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# Mechanistic insights into ER-associated protein degradation

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## Abstract

Misfolded proteins of the endoplasmic reticulum (ER) are discarded by a conserved process, called ER-associated protein degradation (ERAD). ERAD substrates are retro-translocated into the cytosol, polyubiquitinated, extracted from the ER membrane, and ultimately degraded by the proteasome. Recent *in vitro* experiments with purified components have given insight into the mechanism of ERAD. ERAD substrates with misfolded luminal or intra-membrane domains are moved across the ER membrane through a channel formed by the multi-spanning ubiquitin ligase Hrd1. Following polyubiquitination, substrates are extracted from the membrane by the Cdc48/p97 ATPase complex and transferred to the proteasome. We discuss the molecular mechanism of these processes and point out remaining open questions.

## Introduction

Misfolded ER proteins are removed by ERAD, an evolutionarily conserved process in which substrates are retro-translocated into the cytosol, polyubiquitinated, and degraded by the proteasome (for review, see ref. [1]). Work in *S. cerevisiae* resulted in the simple concept that misfolded ER proteins are degraded by three different ERAD pathways (ERAD-L, -M, and -C), depending on whether their misfolded domain is localized in the ER lumen, within the membrane, or on the cytosolic side of the ER membrane [2–4]. These pathways involve distinct ubiquitin ligases. ERAD-L substrates use the RING-finger ligase Hrd1 in complex with three other membrane proteins (Hrd3, Usa1, and Der1) and a luminal protein (Yos9) [2,5,6]. ERAD-M substrates also use Hrd1 and Hrd3, but not Der1 [2], and only in some cases Usa1 [7]. ERAD-C substrates require Doa10, another RING-finger ligase [8]. However, it should be noted that Doa10 also recognizes some substrates by features within their membrane-spanning region (e.g. ref. [9]). A fourth pathway is responsible for the degradation of misfolded inner nuclear membrane proteins and utilizes a ubiquitin ligase consisting of three proteins (Asi1,2,3) [10,11]. Again, the Asi complex seems to recognize substrate features within the membrane. It remains unclear how proteins are targeted to the

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**Conflict of interest**

The authors declare no conflict of interest.

different pathways, as ERAD-M, -C and Asi all handle trans-membrane protein substrates. All ERAD pathways converge on the cytosolic side of the ER membrane, where they require other components of the ubiquitination machinery and an ATPase complex consisting of the AAA+ ATPase Cdc48 (called p97 or VCP in mammals) and a heterodimeric cofactor, consisting of Ufd1 and Npl4 [12–17]. In this review, we focus on the Hrd1-dependent ERAD-L and –M pathways. Although much of the mechanistic work has been done with *S. cerevisiae* components, the conclusions are likely applicable to all eukaryotes.

## Overview of ERAD-L and –M

ERAD-L begins with the recognition of a misfolded protein domain in the ER lumen, which is best understood for misfolded glycoproteins (for review, see [18]). When these proteins linger too long in the ER, the N-linked glycan is trimmed to generate a terminal α-1,6-linked mannose residue (Figure 1, step 1). This sugar is recognized by the lectin Yos9p (step 2) [19,20], which in turn is bound to the luminal domain of Hrd3p [19,20]. In addition, Hrd3 probably recognizes a misfolded substrate segment around the glycan-attachment site, thus providing a second binding site for the substrate. Hrd3 also recognizes non-glycosylated ERAD-L substrates, but whether Yos9 is also involved in this case is unclear. How a substrate is transferred to downstream components is unknown, but ultimately the polypeptide inserts into the membrane channel formed by the Hrd1 ligase (step 2; see below). Membrane insertion also requires the multi-spanning membrane protein Der1 [21,22]. ERAD-M substrates probably enter the Hrd1 channel sideways from the lipid phase (step 3). In both pathways, substrates are then polyubiquitinated on the cytoplasmic side of the ER membrane by Hrd1p [5,6], which recruits ubiquitin-conjugating enzymes, with Ubc7 being the best studied (step 4). Ubc7 cooperates with Cue1, which serves as membrane anchor and activator.

