the possibility of the regulation of cargo-trafficking machinery interaction by reciprocal ubiquitylation status (Oldham et al., 2002; Polo et al., 2002).

In addition to conjugation, substrate ubiquitylation status may also be regulated by ubiquitin cleavage, which is performed by deubiquitylating enzymes (DUBs). The human genome encodes for ~80 DUBs, which can be divided into five classes on the basis of differences in the catalytic domain (Amerik and Hochstrasser, 2004; Nijman et al., 2005). Four classes of DUBs—the ubiquitin COOH-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), Machado-Joseph disease protein domain proteases, and ovarian tumor proteases—are cysteine proteases, whereas the JAMM motif proteases are metalloproteases. Although DUB function does not neatly segregate along class lines, a few generalizations can be made. The substrates for UCHs tend to be small peptides (20–30 amino acids), and UCHs primarily function in the recycling of ubiquitin. The USPs are the largest group, with ~55 members in humans (Nijman et al., 2005). They contain a characteristic catalytic core, which is defined by a small number of motifs flanked by large NH<sub>2</sub>- and/or COOH-terminal extensions. Except for the catalytic core motifs, which only extend over ~100 amino acids, there is no other homology between USP family members.

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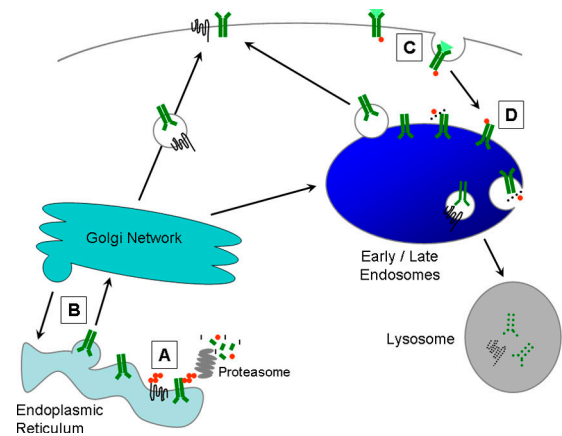
Abbreviations used in this paper: DUB, deubiquitylating enzyme; EGFR, EGF receptor; MVB, multivesicular body; STAM, signal-transducing adaptor molecule; UCH, ubiquitin COOH-terminal hydrolase; USP, ubiquitin-specific protease.

The divergent NH<sub>2</sub>- and COOH-terminal extensions are proposed to impart substrate specificity. Along with the substrate-specific E3 ligases, USPs are the only classes of ubiquitin-modifying enzymes to have expanded significantly throughout evolution (Semple et al., 2003). Information on the other three classes of DUBs is fairly sparse and relatively recent, preventing generalizations; however, individual members of the ovarian tumor protease and JAMM classes are involved in the regulation of protein trafficking (as detailed below). Protein trafficking is regulated by DUBs in several ways, such as maintaining cellular levels of free ubiquitin, antagonizing the degradation of trafficking proteins in the ubiquitin–proteasome system, and regulating nonproteasome-dependent functions of monoubiquitin or multiple monoubiquitin signals.

### Regulation by DUBs of protein trafficking in the exocytic pathway

Secreted and membrane proteins enter the exocytic pathway at the ER. The ubiquitin pathway is a major regulator of protein quality at this juncture, as misfolded proteins inserted into the ER are very rapidly recognized, ubiquitylated, and degraded by proteasomes in the cytoplasm (Meusser et al., 2005). Whereas some common characteristics of misfolded proteins may be recognized by the ubiquitylation machinery, it has recently been shown that one DUB, USP4, plays a very substrate-specific role at the ER (Fig. 1 A). USP4 associates with the cytosolic COOH terminus of the A<sub>2a</sub>-adenosine receptor, a Gs-coupled receptor (Milojevic et al., 2006). This regulates the quality control of A<sub>2a</sub>-adenosine receptor, facilitating the passage of the receptor through the ER and Golgi and resulting in increased A<sub>2a</sub>-adenosine receptor at the plasma membrane. Several controls showed that this increase was caused by facilitated transport through the exocytic pathway and not by recycling from the endocytic pathway (Milojevic et al., 2006). This interaction was specific, as another DUB, USP14, could not substitute for USP4, and USP4 had no effect on the trafficking of other G protein-coupled receptors. As >50% of the A<sub>2a</sub>-adenosine receptor is degraded at the ER, the cis-regulatory function of USP4 has the potential to be a significant and specific regulator of A<sub>2a</sub>-adenosine receptor function at the plasma membrane.

