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The evolving role of ubiquitin modification in endoplasmic reticulum-associated degradation

G. Michael Preston[1] and **Jeffrey L. Brodsky**[2]

[1]Department of Cell Biology, University of Pittsburgh, Pittsburgh, PA 15260, U.S.A

[2]Department of Biological Sciences, University of Pittsburgh, A320 Langley Hall, Pittsburgh, PA 15260, U.S.A

Abstract

The endoplasmic reticulum (ER) serves as a warehouse for factors that augment and control the biogenesis of nascent proteins entering the secretory pathway. In turn, this compartment also harbors the machinery that responds to the presence of misfolded proteins by targeting them for proteolysis via a process known as ER-associated degradation (ERAD). During ERAD, substrates are selected, modified with ubiquitin, removed from the ER, and then degraded by the cytoplasmic 26S proteasome. While integral membrane proteins can directly access the ubiquitination machinery that resides in the cytoplasm or on the cytoplasmic face of the ER membrane, soluble ERAD substrates within the lumen must be retrotranslocated from this compartment. In either case, nearly all ERAD substrates are tagged with a polyubiquitin chain, a modification that represents a commitment step to degrade aberrant proteins. However, increasing evidence indicates that the polyubiquitin chain on ERAD substrates can be further modified, serves to recruit ERAD-requiring factors, and may regulate the ERAD machinery. Amino acid side chains other than lysine on ERAD substrates can also be modified with ubiquitin, and post-translational modifications that affect substrate ubiquitination have been observed. Here, we summarize these data and provide an overview of questions driving this field of research.

Introduction

Proteins exhibit a wide variety of structural and chemical features, which are essential for their function. To attain these features, cotranslational and post-translational modifications occur, along with protein folding. Protein heterogeneity in the cell is made even more complex because some proteins are transported into intracellular organelles. The unique chemical environments within these organelles are often encountered concomitant with cotranslational and post-translational events. For example, the endoplasmic reticulum (ER) receives approximately one-third of all newly synthesized proteins in eukaryotes [1]. Not only do these substrates traverse or become embedded within a lipid bilayer, but also they encounter a more oxidizing and calcium-rich environment compared to the cytoplasm [2–4].

Correspondence: Jeffrey L. Brodsky (jbrodsky@pitt.edu).

Competing Interests

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Moreover, the machinery to catalyze specific post-translational modifications resides in the ER. Therefore, proteins translocated into the ER may acquire N-linked glycans, lipid appendages, and/or disulfide bonds [5–9]. In the absence of these modifications, protein folding in the ER is abrogated.

The protein-folding 'problem' is made even worse by the fact that nascent cotranslationally inserted polypeptide chains enter the ER in an N- to C-terminal fashion [10, 11]. Should folding require more C-terminal residues, the folding of N-terminal portions is delayed [12, 13]. In addition, membrane proteins must fold in three unique environments: the ER lumen, the lipid bilayer, and the cytoplasm. Owing to the fact that the native and unfolded states of many proteins are differentiated by only a few kCals per mole [14, 15], and that genetic mutations or stochastic errors in amino acid incorporation might take place, protein folding is quite error-prone. If uncorrected, proteins may aggregate in the ER, leading to compromised cellular and organelle homeostasis [16–20].

Fortunately, the ER is replete with molecular chaperones and enzymes that directly facilitate protein folding [21, 22]. Molecular chaperones capture unfolded polypeptides by virtue of their ability to bind amino acid patches containing exposed hydrophobic side chains [23–26]. Moreover, eukaryotes have evolved two systems to temper the potentially toxic effects of misfolded proteins. First, the unfolded protein response (UPR) may be induced, which leads to: (1) the induction of factors that increase the protein-folding capacity of the ER; (2) expanded ER volume; and (3) the transport of unfolded proteins to other compartments, such as the vacuole/ lysosome in which they may be degraded [27–29]. Second, an ER-resident protein ensemble directly selects, exports, and degrades misfolded proteins in the ER. This second pathway is known as ER-associated degradation (ERAD), and components of the ERAD machinery are also induced by the UPR [30–34]. Together, the UPR and ERAD constitute two complementary legs of the ER quality control apparatus. However, growing evidence indicates that ERAD also targets properly folded proteins in order to regulate: (1) metabolically controlled enzymes, (2) transcription factor activity, and (3) the amount and thus activity of a plasma membrane metal transporter [35–40].

ERAD substrates are selected by molecular chaperones and by chaperone-like lectins. Once selected, soluble substrates that completely reside within the ER must be retrotranslocated, so that at least a portion of the protein becomes exposed to the cytoplasm. Here, the substrate is modified with the 76 amino acid peptide, ubiquitin. Ubiquitin modification of proteins can be important for protein trafficking decisions (for review, see ref [41]) and as shown recently for the folding and ER exit of a membrane protein [42]. During ERAD, the subsequent acquisition of a polyubiquitin chain helps recruit an ATP-dependent 'engine', known as p97 in mammalian cells or Cdc48 in yeast, which extracts ERAD substrates from the ER [43–45]. The cytoplasmic portion(s) of misfolded integral membrane proteins are also modified with ubiquitin. After or concordant with the complete retrotranslocation of the selected and modified soluble or integral membrane proteins, ERAD substrates are degraded by the 26S proteasome [46–49]. The proteasome is a multicatalytic protease that recognizes polyubiquitinated proteins, which leads to the unfolding and spooling of captured substrates into a chamber in which three proteolytic activities (tryptic, chymotryptic, and caspase-like)

reside [50–52]. Because a subpopulation of proteasomes associates with the ER membrane [53], retrotranslocated ERAD substrates are efficiently degraded.

In this review, we will discuss the step in the ERAD pathway that represents an important decision-making point: the acquisition of a polyubiquitin tag. We will focus on the link between ubiquitination and ERAD by describing many of the initial findings that established which key factors perform this crucial function. We will also discuss how the field has expanded since those initial discoveries, along with some of the variations on the protein ubiquitination theme that have been identified. Finally, we will briefly introduce what we believe are critical questions in future research on the ubiquitination of ERAD substrates.

The ubiquitination pathway

The ubiquitination of a misfolded protein can represent a rate-limiting step during ERAD. But, before the substrate can be ubiquitinated, cells utilize an E1 ubiquitin-activating enzyme that hydrolyzes ATP to create a thiol-ester bond with the C-terminal carboxylate in ubiquitin (Figure 1) [54–56]. After activation, an E2 ubiquitin-conjugating enzyme is utilized to transfer ubiquitin to a substrate that may be bound to an E3-ubiquitin ligase. In other cases, the substrate may be linked to the E3 by a molecular chaperone (see below). Currently, only 1 E1 ubiquitin-activating enzyme and 11 E2 ubiquitin-conjugating enzymes have been identified in yeast, whereas there are 2 E1 ubiquitin-activating enzymes and 35 E2 ubiquitin-conjugating enzymes that have been identified in humans [57, 58]. In contrast, there are roughly 80 and 600 putative E3s in yeast and humans [59].

Among these many ligases, three classes of E3s function in ERAD: (1) RING domain, (2) HECT domain, and (3) U-box domain E3s. RING domain and U-box domain E3s facilitate the transfer of ubiquitin from the E2 ubiquitin-conjugating enzyme to the substrate, whereas HECT domain E3s are directly ubiquitinated before the ubiquitin is transferred [60–64]. Once the substrate becomes mono-ubiquitinated, a ubiquitin chain can be synthesized and elongated. The most prominent Lys in ubiquitin that is elongated in this manner and used to target substrates for degradation is Lys-48 [56, 65–67]. However, substrate ubiquitination via Lys-11-derived isopeptide linkages are also recognized by the proteasome [68]. A more recently discovered class of components of the ubiquitination machinery are the ubiquitin chain elongation factors, called E4s [69]. As the name implies, the activity of the E4 elongates the polyubiquitin chain in order to more effectively recruit factors that facilitate substrate degradation and expedite proteasomal degradation [70–72].