In the next step, the cytosolic Cdc48 ATPase complex is recruited to the membrane by an interaction of the polyubiquitin chain with the cofactor Ufd1/Npl4 (step 5). Recruitment of Cdc48 is also facilitated by an interaction with the membrane protein Ubx2 [23,24]. The Cdc48 ATPase then uses the energy of ATP hydrolysis to progressively pull the substrate out of the membrane [25,26]. Once the substrate is fully removed from the ER, Cdc48 probably dissociates from the Ubx2 anchor, diffusing away from the membrane with its associated substrate (step 6). The substrate is then released from Cdc48 in a process that requires trimming of the polyubiquitin chain by a deubiquitinase (DUB) (step 6). Finally, the substrate is passed on to the proteasome for degradation (step 7). Below, we discuss several of these steps in more detail.

## Hrd1 forms a retro-translocation channel

The identity of the retro-translocation channel was controversial for many years. Based on mutations, pull-down experiments, and interaction with the proteasome, it was postulated that the Sec61 channel, which normally allows polypeptides to move from the cytosol into the ER lumen, can function in reverse (for review, see [27]). However, Sec61 mutations may indirectly affect the biosynthesis of ERAD components, and ERAD substrates interact only weakly with Sec61 [28]. Furthermore, the Sec61 channel contains a plug domain that is

normally displaced by a signal sequence when a secretory protein inserts from the cytosolic side [29,30]. It is difficult to envision how the channel would open from the luminal side of the ER membrane in ERAD. Derlin-1, a mammalian homolog of Der1, was also proposed as a channel, based on the fact that it is a multi-spanning membrane protein that has interaction partners on both sides of the ER membrane [31,32]. However, Hrd1 has clearly emerged as the top candidate for forming a retro-translocation channel.

Initial evidence for Hrd1 forming a channel came from the observation that its overexpression in yeast makes the other components of the Hrd1 complex dispensable for the degradation of ERAD-L and –M substrates [28,33,34]; all downstream components, such as the ubiquitination machinery and the Cdc48 ATPase complex were still required. The existence of a Hrd1 channel was also supported by photo-crosslinking experiments that showed substrate in close proximity of Hrd1 during retro-translocation [28].

*In vitro* experiments with purified proteins later showed that Hrd1 binds and polyubiquitinates soluble ERAD-L substrates [26]. Retro-translocation was recapitulated with proteoliposomes that contained both Hrd1 and a single-spanning substrate protein with a large misfolded domain [35]. When the ubiquitination machinery was added, a segment that was initially inside the lumen of the vesicles was polyubiquitinated by Hrd1, suggesting that it moved across the membrane to the outside of the liposomes. Hrd1 also underwent efficient auto-ubiquitination in the same reaction. Auto-ubiquitination of Hrd1 was postulated to be the trigger for retro-translocation of the substrate, because there was no lysine of the substrate accessible to Hrd1 at the beginning of the reaction, but ubiquitination *per se* was required. Indeed, several studies showed that ubiquitination is still required when all lysines in a substrate are removed [35–38]. The crucial modification seems to occur in the RING finger of Hrd1, as mutation of lysines in this domain prevented retro-translocation *in vitro* and the degradation of ERAD-L substrates *in vivo* [35].

These results led to a model in which auto-ubiquitination of Hrd1 opens the channel for ERAD-L substrates [35]. A polypeptide substrate would then be able to slide back and forth through the channel, but attachment of a polyubiquitin chain could prevent its back-sliding into the ER lumen. Interestingly, the lysine mutations in the RING finger domain do not have a strong effect on ERAD-M substrates (R. Baldridge, personal communication). Thus, ERAD-M substrates may not require auto-ubiquitination of Hrd1, perhaps because a segment of the polypeptide is already in the cytosol.

## Structure of the Hrd1 channel

An electron cryo-microscopy (cryo-EM) structure was recently determined for Hrd1 bound to the luminal domain of Hrd3, a complex that is sufficient for ERAD-M [39]. The complex contains two symmetry-related molecules of Hrd1 and Hrd3 (Figure 2A). The two Hrd3 molecules form an arch on the luminal side. Hrd1 contains eight trans-membrane (TM) segments (Figure 2A), rather than six, as previously assumed [40]. Five of these TM segments form a funnel that extends from the cytosol almost to the luminal side of the membrane (Figure 2A). Trans-membrane segment 1 (TM1) of each Hrd1 molecule laterally seals the funnel of the neighboring Hrd1 molecule on the cytosolic side (Figure 2A; lower