The regulation of traffic between the ER and cis-Golgi is also affected by DUB activity, but this time it involves trans-regulation. In yeast, the DUB Ubp3 participates in the stabilization of two trafficking proteins, Sec23, a COPII subunit protein, and β'-COP, a COPI subunit, that are required for ER to Golgi transport and Golgi to ER retrograde transport, respectively (Fig. 1 B; Cohen et al., 2003a,b). In this role, Ubp3 has an essential cofactor, Bre5, and deletion mutants of either result in defects in the bidirectional transport between the ER and Golgi. Ubp3 appears to be essential for cleavage of the isopeptide bond between Gly76 of ubiquitin and the ubiquitylated lysine residue of the Sec23 and β'-COP substrates. Accumulation of monoubiquitylated forms of Sec23 and β'-COP and an increased turnover of these proteins are seen in both *ubp3Δ* mutants and *bre5Δ* mutants. The enzyme–substrate relationship between Ubp3 and Sec23 was further confirmed by demonstrating direct protein–protein interaction and the inability of a catalytically inactive



**Figure 1. DUBs influence membrane protein trafficking at a variety of trafficking locations.** Conjugation of ubiquitin (red circles) is involved in the trafficking of various cargo, including receptor tyrosine kinases (green) and G protein-coupled receptors (black curved lines). (A) The DUB USP4 regulates ubiquitin–proteasome-mediated degradation of misfolded forms of a Gs-coupled receptor at the ER. (B) The yeast DUB Ubp3 functionally regulates proteins that are necessary for vesicular transport from the ER to Golgi and also Golgi to ER retrograde transport. (C) At the plasma membrane, ubiquitylation, which often occurs in response to receptors binding ligand (triangles), acts as an internalization signal and as a regulatory modification on the protein-trafficking machinery. Epsin, a component of the clathrin-mediated endocytosis machinery, is regulated by FAM/USP9X. (D) After internalization protein cargo is trafficked through multiple endosomes, it is eventually recycled to the plasma membrane or degraded at the lysosome. Recycling to the plasma membrane occurs if the cargo is deubiquitylated (represented by a black dashed line) before ubiquitin signals the sorting of the cargo into internal vesicles. Sorting cargo into internal vesicles destined for lysosomal degradation. Deubiquitylation of the cargo during sorting is necessary for ubiquitin recycling. Two DUB–cargo interactions that antagonize receptor down-regulation have been proposed: AMSH and EGFR or UCH37 and type I TGF-β receptor. In contrast, USP8 appears to facilitate the down-regulation of EGFR. DUBs also regulate the endosomal trafficking machinery indirectly. The overexpression of Ubp1 in yeast results in impaired lysosomal trafficking of at least two cargo proteins but does not alter their ubiquitylation status.

mutant to eliminate the monoubiquitylated form of Sec23 in *ubp3Δ* cells (Cohen et al., 2003a). Notably, the monoubiquitylated Sec23 did not dissociate as freely as wild type from the ER once bound, and it failed to interact with another component of the COPII complex (Cohen et al., 2003a). Thus, ubiquitylation leads to the down-regulation of Sec23 not only by leading to its degradation but also through altering its biochemical properties such that it can no longer perform its function in ER to Golgi transport. Data concerning whether such relationships have been conserved by mammalian cells is preliminary but promising. Both Ubp3 and its cofactor Bre5 have human homologues (UBP10 and G3BP1/2 [Ras–GTPase-activating protein SH3 domain-binding protein], respectively). The ability of G3BP to modulate the deubiquitylating activity of UBP10 has been demonstrated in vitro on artificial substrates (Soncini et al., 2001), and a yeast two-hybrid assay has been used to demonstrate protein interaction between USP10 and bovine β'-COP, whereas no interaction was observed between USP10 and five other components of the COPI complex (Cohen et al., 2003b). Thus, it appears likely that the functional relationship between a specific USP-type DUB and protein transport between the Golgi and ER is evolutionally conserved.

DUBs also influence the exocytic pathway in a more general manner by regulating the dynamics of organelle reassembly. The VCIPI35 DUB is an essential cofactor for p97–p47-mediated Golgi and ER reassembly (Wang et al., 2004; Kano et al., 2005), a process that is required after cell division. For Golgi reassembly, although the role of VCIPI35 is dependent on its DUB activity, it acts independently of the proteasome. However, a ubiquitin mutant that cannot bind p97–p47 was found to inhibit Golgi reassembly when added to the system before disassembly (Wang et al., 2004). It may be inferred from this that a ubiquitylation-dependent interaction between p97–p47 and an unknown protein, which becomes ubiquitylated before Golgi disassembly, is required for Golgi reassembly. This as yet unidentified protein is clearly a candidate for a VCIPI35 substrate. Regardless of substrate identity, VCIPI35's involvement in Golgi and ER reassembly demonstrates that a DUB can functionally participate in membrane fusion events, which are key components of membrane protein-trafficking events.