After an ERAD substrate has been adequately ubiquitinated (Figures 2 and 3), the p97/Cdc48 complex is recruited to the substrate by its cofactors, Ufd1, Npl4, and in some cases Ubx2 (UBXD8 in mammalian cells) [73, 74]. Interestingly, the viral protein, US11, which promotes the ERAD of specific substrates (see below), interacts with p97 in a ubiquitin-independent manner in mammalian cells [75]. However, most p97/Cdc48 substrates are ubiquitinated. The recruitment of the p97/Cdc48 complex leads to substrate ‘retrotranslocation’ (or ‘dislocation’ for membrane proteins) from the ER and into the cytosol, where it is bound and shuttled to the 26S proteasome by delivery factors, such as Rad23 and Dsk2, in yeast [76]. Interaction of Rad23 with the Cdc48–Ufd1–Npl4 complex is

mediated by another Cdc48 cofactor, the E4 Ufd2 [72]. Once Rad23 and Dsk2 bind the Cdc48 complex, they link the complex to the proteasome through a component that resides on the proteasome ‘cap’ (also known as the 19S particle or PA700), Rpn1 [77]. In addition to binding by Rad23–Dsk2–Rpn1, another component of the 19S cap, called Rpn10, can bind ubiquitin in an Rad23-independent manner [78]. After substrate binding to the 19S particle of the proteasome, the proteasome-associated deubiquitinating enzymes (DUBs), Ubp6 and Rpn11, cleave the ubiquitin chain from the substrate, and the substrate is subsequently threaded into the 20S core particle and degraded [79–83]. Entry into the 20S particle requires the activity of six AAA-ATPases that drive substrate entry into the core and facilitate the opening of an aperture that otherwise retains the 20S particle in a closed state [84, 85].

The first link between the ubiquitin pathway and ERAD

Ubiquitination was first identified as an ATP-dependent process important for protein degradation in the late 1970s/early 1980s [86, 87]. However, it was not until the 1990s that a component of the ubiquitination machinery was discovered at the ER [88]. This factor is an ER membrane-resident E2 ubiquitin-conjugating enzyme, Ubc6. It was shown that deleting *UBC6* leads to the rescue of a *sec61* mutant allele that is defective for protein translocation, or entry into the ER. In the absence of Ubc6, the mutant Sec61 channel has a longer half-life, which allows for partial rescue of protein translocation.

Other early discoveries led to the identification of proteins in the ER that were ubiquitinated. At least initially these were substrates that trafficked through the secretory pathway, but were retained in this compartment due to errors in folding or maturation. One of the most notable substrates identified was the cystic fibrosis transmembrane conductance regulator (CFTR), whose failure to properly fold and mature due to rapid degradation is the cause of cystic fibrosis (CF) [47, 49]. Degradation of both immature forms of the wild-type and the ΔF508 mutant form of CFTR, which accounts for the majority of CF cases, was shown to be dependent on both the E1 ubiquitin-activating enzyme and the 26S proteasome for degradation. CFTR and ΔF508 were also directly shown to be ubiquitinated.

Substrates used to identify additional components of the ubiquitination machinery in the yeast ER included a mutated form of a vacuole-targeted protein, carboxypeptidase yscY, which was subsequently termed CPY* [46]. Analyses of CPY* degradation uncovered the importance of an E2 ubiquitin-conjugating enzyme, Ubc7, as well as the proteasome in removing this trapped protein from the ER [46, 89, 90]. In parallel, an enzyme that catalyzes the rate-limiting step in cholesterol synthesis, known as hydroxymethylglutaryl coenzyme A reductase, was known to be metabolically regulated and degraded in the ER [91, 92]. By examining the genetic requirements for the degradation of the yeast homolog, Hmg2, three *HRD* genes that are important for the degradation of Hmg2 at the ER were identified [38]. Two of these *HRD* genes, *HRD1* and *HRD3*, are vital for the ubiquitination and degradation of many other ERAD substrates (Table 1) [93–95]. Hrd1 is one of the major ER membrane-resident E3s involved in ERAD in yeast and is the central component of the Hrd1 complex, which is conserved between yeast and mammals (see below). Hrd3 is an ER membrane-resident protein that interacts with Hrd1. When *HRD3* is deleted, Hrd1 degradation

increases, leading to a loss of function of the Hrd1 complex [94, 96, 97]. Finally, the major histocompatability complex class I (MHCI) protein was utilized to identify a novel mechanism of immune evasion by human cytomegalovirus (HCMV). A virally encoded, ER-localized protein, US11, triggered nascent MHCI heavy chain transport from the ER into the cytosol, where it is acted upon by a cytosolic *N*-glycanase, ubiquitinated, and subsequently, degraded by the 26S proteasome [98, 99]. Together, these substrates have proved essential for continued analysis of the factors important for ubiquitination and degradation during ERAD.

Expanding the links between ubiquitination and ERAD

Identification of contributing E3s and their associated complexes

While these initial breakthroughs were crucial to better understand the importance of protein ubiquitination in ERAD, the years that followed led to the identification of additional factors, complexes, and substrates associated with this process (Table 1). In yeast, there are two primary E3s, Hrd1 and Doa10, that are required for ubiquitination and degradation; however, there are currently eight E3s that play some role during the ubiquitination of ERAD substrates in yeast. In mammals, there are four E3s, Hrd1, TEB4, gp78, and CHIP, that are associated with the degradation of several notable ERAD substrates, but ~19 E3s have been linked more generally to ERAD (Table 1). The expanded number of E3s involved in ERAD in mammals when compared with yeast is most probably due to the greater number of potential substrates (e.g. transmembrane proteins) in mammalian cells.

While Hrd1 was the first E3 linked to the ERAD pathway, it was appreciated later that the Hrd1 complex (Figure 2) ubiquitinates substrates that possess misfolded regions in their ER luminal (ERAD-L) or membrane-spanning domains (ERAD-M) [38, 93, 95, 100, 101]. In yeast, the Hrd1 complex consists of Hrd3, Usa1, Der1, Dfm1, Yos9, Kar2, Ubc7, and Cue1 [97, 102]. While these factors are all known to be a part of the Hrd1 complex, not all of them are considered components of the core Hrd1 complex. When the complex was purified, Hrd3, Usa1, Der1, and Yos9 copurified, thus defining components of the core complex [102]. As noted previously, Hrd3 stabilizes the Hrd1 oligomer, but also binds glycosylated misfolded luminal substrates by interacting with a luminal lectin, Yos9, as well as nonglycosylated misfolded substrates via an Hsp70 molecular chaperone, Kar2 [94, 103–105]. Until recently, it was difficult to study the role of Hrd3 in ERAD due to the increased turnover of Hrd1 when *HRD3* is deleted. However, when the ubiquitin-like domain of Usa1 is removed, Hrd1 remains stable and a direct role for Hrd3 in ERAD was established [106]. Der1 also binds soluble luminal substrates and interacts with Hrd1 through Usa1 [107, 108]. In addition, a Der1 homolog, Dfm1, interacts with Hrd1 and the E3 ligase, Doa10, and helps degrade a Doa10 substrate, Ste6* [73, 109]. In a mechanism believed to be independent of ERAD, Dfm1 also interacts with Cdc48 in the absence of Ubx2 [109, 110]. As the Hrd1 complex engages substrates, Ubc7 is recruited to the ER membrane by a transmembrane protein, Cue1 [111, 112]. In addition to Ubc7, another E2, Ubc1, ubiquitinates select ERAD substrates [93, 113]. Hrd1 also recruits the ER membrane protein, Ubx2, to the complex, which anchors Cdc48 to the membrane [73, 74]. Ubx2 anchoring facilitates efficient substrate retrotranslocation. Some evidence suggests that Der1 and the mammalian homolog

(also see below) may act as the retrotranslocation channel for substrates, or at least are intimately associated with the retrotranslocation process [114–118], while other evidence suggests that Hrd1 could serve as the channel [119, 120]. In either case, this would position the ubiquitination machinery near the site of retrotranslocation.

Mammalian homologs for many of the components discussed above were subsequently identified (Figure 3). The mammalian homolog of Hrd1, also called HRD1 or synoviolin [121, 122], is important to ubiquitinate several ERAD substrates (Table 1). Other mammalian homologs of yeast Hrd1 complex members have been identified as well: SEL1L (Hrd3), HERP (Usa1), DERLIN-1, DERLIN-2, DERLIN-3 (Der1), OS-9 and XTP3-B (Yos9), BiP (Kar2), UBE2G2 (Ubc7), and Aup1 (Cue1) [102, 116, 123–129].