panels). The aqueous cavity of Hrd1 is reminiscent of that in the Sec61 channel and its prokaryotic homolog, the SecY channel, which allow protein transport in the opposite direction [29,30]. Deep cytosolic invaginations are also seen in the bacterial YidC protein and its homologs in plants and mitochondria (Figure 2B) [41,42]. These proteins allow hydrophobic TM segments to move from the cytosol into the lipid bilayer, whereas Hrd1 facilitates the reverse process during ERAD-M (Figure 2B). Given that the known protein-conducting conduits have large aqueous cavities, it seems that the thinning of the membrane might be a general principle to reduce the energy barrier for polypeptide movement in or out of a membrane.

## Open questions about the function of Hrd1 and its partners

Although Hrd1 has emerged as the best channel candidate for ERAD-L and –M, its function is still poorly understood. One issue is whether the same Hrd1 complex is involved in both ERAD pathways. In fact, it seems possible that a complex comprising Hrd1, Hrd3, Usa1, Der1, and Yos9 would function in ERAD-L, whereas a distinct Hrd1/Hrd3 sub-complex would mediate ERAD-M. Whether Hrd1 functions as a dimer or monomer in these pathways is also unclear. In the current Hrd1/Hrd3 structure, two Hrd1 channels are paired [39], but Hrd1 oligomerization *in vivo* actually requires Usa1 [7,28]. Perhaps, Hrd1 auto-ubiquitination and substrate binding cause the dissociation of the Hrd1 dimer into monomers [28].

Other unresolved questions concern the path of the substrate across the membrane and the role of the other components of the Hrd1 complex. Der1 is an inactive relative of rhomboid proteases [43] and recruits substrates from the ER lumen. Perhaps, it replaces the second Hrd1 molecule and sits at the lateral gate of a monomeric Hrd1 channel, allowing luminal substrates to enter sideways, rather than directly from the luminal aqueous phase. Because Usa1 physically links Der1 and Hrd1, it may position Der1 for substrate transfer [7,22,28]. The exact function of Yos9 and Hrd3 in substrate recognition also needs to be defined. Hrd3 probably not only binds substrate, but also inhibits the activity of Hrd1, as in its absence, Hrd1 undergoes excessive auto-ubiquitination and degradation [33,44]. How Hrd3 would regulate Hrd1 activity is unclear, particularly because the RING finger domains of the Hrd1 molecules are invisible in the density map of the Hrd1/Hrd3 complex (Figure 2A). In one model, substrate binding to Hrd3 would relieve the inhibition of Hrd1, allowing auto-ubiquitination and opening of the channel. Finally, it remains unclear how auto-ubiquitination of Hrd1 would be reversed to reset the channel for the next round of ERAD-L. The unidentified DUB must be distinct from the relatively well characterized enzyme Otu1, a DUB involved in the trimming of polyubiquitinated substrate on the Cdc48 ATPase complex (see below).

The answer to several of these questions will likely come from cryo-EM structures of the Hrd1 complex and from experiments that test the binding and translocation of defined substrates. It will be a major challenge to generate translocation intermediates, which have been instrumental in elucidating the mechanism of “forward translocation” through the Sec61/SecY channel [45].

Hrd1 is probably a member of a larger class of channel-forming ubiquitin ligases in the ER, which includes Doa10 and the Asi complex. In mammals, Hrd1 has a close homolog (gp78), and both proteins show sequence similarity in their putative channel-forming TMs 3–6 to the ligases RNF145 and RNF139 (alternately called TRC8) [39]. Future experiments need to test whether they all form protein-conducting channels that allow proteins to move from the ER into the cytosol.

## Mechanism of the Cdc48 ATPase complex

The Cdc48 ATPase consists of an N-terminal domain (N domain) and two ATPase domains (D1 and D2) [46]. Six Cdc48 monomers form a double-ring structure, with a “cis” and a “trans” side and a central pore (Figure 3). The N domains are coplanar with the D1 ring when the D1 ATPases are in the ADP-bound state (“down-conformation”), and in an “up-conformation” when in the ATP-bound state (Figure 3; stage 1) [47]. The hexamer associates on its “cis side” with one copy of the Ufd1/Npl4 (UN) cofactor complex [48].