### Regulation of protein trafficking in the endocytic pathway by DUBs

The endocytic pathway begins with the internalization of plasma membrane proteins, which is followed by multiple sorting events at the early/recycling endosome and the late endosome/MVB compartments. These ultimately either return the protein to the plasma membrane or deliver it to the lysosome for degradation. Most, if not all, plasma membrane proteins in yeast are endocytosed in a ubiquitin-dependent manner, indicating that ubiquitylation is an ancient signal for trafficking (Hicke and Dunn, 2003). In mammalian cells, the best-studied examples of ubiquitin-dependent endocytosis involve ligand-activated receptor tyrosine kinases. Both E3 ubiquitin ligases and DUBs regulate endocytic traffic both in cis and trans.

Epsin is a trafficking accessory molecule involved in both clathrin-mediated endocytosis (Ford et al., 2002) and the internalization of ubiquitylated cargo in a clathrin-independent manner (Fig. 1 C; Chen and DeCamilli, 2005; Sigismund et al., 2005). That epsin may be regulated by a DUB was first indicated by genetic studies in *Drosophila melanogaster*, where mutations in the liquid facets (*lqf*, *Drosophila* epsin) gene were dominant enhancers of the eye defect observed in mutants of the DUB fat facets (*faf*), a USP-type DUB (Cadavid et al., 2000). Two experiments support the enzyme substrate relationship between *faf* and *lqf*. First, the overexpression of *lqf* replaced the requirement of *faf* during eye development, as would be expected if *faf*'s role was to stabilize *lqf* (Cadavid et al., 2000). Also, the deubiquitylation of *lqf* by wild-type *faf* but not a catalytically inactive mutant stabilized *lqf* (Chen et al., 2002). Consequently, it has been concluded that in *Drosophila*, *faf* opposes the ubiquitin–proteasome-mediated degradation of *lqf*. Subsequently, it was found that *lqf* is essential for directing the endocytosis and subcellular localization of Delta in Delta/Notch signal-sending cells such that Delta can be activated (Wang and Struhl, 2004). Perturbation of this function is the basis for the eye phenotype of *faf*-null and *lqf*-null *Drosophila* mutants (Overstreet et al., 2004).

The relationship between the homologues of *lqf* (epsin1) and *faf* (FAM/USP9X) is conserved in higher vertebrates but is likely to have functional differences. Epsin1 does not appear to be polyubiquitylated and degraded by the proteasome but is instead monoubiquitylated (Oldham et al., 2002). The levels of monoubiquitylated epsin1 decrease simultaneously with a global decrease in ubiquitylated proteins upon calcium-induced depolarization in rat synaptosomes or stimulation of calcium signaling in nonneuronal cell types (Chen et al., 2003). Loss of monoubiquitylated epsin1 was specifically prevented by siRNA-mediated FAM/USP9X knockdown. It was proposed that the ubiquitylation of epsin may prevent its interaction with several binding partners, including lipids, AP-2, and clathrin; therefore, FAM-mediated deubiquitylation activates epsin (Chen et al., 2003).

Curiously, epsin is not the only link between the FAM/USP9X DUB and membrane protein trafficking. FAM colocalizes with markers for several protein-trafficking compartments, including the TGN and late endosomes, and there is strong circumstantial evidence linking FAM/USP9X to trafficking of the E-cadherin– $\beta$ -catenin complex in epithelia (Murray et al., 2004). Additionally, doublecortin, an essential neural protein that associates with microtubules and clathrin adaptor proteins AP-1 and AP-2 (Friocourt et al., 2001), is a binding partner but not a substrate of FAM (Friocourt et al., 2005).