Perhaps not surprisingly, mammalian HRD1 complex members have also been implicated in the ubiquitination of ERAD substrates (Table 1). As in yeast, HRD1 functions in the degradation of both glycosylated (utilizing EDEM1, DERLIN-2, DERLIN-3, OS-9, XTP3-B, and SEL1L) and nonglycosylated substrates (utilizing BiP, HERP, and DERLIN-1) [30, 130–133]. While HRD1 activity for glycosylated substrates is generally thought to function in a SEL1L-dependent manner [123, 127], another HRD1 regulator, the ER membrane-resident protein FAM8A1, binds and regulates HRD1 function in an SEL1L-independent manner [134]. After substrate association with the HRD1 complex, AUP1, which contains a Cue domain homologous to that found in the yeast Cue1 protein, binds the complex and recruits UBE2G2 through its G2BR domain [127, 128, 134–137]. For some substrates, HRD1 also utilizes the Ubc6 homolog, UBE2J1 [138]. Which of these E2 ubiquitin-conjugating enzymes is the predominant enzyme for HRD1 is an open question. Mammalian HRD1 also binds to other factors that play a role in ERAD. For example, Hrd1 associates with UBXD2 and UBXD8, which function similarly to yeast Ubx2 in anchoring p97 to the ER membrane. Furthermore, UBXD2 recruits the mammalian Dsk2 homolog, called UBIQUILIN [127, 139–142], to the Hrd1 complex, whereas UBXD8 recruits a cytosolic chaperone, BAG6 [143, 144]. Recently, UBIQUILIN and BAG6 were shown to function as chaperones as well, suggesting that they play an important role as holdases for dislocated proteins prior to degradation [143,145]. Yet, another factor that binds the HRD1 complex and recruits p97 is VIMP (VCP-interacting membrane protein) [114]. VIMP localizes to the HRD1 complex via association with DERLIN-1 and subsequently binds p97. HRD1 and DERLIN-1 are also capable of binding p97, suggesting that they contribute to the maintenance of p97 residence at the ER membrane [115, 146].

The other ERAD-associated E3 complex in yeast centers around the nuclear membrane/ER membrane-localized E3, Doa10 [147]. While Hrd1 functions in collaboration with a variety of other proteins, Doa10 acts predominantly with three different components in a ubiquitination complex: Ubc6, Ubc7, and Cue1 [102, 147, 148]. Recent data suggest that Ubc6 initially monoubiquitinates the substrate, whereas Ubc7 creates the polyubiquitin chain by adding subsequent ubiquitin moieties [149]. In contrast with Hrd1, which ubiquitinates substrates containing misfolded lesions in the ER lumen and ER membrane, Doa10 substrates contain a cytosolic lesion (ERAD-C) [101]. As a result, cytosolic chaperones, such as an Hsp70, Ssa1, and the cytosolic Hsp40s, Ydj1 and Hlj1, are also important for the degradation of Doa10-dependent substrates [71,150–153]. Like Hrd1,

Doa10 recruits Ubx2 to the complex, allowing for increased efficiency of substrate retrotranslocation by Cdc48 [73, 74]. In the mammalian system, TEB4/MARCH-VI [154, 155] is the Doa10 homolog [154, 156–158]. TEB4/MARCH-VI acts in a complex with the E2 ubiquitin-conjugating enzyme, UBE2G1 [154]. Similar to the situation observed in yeast, cytosolic chaperones, such as HSC70, DNAJB12, and HDJ2 [159–163], can help degrade some ERAD substrates and UBXD8 can aid in substrate retrotranslocation.

The mammalian E3, gp78 [164], is another major E3 ligase for ERAD substrates in mammalian cells. gp78 is an ER membrane protein that is similar to HRD1 and utilizes UBE2G2. This interaction is through an embedded G2BR domain [137]. While gp78 clearly functions as an E3 during ERAD, there is also evidence that it acts as an E4 in co-operation with other E3s [165]. Additional evidence suggests that gp78 participates in substrate retrotranslocation downstream of HRD1 [166]. The diversity in gp78 function may reflect substrate specificity and partner specificity, which represent an important area of future study. Regardless, similar to HRD1 and TEB4/MARCH-VI, gp78 also recruits UBXD2 or UBXD8 to help cement p97 at the ER membrane. Alternatively, gp78 can directly interact with p97 through its VIM (VCP-interacting motif) domain [115, 167].

One of the earliest ERAD-requiring E3s identified in mammalian cells was the cytosolic HSC70-interacting protein, CHIP. While several ERAD substrates were identified that require CHIP activity, the best-studied substrate is CFTR [161]. Interestingly, CHIP requires HSC70 as a cofactor for function, but CHIP can also act in collaboration with other E3s, such as PARKIN [161, 168, 169]. This may reflect the ability of CHIP to act cooperatively, or it may reflect an E4-type activity. For example, while RMA1 and CHIP both facilitate mutant CFTR degradation, the dependence on each E3 was linked to the subdomain in which the mutation resided [170]: RMA1 recognizes mutations early in the protein sequence and in the transmembrane domains of CFTR, whereas CHIP is believed to recognize cytoplasmic domains and the C-terminal second nucleotide-binding domain in CFTR.

Several additional E3 ubiquitin ligases have been identified that act on ERAD substrates in both yeast and mammalian cells (Table 1). Many of these enzymes are required for the degradation of only a few substrates, yet this might again reflect the fact that the universe of identified and characterized ERAD substrates is relatively small (see Future Work). In yeast, the cytosolic E3, Ubr1, is required along with Hrd1 and Doa10 during the degradation of Ste6* and CFTR, and employs the cytosolic E2, Ubc2 [171]. However, Ubr1 is better known for its role in degrading unstable N-end rule substrates [172]. In addition, Rsp5, a cytosolic/Golgi-resident E3, ubiquitinates overexpressed CPY* and other select substrates when cells are exposed to oxidative stress [173, 174]. Finally, in yeast, the nuclear membrane E3 complex, consisting of Asi1, Asi2, and Asi3, plays a role in the destruction of Erg11, a sterol synthesis component that resides in the ER [175, 176].

Roles for specialized E3s in ERAD have also been studied in mammalian cells. Some of these enzymes are important only under select conditions. For instance, $SCF^{\beta\text{-}TrCP}$ and TMEM129 are utilized by viruses to ubiquitinate ER-localized factors associated with the immune system [177–179]. Other ligases recognize specific protein classes: the cytosolic E3s SCF^{Fbx2} and SCF^{Fbx6} target glycosylated ERAD substrates in the cytosol and modify

them prior to proteasomal degradation [180, 181]. It is unknown if these E3s recognize retrotranslocated, glycosylated ER-resident proteins that have been missed by the cytoplasmic glycosidase, PNGase, which normally removes ER-catalyzed *N*-glycans prior to ERAD [182, 183]. Other E3s connected to the ERAD pathway include TRC8, RNF170, RNF185, TRIM13, SMURF1, NIXIN/ZNRF4, and NRDP1 (Table 1). One E3, RNF103, is an ER-resident ligase that has yet to be linked to substrate degradation. However, the factor regulates protein ubiquitination levels, interacts with p97 and DERLIN-1, and is autoubiquitinated [184]. Once again, this may reflect the dearth of characterized, potential ERAD substrates that can arise due to errors in secretory pathway folding.

The importance of ubiquitin chain extension (E4) and DUB enzymes during ERAD

For maximal binding to the 26S proteasome, a substrate needs a polyubiquitin chain of at least four ubiquitin molecules, but increasing the length beyond four molecules more modestly increases proteasome affinity [185, 186]. Some recent evidence indicates that the addition of multiple single ubiquitin moieties on a proteasomal substrate is sufficient for proteasome targeting [187–189]. As a result, varying the ubiquitin chain length could regulate degradation efficiency and modify the speed at which ubiquitinated substrates are degraded [190]. In general, however, the effect of chain length on the rate of substrate degradation has not been satisfactorily investigated. But, in order to control ubiquitin chain length, cells possess a multitude of factors that either increase (E4s) or decrease (DUBs) the length of the chain. While it is possible the extension of the ubiquitin chain is linked to increased degradation, trimming can rescue ubiquitinated substrates or — in some cases — facilitate degradation. For instance, if a substrate is deubiquitinated after p97/Cdc48 activity, it could aggregate in the cytoplasm or might subsequently be re-ubiquitinated by a cytoplasmic E3. Alternatively, if a substrate is deubiquitinated prior to p97/Cdc48 activity, it could remain in the ER membrane and potentially traffic to the Golgi or be re-ubiquitinated by ER-resident E3s. These different fates may be dictated by the timing of DUB activity. However, deubiquitination prior to p97/Cdc48 activity has also been suggested to allow substrate egress through the p97/Cdc48 hexamer [191]. Definitive proof of this model awaits the reconstitution and structural analysis of p97/Cdc48-dependent degradation. Regardless, of the ERAD-associated DUBs, several are associated with p97/Cdc48, whereas others reside in the ER membrane/cytoplasm (Table 1). This difference in localization/interaction partners could suggest alternative steps at which these DUBs act, but, to date, this has not been satisfactorily addressed.