Much of our knowledge about the mechanism of the Cdc48 ATPase in ERAD comes from the use of simplified *in vitro* systems, in which artificial, polyubiquitinated substrates were used [25,49]. These and other experiments resulted in a model for substrate processing by Cdc48 (Figure 3). First, the UN cofactor binds the polyubiquitin chain attached to the substrate (Figure 4; stage 2). This interaction decreases ATP hydrolysis of the D1 domains, thus favoring their ATP-bound state with the N domains in the up-conformation. At the same time, ATP hydrolysis by D2 is stimulated, resulting in the translocation and unfolding of fluorescent model substrates (stages 2–4) [25,50]. The polypeptide is pulled through the central pore of the double-ring ATPase, as demonstrated by photocrosslinking to internal amino acid positions in the rings [25]. Furthermore, when Cdc48 was fused to the protease domain of FtsH to generate an artificial proteasome, the substrate was degraded [25], indicating that the polypeptide completely exited Cdc48 at its trans-side and entered the associated protease.

Substrate release from the Cdc48 complex requires a DUB, as complete translocation is otherwise counteracted by the association of the polyubiquitin chain with the UN cofactor at the cis side. *In vitro* experiments show that Otu1, a DUB which binds to the N domain via its Ubx-like domain, trims the polyubiquitin chain [25]. However, there must be other DUBs that can replace Otu1, as the deletion of OTU1 in *S. cerevisiae* has no effect on ERAD [26]. Surprisingly, only a minority of the substrate molecules released from Cdc48 *in vitro* lose all ubiquitins; most retain an oligoubiquitin chain with up to ten ubiquitin molecules, which is also pulled through the central pore (stages 4–6). These ubiquitin molecules are probably sequentially unfolded and refold when they emerge on the trans-side. These results also imply that at least two polypeptide strands can be accommodated inside the pore, indicating that the pore is wider during translocation than seen in current structures of the resting ATPase. In addition, the carbohydrate chain of glycosylated ERAD substrates must also be accommodated inside the pore, because the N-glycanase Png1 binds to the C-terminus of Cdc48 and probably has only access to the chain after its translocation through the ATPase rings [51].

How substrates are transferred from Cdc48 to the proteasome is also still unclear. Many of the substrate molecules released from Cdc48 *in vitro* contain four or more ubiquitin molecules [25], and could therefore be processed by the 26S proteasome [52]. They are probably transferred from Cdc48 to the proteasome indirectly through the shuttling factors Rad23 and Dsk2, which each have both ubiquitin- and proteasome-binding domains [53,54]. Archaeal Cdc48 homologs and certain mutants of eukaryotic p97 can cooperate with the 20S proteasome to degrade substrate, without involvement of the 19S regulatory subunit [55,56]. Future experiments need to address whether this pathway is used by wild type p97 for some polyubiquitinated substrates. The transfer of substrates to the proteasome probably involves other Cdc48 cofactors. For example, Ufd3 binds to the C-terminal tail of Cdc48 and prevents abnormal ubiquitin degradation [57,58], perhaps by interfering with the Cdc48-20S interaction. The Ufd2 cofactor extends the polyubiquitin chain on substrates [59] and might thus rescue substrates whose chains are too short for degradation by the 26S proteasome. Future experiments need to clarify how substrates are processed by these cofactors and transferred to the proteasome.