Other DUBs may regulate the endocytic traffic of specific cargo at the plasma membrane. In mammalian cells, activated G protein–coupled receptors associate with  $\beta$ -arrestin, and both proteins are ubiquitylated before internalization of the complex. In this instance, ubiquitylation of the  $\beta$ -arrestin adaptor protein rather than the receptor itself is required for receptor internalization (Shenoy et al., 2001). Different G protein–coupled receptors show different recycling kinetics that correlated with the ubiquitylation status of the associated  $\beta$ -arrestin. Furthermore, overexpression of a  $\beta$ -arrestin–ubiquitin fusion, such that  $\beta$ -arrestin could not be deubiquitylated, slowed the recycling kinetics of fast recycling receptors and also led to enhanced receptor internalization and degradation (Shenoy and Lefkowitz, 2003). This study strongly suggests that fast recycling receptors recruit DUBs to act on  $\beta$ -arrestin. In yeast, the soluble form of Ubp1 may also function at the plasma membrane or sorting endosome to deubiquitylate an as yet unidentified component of the protein-trafficking machinery and recycle ubiquitylated proteins. This was based on the observation that the overexpression of soluble Ubp1 disrupted the lysosomal trafficking of the ATP-binding cassette transporter protein Ste6 as well as the  $\alpha$ -factor receptor Ste2 even though Ubp1 did not alter the ubiquitylation status of either cargo (Schmitz et al., 2005). However, Ubp1 deubiquitylating activity was required as a catalytically inactive form and had no effect on either plasma membrane protein (Schmitz et al., 2005).

A subsequent critical junction for protein sorting in the endocytic pathway occurs at the endosomes. Precisely how protein sorting at the endosomes is regulated is far from clear, except to say that it involves the interdependent interactions between individual components of large multiprotein complexes. Ubiquitin influences endosomal sorting at three



levels: (1) if cargos remain ubiquitylated, they are ultimately fated for degradation in the lysosome; (2) many of the accessory proteins are ubiquitylated; and (3) they may also contain motifs that bind ubiquitin or ubiquitin-like domains on cargo or other accessory proteins. Delineating a precise role for DUBs is difficult, as for any protein in these processes, but several recent studies have made significant progress in understanding the role of two: USP8 (UBPY) and AMSH (associated molecule with the SH3 domain of STAM), a JAMM-class DUB.

Knockdown of USP8 levels using siRNA significantly inhibits the down-regulation of ligand-activated growth receptors such as EGF receptor (EGFR) and Met (McCullough et al., 2004; Bowers et al., 2006; Row et al., 2006). Conversely, AMSH negatively regulates EGFR down-regulation and is proposed to recycle the EGFR at the sorting endosome (McCullough et al., 2004; Row et al., 2006). The molecular mechanism underlying these opposing effects is hinted at by the observation that USP8 and AMSH bind a central SH3 domain of STAM proteins in a mutually exclusive manner (Kato et al., 2000). STAM and its constitutive binding partner Hrs participate in recognizing ubiquitylated cargo on early endosomes (Bilodeau et al., 2003), leading to the sorting of such cargo into internal vesicles at the MVB (Fig. 1 D). Overexpression of a STAM2A mutant lacking its SH3 domain interfered with the lysosomal degradation of PDGF and its receptor (Takata et al., 2000; McCullough et al., 2004). A proportion of USP8 and AMSH colocalize with STAM at endosomes (McCullough et al., 2004; Row et al., 2006). The functional relevance of the interaction between USP8, AMSH, and STAM in the endocytic trafficking of activated growth factors is supported by other observations, including that catalytically inactive AMSH, a potential “substrate trap” mutant, resulted in the accumulation of ubiquitin on endosomes, an increased association with STAM, and the generation of a minor product consistent with ubiquitylated STAM (McCullough et al., 2004). Further interactions between AMSH and other trafficking accessory proteins such as clathrin heavy chain and a component of ESCRT III indicate that AMSH is “deeply integrated as a hub protein within the MVB-sorting protein interaction network” (McCullough et al., 2006). It was also observed that the associations between AMSH and trafficking machinery was reinforced by the simultaneous binding of STAM, with the subsequent activation of AMSH coupled to its association with the MVB-sorting machinery (McCullough et al., 2006).

The role of USP8 in facilitating the passage of EGFR and Met to the lysosome has been supported by the observation that USP8 can deubiquitylate monoubiquitylated growth factor receptors as well as act on both K48- and K63-linked ubiquitin chains *in vitro* (Row et al., 2006). USP8 is recruited to endosomes upon EGF stimulation but shows no association with endosomes in starved cells in contrast to AMSH (Row et al., 2006). Interestingly, when USP8 is depleted, STAM becomes destabilized, which is a process dependent on the proteasome (Row et al., 2006). Therefore, part of USP8's function may be to maintain STAM levels. However, it is proposed that USP8 might regulate multiple components in the endocytic pathway, such as the growth factor receptors themselves. Precisely where in the endocytic pathway a DUB might deubiquitylate a recep-