The major DUB involved in ERAD in yeast is Otu1. Otu1 was not implicated in ERAD until it was shown to interact with Cdc48, which occurs through its ubiquitin regulatory X (UBX)-like domain [192]. Otu1 binding to the N-terminus of Cdc48 does not interfere with binding of the Cdc48 cofactors, Npl4 and Ufd1, which are vital to recognize ubiquitinated substrates [75, 193, 194]. These data suggest that different members of the Cdc48 hexameric ring bind Otu1 versus Npl4/Ufd1. While Otu1 exhibits deubiquitination activity both *in vivo* and *in vitro*, another ubiquitin chain modifier, Ufd3, does not appear to catalyze substrate deubiquitination [192]. However, in *ufd3*Δ cells, the level of free ubiquitin is significantly

decreased [195]. The effect on free ubiquitin levels may be linked to the Ufd3-binding site on Cdc48, as Cdc48 cannot bind both Ufd3 and the E4, Ufd2, at the same time [192]. Interstingly, this site is different from the Otu1-binding site, as Cdc48 can associate with both the Otu1 and Ufd3 DUBs simultaneously. One view is that the binding of Otu1 and Ufd3 to the Cdc48 complex primes this enzyme to inhibit ubiquitination, and thus degradation, of ERAD substrates. How this critical step might be further regulated is unknown.

As might be anticipated, there are many more ERAD-associated DUBs in mammalian cells than in yeast. YOD1, the mammalian homolog of Otu1, also interacts with p97 through its UBX domain [191]. YOD1 deubiquitinates known ERAD substrates and associates with DERLIN-1 and UBXD8, two components of the HRD1 complex (see above). It was suggested that YOD1 co-operates with the p97 complex to efficiently retrotranslocate ubiquitinated substrates, which may be prevented from being threaded through the central p97 pore (also see above); alternatively, YOD1 may act upstream of retrotranslocation by controling ubiquitin chain length to optimize p97 recruitment [191,196].

An additional DUB in the mammalian ERAD pathway is ATAXIN-3. ATAXIN-3 aggregates upon extension of its polyglycine domain, which leads to Machado–Joseph Disease (also known as spinocerebellar ataxia type 3) [197]. ATAXIN-3 localizes to both the nucleus and the cytoplasm, where it interacts with substrate recognition factors for the 26S proteasome [198]. ATAXIN-3 was subsequently shown to bind p97 through an Arg/ Lys-rich region [199, 200]. Currently, the role that ATAXIN-3 plays in ERAD is not completely clear. Some evidence suggests that ATAXIN-3 removes the ubiquitin chain on ERAD substrates to increase protein half-life, which allows more time for substrate folding [201]. An alternative hypothesis is that ATAXIN-3 acts after retro-translocation to support the degradation of ERAD substrates [202]. One reason for this discrepancy may arise due to differences in methodology, which employed either overexpression studies *in vivo* or the use of purified complexes in *in vitro* reactions. Interestingly, ATAXIN-3 itself is modified by ubiquitination, which appears to increase DUB activity [203].

Other mammalian DUBs associated with the ERAD pathway include USP13, USP19, and USP25 (Table 1). USP13 interacts with p97, UFD1, NPL4, and UBXD8, and upon USP13 deletion, cells are more susceptible to ER stress. Moreover, deletion of the gene encoding USP13 leads to the accumulation of an ERAD substrate, TCRαGFP [204]. In another study, USP13 was shown to associate with gp78 and deubiquitinate a component of the ERAD complex, keeping the complex from becoming inactivated [205]. This result is in line with other studies implicating E3 and DUB activity in regulating the ERAD machinery in addition to or in contrast with the modification of ERAD substrates (also see below). In turn, USP19 has seven isoforms that arise from alternative splicing. Some of these isoforms contain a C-terminal transmembrane domain, which targets them to the ER membrane. The ER membrane-localized population rescues the degradation of two ERAD substrates, TCRα and ΔF508 CFTR [206]. Interestingly, expression of a catalytically inactive USP19 mutant partially rescued TCRα, suggesting a DUB-independent role during ERAD. However, Ye and colleagues have provided evidence that the role of USP19 during ERAD is dependent on its overexpression [207]. Of note, wild-type levels of USP19 are mostly cytosolic, lack

association with ERAD factors, and fail to alter the degradation of ERAD substrates. Finally, USP25, which interacts with p97 as well as HRD1, decreases ubiquitination of the ERAD substrate CD3δ [208].

As mentioned above, a class of E3-like ligases, known as E4s, catalyzes the extension of ubiquitin chains. For ERAD, the best characterized member of this family is the yeast protein Ufd2. In the absence of Ufd2 activity, the length of ubiquitin chains assembled by the E1–E2–E3 complex onto a series of ERAD substrates is insufficient for protein turnover both *in vitro* and *in vivo* [69]. Owing to its interaction with Cdc48, Ufd2 facilitates the degradation of several substrates [72]. As noted in the previous section, Ufd2 competes with Ufd3 for binding to Cdc48, which modulates protein turnover [192]. Two mammalian Ufd2 homologs, UBE4A and UBE4B, exist, but little is known regarding their role in ERAD. Nevertheless, it has been shown that UBE4B can recognize K48 linkages and interact with p97 [209, 210].

Variations on a theme

Alternatives to Lys-48 ubiquitin linkage in ERAD

While the Lys-48 polyubiquitin linkage is predominantly used for proteasomal degradation, polyubiquitin chains appended to other lysine residues are recognized by the 26S proteasome. In yeast, ubiquitin chains containing Lys-6, Lys-11, Lys-23, Lys-29, and Lys-33 linkages all accumulate to varying levels when the proteasome is inhibited, whereas Lys-63 ubiquitin chain levels are unaffected [68]. Of the non-Lys-48 polyubiquitin chains, Lys-11-based chains are the most affected upon proteasomal inhibition; in addition, only Lys-11 chains increase when Rad23 and Dsk2 were deleted. Interestingly, the yeast E2, Ubc6, is autoubiquitinated with Lys-11 isopeptide moieties [68]; Doa10 and Ubc6 are responsible for a subpopulation of these cellular Lys-11 chains [68]. Lys-11-linked polyubiquitin chains are also induced upon ER stress [68]. When similar experiments were performed in mammalian cells, Lys-11, Lys-29, and Lys-48 linkages rose when the proteasome was inhibited [211]. Of these, the majority of the identified ubiquitin chain linkages that accumulated were Lys-11 and Lys-48. It is worth noting, however, that the accumulation of polyubiquitin moeities containing chains other than those attached via Lys-48 may represent mixed linkages. How these are built and assembled is an area of active research [212].

A mechanism for lysine-independent ubiquitination was first identified with viral E3s. For example, the viral E3 ligase, MIR1, promotes the ubiquitination and degradation of a modified MHCI that has an artificial glycine/alanine cytoplasmic domain containing only a single cysteine [213]. Since cysteine is the catalytic residue in E1s, E2s, and some E3s, this residue can clearly become ubiquitinated. Alternatively, another viral E3, mK3, can ubiquitinate lysine-less MHCI by conjugating ubiquitin to serine and threonine residues [214]. Yet another viral protein, the HIV protein, VPU, utilizes the $SCF^{\beta\text{-}TrCP}$ E3 ligase to modify two substrates, tetherin and CD4, by utilizing lysine, serine, and threonine [215, 216]. Additional evidence obtained investigating the ubiquitination of tetherin by $SCF^{\beta\text{-}TrCP}$ suggested that tyrosine residues in the cytoplasmic domain, or the free amino group in the N-terminus, might be targeted [217]. Since the exact nature of polyubiquitin chains in ERAD substrates is rarely investigated, it is possible that these phenomena are actually quite

common. In fact, TCRα, which contains only five cytoplasmic residues (RLWSS), is modified by HRD1 on the serine side chains [218]. HRD1 also modifies serines, threonines, and lysines on the immunoglobulin Ns1 LC, which is a trapped ER luminal ERAD substrate [219]. Moreover, treatment of mini-HC (γ V_H–C_H1) and NHK α1-antitrypsin with sodium hydroxide decreased the amount of ubiquitin appendages, suggesting the presence of serine/threonine ubiquitin conjugates on these ERAD substrates. Finally, purified p97 protein complexes from cells treated with an ER stressor and a proteasome inhibitor harbored increased levels of sodium hydroxide-labile species [219]. These data suggest that serine/threonine ubiquitination rises during stress, and that this modification plays an important role to mitigate cellular stress *in vivo*.

ERAD substrates are not only modified with ubiquitin, but also there is evidence that two other protein modifications affect ERAD substrate stability: attachment of NEDD8 and SUMOylation. NEDD8 is a ubiquitin-like protein linked to cullins, which activate select E3 ligases [220]. A role for NEDD8 in ERAD has been suggested for the ERAD substrate ΔF508 CFTR [221]. During SUMOylation, SUMO (small ubiquitin-like modifier) is conjugated to lysines through the action of dedicated SUMO E1, E2, and E3 enzymes in a reaction analogous to the ubiquitin-conjugation cascade [222, 223]. One difference between the SUMO and ubiquitin pathways is that there are three SUMO isoforms in mammals, and only SUMO-2 and SUMO-3 form chains. While SUMOylation is required to regulate numerous cellular events, SUMO tags can also recruit an E3, RNF4, which leads to mixed SUMO/ubiquitin chains [224–226]. One prominent ERAD substrate modified by SUMOylation is CFTR [227]. While many E3s facilitate degradation of the disease-associated ΔF508 mutant form of CFTR (Table 1), SUMOylation is also evident. Specifically, ΔF508 is bound by the small heat-shock protein, HSP27, which recruits the E2 SUMO-conjugating enzyme, UBC9. The HSP27/UBC9 complex recognizes a non-native fold in ΔF508 CFTR, which triggers SUMOylation [228]. In turn, the poly-SUMO tag recruits RNF4, which polyubiquitinates CFTR [227]. There is still much more to be explored about this modification — and the co-ordinated interaction and assembly of ubiquitin and SUMO chains — and it is likely that a growing number of ERAD substrates will be discovered that are similarly SUMOylated.