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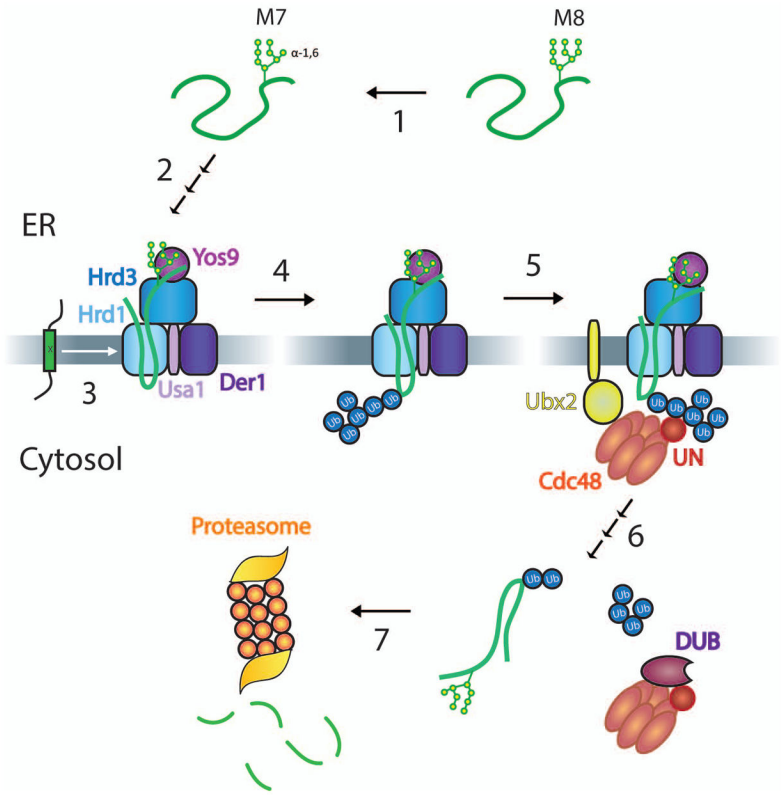


**Figure 1. Overview of ERAD-L and –M**

The scheme shows different steps in ERAD-L. Step 1: The N-glycan chain of a misfolded luminal glycoprotein is trimmed from eight to seven mannoses (M8 and M7, respectively) by glycosidases. Step 2: The generated terminal α1,6-linked mannose residue binds to Yos9, and the misfolded segment around the glycan attachment site binds to Hrd3. The substrate inserts into the Hrd1 channel with the help of Der1, which associates with Hrd1 through Usa1. Step 3: ERAD-M substrates are misfolded in their membrane-spanning segments (indicated by an "x") and enter Hrd1 sideways. Step 4: Both ERAD-L and ERAD-M substrates are polyubiquitinated by Hrd1. Step 5: The Cdc48 ATPase is recruited to the ER membrane by binding of the Ufd1/Npl4 (UN) cofactor to the ubiquitin chain and by Cdc48 binding to Ubx2. Step 6: Cdc48 uses ATP hydrolysis to pull the polypeptide substrate out of the membrane, the complex of Cdc48 ATPase and substrate leaves the membrane, and a DUB trims the ubiquitin chain, allowing release of the substrate from Cdc48. Step 7: The substrate is degraded by the proteasome.