tor is critical, as it could result in opposite effects. If USP8 deubiquitylates EGFR at the MVB, this facilitates EGFR's progression toward degradation in the lysosome and, thus, aids receptor down-regulation (Bowers et al., 2006; Row et al., 2006). However, it has also been suggested that USP8 might be active at the sorting endosome, in which case the deubiquitylated receptor is recycled to the plasma membrane and is ready for another round of signaling (Mizuno et al., 2005). It is not clear why the data of Mizuno et al. (2005) showed an opposite effect on EGFR by USP8, but it is supported by their observations that upon EGF stimulation, USP8 directly binds EGFR. Another DUB UCH37 has been suggested to deubiquitylate activated type I TGF- $\beta$  receptor, thereby preventing its down-regulation, but it was not shown whether this occurs at the plasma membrane or at the sorting endosome (Fig. 1 D; Wick et al., 2005).

In the ubiquitin–proteasomal system, coupling of DUBs to the proteasome is necessary for ubiquitin recycling (Amerik and Hochstrasser, 2004), and one yeast DUB has been shown to play an analogous role at a late stage of the ubiquitin–lysosome system. Doa4 (UBP4) was one of the first identified yeast DUBs, and it was noted that in *doa4* $\Delta$  mutant cells, many substrates of the ubiquitin–proteasome system were stabilized as a result of a depletion of ubiquitin (Papa et al., 1999; Swaminathan et al., 1999). Interestingly, a screen for genetic suppressors of the *doa4* phenotype identified members of the vacuolar protein-sorting pathway and not components of the proteasomal system (Amerik et al., 2000). Further examination of these mutants led to the conclusion that Doa4 acts at the late endosome/prevacuolar compartment to recover ubiquitin from membrane proteins before their sorting into internal vesicles and subsequent lysosomal degradation (Fig. 1 D; Amerik et al., 2000; Dupre and Haguener-Tsapis, 2001). A vertebrate homologue of Doa4 has not been identified, although deubiquitylation of the specific cargo protein EGFR has been shown to occur before its lysosomal degradation (Alwan et al., 2003). This deubiquitylation event was inhibited by the proteasomal inhibitor lactacystin, which delayed but did not prevent the lysosomal degradation of EGFR, leading to the intriguing possibility that in vertebrates, the deubiquitylating activity of the proteasome might fulfill the role played by Doa4 in yeast.

### Conclusions/future directions

The study of ubiquitylation and membrane protein trafficking is an exciting and relatively recent field. Although the study of DUBs has lagged behind that of ubiquitin ligases, it is already apparent that DUBs play key and varied roles in protein trafficking. Through participation in membrane protein-trafficking events, DUBs regulate protein localization and stability, membrane fusion, signaling pathways, and developmental events. The current deficiencies in our knowledge are many and mostly self-evident, but two major obstacles need to be overcome for significant progress to be made. The first concerns the observation that many DUBs having multiple subcellular locations and substrates/binding partners. The second challenge comes from the observation that many DUBs and E3 ligases are complexed together and often regulate both the activity and stability of themselves and each other as well as common substrates

(Nijman et al., 2005). The possible permutations and combinations inherent in these interactions make drawing simple linear models from over- or underexpression studies nearly impossible.

These complex, reciprocal regulatory networks are reminiscent of other cell signaling pathways, and so an appreciation of what has been learned from phosphorylation signaling pathways will be useful in answering the “where to from here?” question. The regulation of kinases and phosphatases in time and space determine cell signaling dynamics (Kholodenko, 2006), so for the study of DUBs in protein trafficking, it will be critical to include techniques such as fluorescence resonance energy transfer to determine exactly which subcellular pools of DUBs and substrates are interacting. It will also be important to molecularly dissect the DUBs to identify individual localization signals and/or specific substrate-binding sites so that more precise questions can be addressed. The interaction between DUBs and E3 ligases echoes a universal motif found in cellular networks in which kinases, phosphatases, guanine nucleotide exchange factor, and GTPase-activating proteins are in complexes regulating the same substrate (Kholodenko, 2006). Far from representing a futile cycle, such arrangements can provide ultrasensitivity to signaling pathways and are modulated by controlling the localization and recruitment of the different enzymes to the complex (Kholodenko, 2006). Clearly, defining DUB–E3 ligase pairs and the signals that recruit them to specific points of protein trafficking will represent a major step forward along the pathway, for although ubiquitylation may mark the beginning of a protein’s journey, it is not over until DUBs signal the final destination.

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