Post-translational modifications on ERAD complexes

Although this review has focused on protein ubiquitination, Nα-acetylation has also been implicated in ERAD, at least in yeast. The importance of Nα-acetylation in ERAD came from a screen investigating the nature of the Doa10 complex [148]. Based on a genetic screen, *nat3Δ* mutants were found to stabilize a degron, Deg1, that derives from a Doa10-dependent substrate known as Mat2α. Nat3 resides in the NatB complex, which is partially responsible for Nα-acetylation in yeast [229]. Importantly, the degradation of this Nα-acetylated population of Deg1 is also Doa10-dependent [230]. However, the NatB requirement for Deg1 degradation was absent when wild-type Mat2α was examined [231]. Instead, Der1 requires Nα-acetylation and this activity is required for ERAD. Without Nα-acetylation, Der1 itself is ubiquitinated by Hrd1 and subsequently degraded.

As suggested in the preceding section, ERAD activity can also be regulated in response to cellular stress. For example, the ratio of components in the yeast Hrd1 complex is modified when cells are stressed chemically to induce the UPR or by constitutive UPR induction [96]. Inactivation of the Cdc48 complex similarly leads to altered ratios of Hrd1 complex components, which may reflect regulated complex assembly and perhaps increased ERAD efficiency [96]. In support of this hypothesis, autoubiquitination of Hrd1 *in vitro* is sufficient to recruit the Cdc48 complex, which can then engage and retrotranslocate a substrate into the cytosol [119]. Surprisingly, Hrd1 is also retrotranslocated from the ER in a reconstituted system, suggesting that a Hrd1 regulatory factor or a specific modification offsets this destructive event. In mammalian cells, a factor that may regulate the Hrd1 complex is the E2, UBE2J1. UBE2J1 activity decreases the levels of Hrd1 complex members SEL1L, EDEM, and OS-9 [135]. Without UBE2J1 activity, ERAD substrates were degraded more rapidly than in wild-type cells, which is believed to be due to greater amounts and activities of these ERAD-requiring components. Needless to say, numerous other systems probably exist to regulate the activity of the ERAD machinery, which must respond to changes in cellular stress as well as cellular differentiation and growth *in vivo*.

While the examples cited above suggest mechanisms to augment ERAD, negative ERAD regulators also exist. ERAD inhibition might allow more time for proteins to fold, perhaps after a stress response has been rectified. One negative regulator of ERAD in mammalian cells is the p97 cofactor SAKS1 [232, 233]. SAKS1 interacts with p97 through its UBX domain and also requires a functional ubiquitin-associating (UBA) domain to bind polyubiquitin chains [232]. When SAKS1 binds to both a polyubiquitinated substrate and p97, the degradation of an ERAD substrate and a misfolded cytosolic quality control (cytoQC) substrate was attenuated. Another negative regulator of ERAD in mammalian cells is the small p97/VCP-interacting protein (SVIP). SVIP localizes to the ER membrane, where it binds p97 and DERLIN-1 [234]. By associating with p97 and DERLIN-1, SVIP inhibits the ubiquitination and degradation of select gp78-dependent ERAD substrates. These results support the hypothesis that the p97/Cdc48 complex can be regulated by competition for cofactor binding.

Roles for unexpected factors in ERAD

As a result of the long-term search for factors involved in ERAD through genetic and proteomic approaches, it has become increasingly clear that cytosolic factors play important roles in the degradation of aberrant proteins in the ER. One such protein is the 26S proteasome-interacting E3/E4, Hul5 [82, 235]. Hul5 was first shown to be important for the degradation of cytoplasmic substrates [236]. However, by utilizing fusions of two known ERAD substrates, CPY* and Sec61-2, to a membrane-tethered product of the *LEU2* gene (cytosolic 3-isopropylmalate dehydrogenase), Hul5 was found to facilitate the retrotranslocation of an ER membrane-resident protein [237]. Consistent with its activity as an E4, Hul5 was dispensable for the degradation of the CPY* or Sec61-2 portions of the fusion proteins, but instead elongated the ubiquitin chain on the transmembrane-anchored *LEU2* moieties, which increased interaction with the Cdc48 complex. In the absence of Cdc48 complex interaction, the ER membrane-anchored *LEU2* product was stabilized. Furthermore, as discussed earlier in the present study, two E3 ligases that reside in the Golgi

and inner nuclear membrane, respectively, Rsp5 and the Asi complex, aid in the degradation of some substrates. Rsp5 degrades CPY* in a pathway known as Hrd1-independent proteolysis, whereas the Asi complex modifies the ER-resident enzyme, Erg11 [173–176]. It is unclear if Erg11 degradation represents a unique aspect of the ERAD pathway or if the Asi complex prevents Erg11 activity in the nuclear membrane. Overall, cross-talk between components of different cellular quality control pathways is important for the degradation of proteins, regardless of substrate localization.

Along with alternative E3s, molecular chaperones localized in the ER and the cytosol are implicated in ERAD. However, it is unclear whether these chaperones only serve as recognition factors for E3 binding to substrates or if they also serve to bridge the E3 to the substrate. In addition, evidence indicates that Hrd1 binds soluble ERAD substrates through its transmembrane domains during ubiquitination [119], and the integral membrane ERAD substrate, Hmg2, is recognized by Hrd1 transmembrane domains [238]. Some cytosolic chaperones help ubiquitinate transmembrane ERAD substrates. For example, the yeast Hsp70 (Ssa1) and Hsp40 chaperones (Ydj1 and Hlj1) affect the ubiquitination and degradation of several integral membrane substrates that display prominent cytoplasmic domains: Ste6* [71, 239], Pma1-D378S [152], and CFTR [150, 151]. Similar requirements for cytosolic chaperones during ubiquitination were seen in mammalian cells as well [159–161, 240, 241]. Owing to a lack of a requirement for these factors in the degradation of ER luminal substrates, it is believed that they do not function in the retrotranslocation complex, but instead catalyze ubiquitination and/or proteasome targeting.

Future work

Protein ubiquitination was first discovered as a mechanism that controls the steady-state levels of proteins, but only later was this pathway shown to regulate protein quality control [56, 242]. In turn, ER protein quality control was thought to require a luminal protease that could directly dispose of misfolded or improperly modified proteins in this compartment [243]. However, the degradation of the integral membrane CFTR protein [47, 49] and the reconstitution of soluble protein retrotranslocation [48, 244] indicated that the 26S proteasome was responsible for ER protein turnover. It logically followed that ERAD would require the cytoplasmic-localized ubiquitination machinery. Indeed, the first definitions of ERAD derived from the isolation of components required for substrate ubiquitination [38, 46, 88, 98, 99]. Since these early days, our knowledge of the intersection between the ERAD pathway and the ubiquitination machinery has rapidly expanded.

While the field has significantly matured, numerous questions have yet to be explored or remain unanswered. For example, lysines other than Lys-48 that are ubiquitinated have been encountered, and some appear to target substrates to the proteasome. Yet, it remains unresolved how these residues are processed and whether they represent a mechanism to control ERAD efficiency. The presence of mixed ubiquitin chains poses a particularly thorny problem, as their identification and production for *in vitro* analyses represent substantial technical hurdles. Are these alternate chains differentially recognized by components of the proteasome, ubiquitin-interacting factors, and DUBs? There exists a strong potential for specificity, as DUBS and ubiquitin elongation factors in the same family can recognize

distinct ubiquitin linkages [245]. Moreover, the acquisition of different ubiquitin modifications, as well as SUMOylation, may represent responses to specific cellular conditions or stress responses. However, it remains unclear why the cell 'chooses' to build different linkages.

Another important direction for future research involves the expansion of the roles that post-translational modifications play in ERAD efficiency. In addition to the examples discussed above, ERAD facilitators can be tagged with other modifications. For example, Parkin is subject to S-nitrosylation [246], and BiP, the ER luminal Hsp70, is oxidized, ADP-ribosylated, and AMPylated [247–250]. Here too, these modifications may represent responses to cellular stress or alert the ER to conditions that will affect the efficacy of protein biogenesis. As an example, it is noteworthy that the UPR is activated before the proliferation of the ER that accompanies the transition of a plasma cell to a mature, antibody-producing B cell [251].