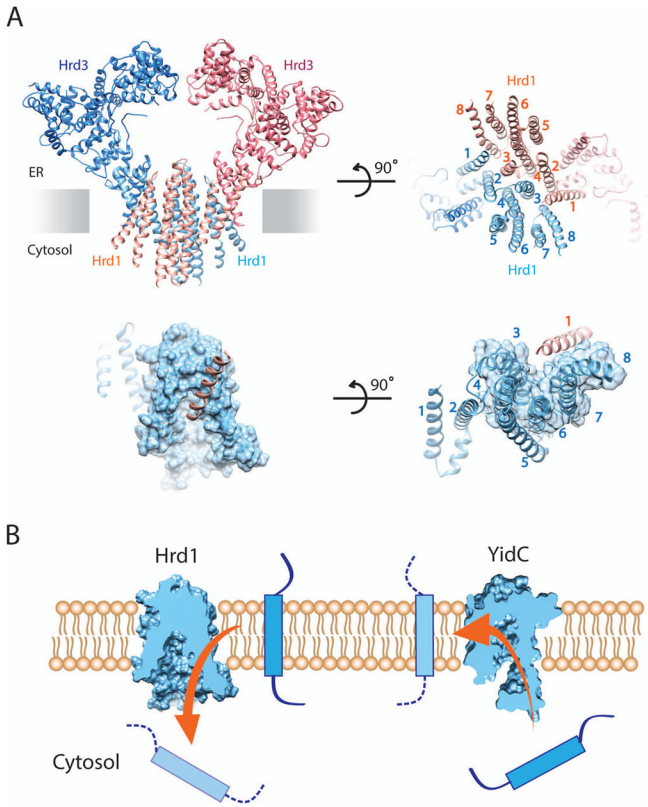


**Figure 2. Structure of a Hrd1/Hrd3 complex**

(A) Model of Hrd1 bound to the luminal domain of Hrd3, based on cryo-EM single-particle analysis [38]. The upper left panel shows a cartoon of the Hrd1/Hrd3 dimer, with the Hrd1 molecules in light blue and salmon, and the Hrd3 molecules in dark blue and red. The upper right panel shows a view from the cytosol. The lower left panel shows a space-filling model of the funnel of one Hrd1 molecule together with TM1 of the other. The lower right panel shows a view from the cytosol. (B) The left panel shows a cut through a space-filling model of Hrd1. Hrd1/Hrd3 allows an ERAD-M substrate to move into the cytosol (arrow). The right panel shows a cut through a space-filling model of the bacterial YidC protein, which allows TM segments to move from the cytosol into the membrane (arrow).

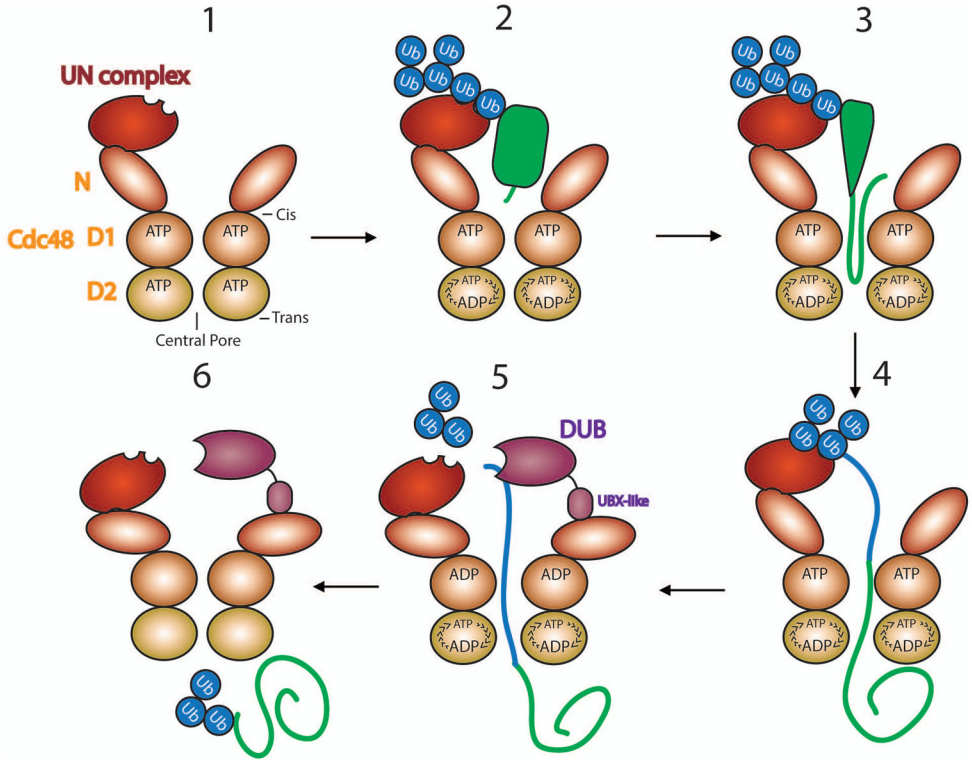


**Figure 3. Substrate processing by the Cdc48 ATPase**

The scheme shows different stages. Stage 1: The Cdc48 ATPase, containing an N domain and two ATPase domains (D1 and D2), forms a hexameric, double-ring structure that associates with one copy of the Ufd1/Npl4 (UN) cofactor. The central pore and the cis- and trans-sides are indicated. Stage 2: The ubiquitin (Ub) chain attached to a substrate (in green) binds to UN. The D1 ATPases are locked in the ATP-bound state with the N domains in the up-conformation, while the activity of the D2 ATPases is stimulated. Stage 3: The substrate is pulled through the central pore, causing polypeptide unfolding. Stage 4: The substrate is moved entirely to the trans-side of the ATPase ring. Ubiquitin is also unfolded and follows the substrate (blue line). Stage 5: ATP hydrolysis in D1 causes movement of the N domains into the down-conformation, allowing a DUB to trim the ubiquitin chain. The DUB Otu1 binds through its UBX-like domain to the N domain. Stage 6: Ubiquitin molecules emerging at the trans-side presumably refold, allowing the substrate to be recognized by shuttling factors and the proteasome.