In theory, only *bona fide* ERAD substrates should be targeted for ubiquitination, whereas wild-type, folded proteins should escape this fate. Which characteristics of substrates lead to their recognition by the molecular chaperones that act upstream of the ubiquitination pathway or by the E3 ligases that directly recognize substrates? The structure (or misfolded state) of a protein should serve as a recognition device [252–254]. Another parameter that may control ERAD substrate recognition and subsequent ubiquitination is the appearance of surface-exposed hydrophobic side chains, which should be enclosed within the structure of properly folded proteins [119,255]. It is also possible that select post-translational modifications or amino acid motifs trigger ERAD when they are exposed [256,257].

Finally, the regulation of the ERAD machinery represents a fertile area for ongoing and future research. Although select examples have been described in this review, we know nothing about how ERAD is regulated in different tissues and how various disease states might alter ERAD efficiency. One report suggested that HER2+ breast cancer cells have a heightened requirement for ERAD, but this was not rigorously demonstrated [258]. Moreover, the pathway should respond to changes in cell, tissue, and organ development, and some hints on the requirement for HRD1 during these events have recently been uncovered [259–261]. But, given the fact that ~8000 proteins pass through the ER in a human cell, and there are numerous misfolded conformations that these proteins can attain, variations on the ERAD theme will most probably become the norm.

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Abbreviations

CF	cystic fibrosis

CFTR	cystic fibrosis transmembrane conductance regulator
CHIP	C-terminus of Hsc70-interacting protein
DUBs	deubiquitinating enzymes
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ERAD-C	ER cytosolic lesion
ERAD-L	ER luminal
HC	heavy chain
HCMV	human cytomegalovirus
HECT	homologous to the E6AP carboxyl terminus
HRD	Hmg CoA reductase degradation
HSP	heat-shock protein
LC	light chain
MHCI	major histocompatability complex class I
RING	really interesting new gene
SCF	Skp1, Cullins, F-box proteins
SUMO	small ubiquitin-like modifier
SVIP	small p97/ VCP-interacting protein
UBX	ubiquitin regulatory X
UPR	unfolded protein response
VIMP	VCP-interacting membrane protein

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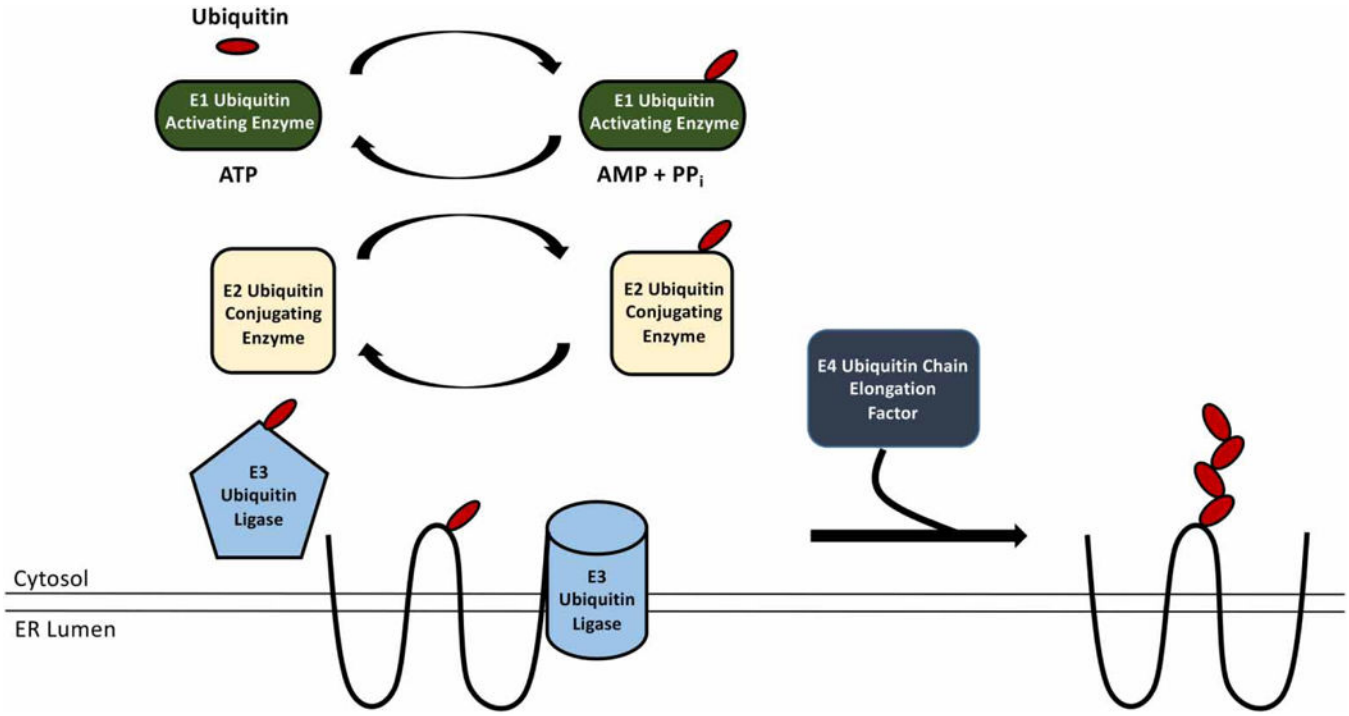

Figure 1. The ubiquitination pathway

The cytosolic E1 ubiquitin-activating enzyme hydrolyzes ATP to activate the ubiquitin molecule. ATP hydrolysis and the formation of a transient AMP-derivative lead to the formation of a thioester bond between the E1 and the C-terminus of ubiquitin. The E1 then transfers ubiquitin to one of the ~11 yeast or the ~35 mammalian E2 ubiquitin-conjugating enzymes. The covalently bound ubiquitin—E2 adduct then binds one of the ~80 yeast or the ~300 mammalian E3 ubiquitin ligases. The E3 enzymes may also be bound to the ERAD substrate and facilitate transfer of ubiquitin to the substrate, or a chaperone intermediate (not shown) may facilitate transfer. Some E3 ubiquitin ligases (e.g. HECT domain E3s) become covalently modified with ubiquitin during ERAD substrate modification, while other E3s (e.g. RING and U-box domain proteins) facilitate the transfer of ubiquitin from the E2 ubiquitin-conjugating enzyme to the substrate. Importantly, select E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases are cytoplasmic, while others reside at the ER membrane. Once a substrate is ubiquitinated by an E3 ubiquitin ligase, other enzymes, such as E4s, may further extend the ubiquitin chain on the ERAD substrate.

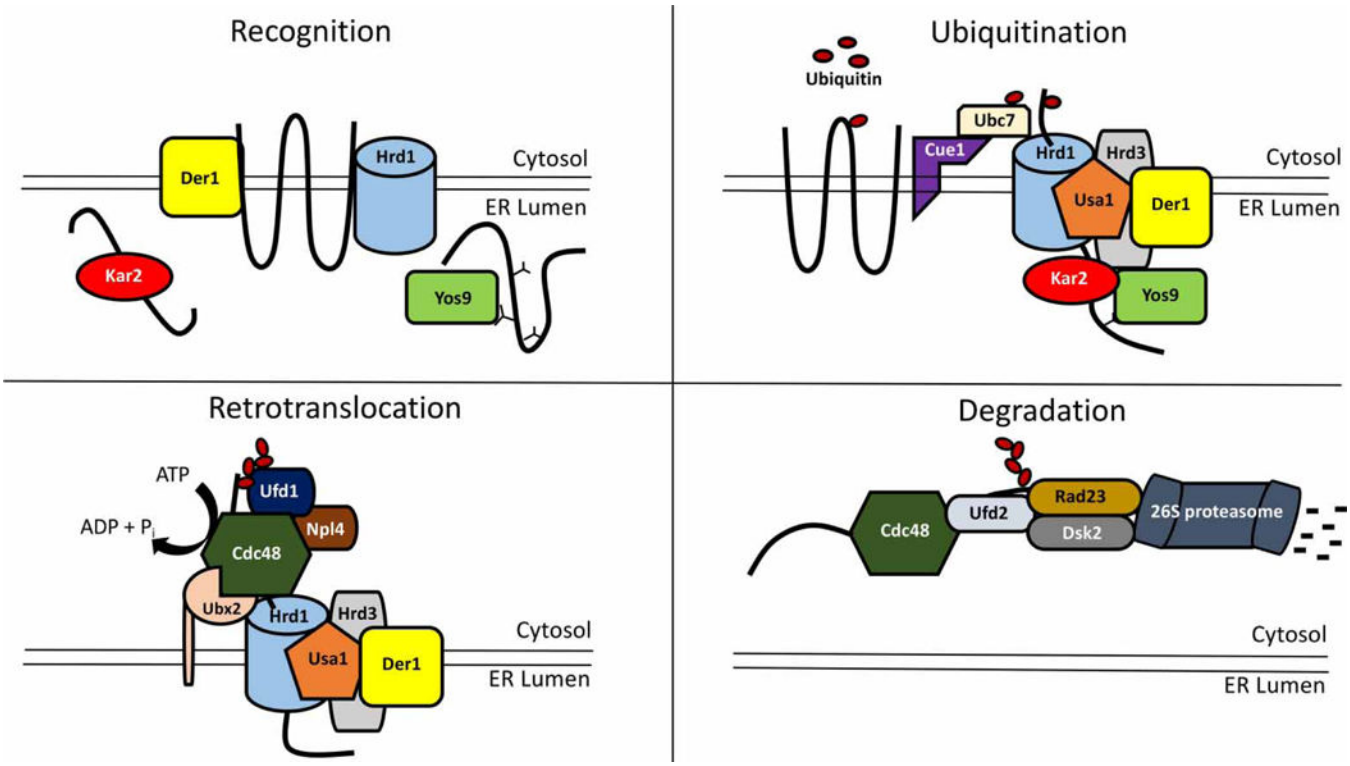

Figure 2. Function of the Hrd1 complex during ERAD in yeast

In the first step during ERAD (‘Recognition’), a misfolded substrate is recognized by a subset of factors, namely Kar2 (ER luminal Hsp70 chaperone), Yos9 (ER luminal lectin), Der1 (transmembrane core Hrd1 complex member), or directly by the E3, Hrd1. Once the substrate has been recognized, the substrate is transferred to the Hrd1 complex for polyubiquitination. Kar2 and Yos9 bind to the Hrd1 core complex member, Hrd3, and the substrate is transferred to Hrd1 (‘Ubiquitination’). Der1 is bound by Usa1, which helps link Der1 to Hrd1. Cue1 is an ER membrane protein that recruits the E2 ubiquitin-conjugating enzyme, Ubc7, to the Hrd1 complex. After the substrate is polyubiquitinated, the dislocation machinery is linked to the complex. This dislocation complex consists of the membrane protein, Ubx2, which helps recruit the AAA+ ATPase, Cdc48. Cdc48 is stabilized at the Hrd1 complex through an interaction with Hrd1 and through an interaction with the Cdc48 cofactors, Ufd1 and Npl4, with the polyubiquitin chain. Assembly is believed to be due to Ufd1 binding to the polyubiquitin chain, as yeast Npl4 lacks a zinc finger domain. Another class of cofactors that bind Cdc48 and affect retrotranslocation includes DUBS, such as Otu1 (not shown here). Once bound to the substrate, Cdc48 hydrolyzes ATP and liberates the substrate from the ER (‘Retrotranslocation’). The Cdc48 cofactor, Ufd2, then extends the polyubiquitin chain and interacts with the ubiquitinated protein shuttles, Rad23 and Dsk2 (‘Degradation’). Although not shown in this figure, ubiquitin chains may be trimmed by DUBS prior to substrate passage through Cdc48 and then extended again by Ufd2. Rad23 and Dsk2 can also interact with the 19S cap of the cytosolic 26S proteasome, which leads to substrate degradation. Another DUB associated with the 19S cap of the proteasome, called Rpn11 (not shown), removes the polyubiquitin chain attached to the ERAD substrate, so it efficiently threads into the core of the 26S proteasome for degradation.

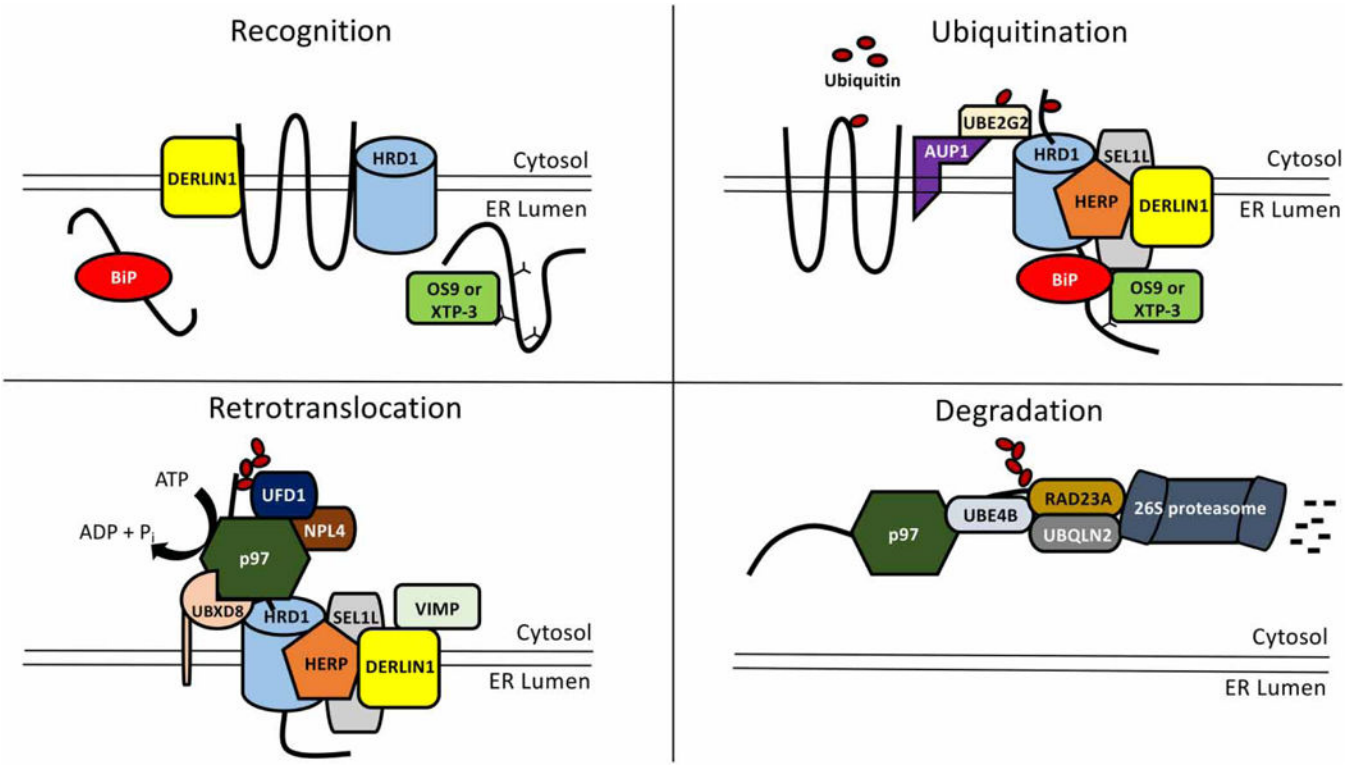

Figure 3. Function of the Hrd1 complex during ERAD in mammalian cells

In the first step during ERAD ('Recognition'), a substrate is recognized by a group of luminal and membrane-associated factors, namely BiP (Hsp70 chaperone), OS9 and XTP-3 (lectins), Derlin1 (transmembrane core Hrd1 complex member), and/or HRD1. While OS9 and XTP-3 recognize misfolded ER luminal glycosylated substrates, they may also recognize nonglycosylated substrates. The substrate is then transferred to the HRD1 complex and polyubiquitinated. BiP, OS9, and XTP-3 bind the HRD1 core complex member, SEL1L, and the substrate is next transferred to HRD1 ('Ubiquitination'). DERLIN1 is bound by HERP1, which helps link Derlin1 to HRD1. The mammalian HRD1 complex can utilize DERLIN2 and DERLIN3 as well. AUP1 is a Cue domain-containing ER membrane protein that recruits the E2 ubiquitin-conjugating enzyme, UBE2G2, to the HRD1 complex. Once polyubiquitinated, the dislocation machinery is recruited to the HRD1 complex. This complex includes the membrane protein, UBXD8, which augments p97 recruitment to the HRD1 complex. Along with UBXD8, the HRD1 complex can utilize another protein, called UBXD2, to recruit p97. p97 is further stabilized at the HRD1 complex through interaction with HRD1 along with the p97 cofactors, UFD1 and NPL4, via the polyubiquitin chain. In an alternative mechanism of p97 recruitment, the cytoplasmic protein, VIMP, binds p97 (not shown) at the HRD1 complex through a VIMP interaction domain in DERLIN1. Another class of cofactors that bind p97 and act during ERAD include DUBS, such as YOD1 (not shown). Once bound to the substrate, p97 hydrolyzes ATP and removes the substrate from the ER ('Retrotranslocation'). After retrotranslocation, the p97 cofactor and a mammalian homolog of Ufd2, UBE4A/B, may extend the polyubiquitin chain and associate with the protein shuttles, RAD23A, and a specific UBIQUILIN, UBQLN2 ('Degradation'). Ubiquitin chains may be trimmed by DUBS prior to substrate transit

through p97 and could then be extended again by UBE4A/B. RAD23A and UBIQUILIN also interact with the 19S cap of the cytosolic 26S proteasome, which facilitates substrate degradation. In addition, there is a DUB associated with the 19S cap of the proteasome, RPN11, which removes the polyubiquitin chain attached to the ERAD substrate, which aids efficient entry of the substrate into the 26S proteasome core.

Table 1

List of cellular factors shown to affect the ubiquitination of ERAD substrates.

Factors	Select substrates	Notes	References
E1 ubiquitin-activating enzymes[1]			
Yeast			
Uba1			[262]
Mammalian			
E1a, E1b		Isoforms resulting from alternative splicing	[263–265]
E2 ubiquitin-conjugating enzymes[2]			
Yeast			
Ubc1	CPY*, Hmg2	Cytosolic E2 that is recruited to the ER membrane by Hrd1	[93, 113]
Ubc6	Sss1, *sec61*, Deg1–Ura3, Pma1–D378N, Ste6*	Integral ER membrane E2	[89, 148, 239, 266, 267]
Ubc7	Hmg2, CPY*, SSS1, *sec61*, Deg1–Ura3, Pma1–D378N, Ste6*	Cytosolic E2 that is recruited to the ER membrane by Cue1	[46, 89, 90, 93, 95, 148, 239, 266–268]
Ubc2	Ste6*	Cytosolic E2 only shown to be important with Ubr1	[171]
Mammalian			
UBE2D1	CFTR, p53, APP	Homolog of yeast Ubc4/5	[161, 265, 269]
UBE2J1	MHCI, CFTR, TCRα, OS-9, EDEM, SEL1L	Homolog of yeast Ubc6	[135, 138, 170, 270]
UBE2K	MHCI	Homolog of yeast Ubc1, used by viral protein US11	[271]
UBE2G2	HERP, gp78, CD3-δ, TCRα, $InsP_3R$	Homolog of yeast Ubc7	[137, 272–274]
E3 ubiquitin ligases			
Yeast			
Hrd1	CPY*, sec61-2, Hmg2, pdr5–C1427Y, Huntingtin, unglycosylated PrP	Integral ER membrane E3	[38, 93, 100, 275–278]
Doa10	Deg1-Ura3, Ubc6, Ste6*, Erg1, α, β, γ subunits of ENaC, Pca1, Zrt1, Sbh2	Integral ER membrane E3	[147, 148, 152, 156, 239, 279, 280]
Rsp5	CPY*	Cytosolic E3 required only when substrates are significantly overexpressed and cells are under oxidative stress	[173, 174]
Ubr1	Ste6*, CFTR	Cytoplasmic E3	[171]
Asi1/Asi1/ Asi3	Erg11	Integral nuclear membrane-localized E3 complex	[175, 176]
Mammalian			
HRD1[3]	HMGR, TCR-α, CD3-δ, MHCI, NHK, APP, α1AT, gp78, NS1 LC, CD95/Fas, p53, CD147	Homolog of yeast Hrd1	[36, 121, 123, 138, 219, 265, 269, 281–285]
TEB4	Type 2 iodothyronine deiodinase (D2), squalene monooxygenase (SM), HMGR	Homolog of yeast Doa10	[154, 156–158]
gp78	CD3-δ, apoB100, CFTR, HMGR, α1AT, Kai1, Cytochrome P450 3A	Homolog of yeast Hrd1	[137, 164, 165, 286–290]
CHIP	CFTR, NCC, Pael-R, cytochrome P450 3A	Cytoplasmic E3	[161, 168, 169, 290–292]
RMA1/ RNF5	CFTR	Integral ER membrane-resident E3	[163, 165, 170]
TRC8	MHCI, SREBP-1, SREBP-2, Heme oxygenase-1, XBP1u, α1 integrin, α2 integrin, α4 integrin, β1 integrin,	Integral ER membrane-resident E3	[37, 293–296]

Factors	Select substrates	Notes	References
	thrombomodulin, PTPRJ and IL-12 receptor β1		
SCF^{Fbx2}	Pre-integrin β1, TCR-α, CFTR	Cytoplasmic/ER membrane-associated E3	[180, 221]
SCF^{Fbx6}	TCR-α	Cytoplasmic/ER membrane-associated E3	[181]
$SCF^{β\text{-}TrCP}$	CD4, Tetherin	Cytoplasmic E3 utilized by HIV protein, Vpu	[177, 178]
RNF4	CFTR	Cytoplasmic/nuclear E3 that recognizes poly-SUMOylated substrates	[227]
RNF103	Autoubiquitination	Integral ER membrane-resident E3	[184]
RNF170	Inositol 1,4,5-trisphosphate (IP_3)	Integral ER membrane-resident E3	[297]
RNF185	CFTR	Integral ER membrane-resident E3 that is homologous to RMA1/RNF5	[298]
PARKIN	Pael-R, mutant glucocerebrosidase	Cytoplasmic E3	[168, 299]
TRIM13	CD3-δ, $Ca_v1.2$	Integral ER membrane-resident E3 that also plays a role in autophagy	[300, 301]
SMURF1	WFS1	Cytoplasmic/nuclear-localized E3	[302]
TMEM129	MHCI	Integral ER membrane-resident E3 utilized by HCMV	[179]
NIXIN/ ZNRF4	Calnexin	Integral ER membrane-resident E3	[303]
NRDP1	ErbB3	Cytoplasmic E3	[304]
SYVN1	CFTR	ER/cytoplasmic E3	[221]
Ubiquitination modifiers			
Yeast			
Cue1	CPY*, Hmg2, KWW	Required for Ubc7 activation and integral ER membrane protein	[101, 111, 112, 305]
Hrd3	Hmg2, CPY*, sec61-2, Pdr5*, unglycosylated PrP	Integral membrane component of Hrd1 complex	[38, 94, 105, 106, 277]
Ssa1	Ste6*, Pma1–D378S, CFTR	Cytosolic Hsp70	[71, 150, 152, 153]
Ydj1	Ste6*, Pma1–D378S	Cytosolic Hsp40 that can be farnesylated	[71, 152, 153]
Hlj1	Ste6*, CFTR	Homolog of Ydj1	[71, 151]
Otu1[4,5]	CPY*, Spt23	Cytosolic deubiquitinating enzyme	[119, 192, 306]
Ufd3[4]	Spt23	Cytosolic ubiquitin chain length regulator	[192]
Ufd2[4]	Ole1, HMG2, ^{DEG1}Sec62	Cytosolic ubiquitin extension enzyme (E4)	[69, 72]
Mammalian			
HDJ2	CFTR, Pael-R	Homolog of yeast Ydj1	[159, 161, 168]
SEL1L	NHK, TTR^{D18G}, $RI_{332,}$ tyrosinase	Homolog of yeast Hrd3	[123, 134, 135]
FAM8A1	NHK, TTR^{D18G}	Hrd1-binding partner believed to regulate Hrd1 function	[134]
HSC70	CFTR, Pael-R, ApoB100, ENaC, NCC	Homolog of yeast Ssa1	[160, 161, 168, 240, 241, 307]
DNAJB12	CFTR	Integral ER membrane-resident Hsp40	[162, 163]
USP13[4]	Ubl4A	Deubiquitinating enzyme	[204, 205]
USP19	CFTR, TCRα	Integral ER membrane-resident deubiquitinating enzyme	[206]
USP25	CD3-δ, APP, CFTR	Integral ER membrane-resident deubiquitinating enzyme	[208]

Factors	Select substrates	Notes	References
AUP1	MHCI, NHK, HMG CoA	Integral ER-membrane Cue domain-containing protein that recruits Ube2g2 to the ER membrane and lipid droplets	[128, 308]
YOD1[4,5,6]	RI_{332}, NHK, TCRα	Homolog of yeast Otu1	[191]
ATAXIN-3[4]	TCRα, CD3-δ, BACE457Δ	Deubiquitinating enzyme	[201, 202, 204]
UBE4B[4]		Homolog of yeast Ufd2	[69, 72]
NEDD8	CFTR	Cytoplasmic ubiquitin-like protein	[221]

[1] Substrates for the E1 are the E2s.

[2] Substrates for the E2s are the E3s.

[3] Some ERAD E3s facilitate the degradation of cytosolic proteins [254, 309].

[4] These components can recognize and bind to the Cdc48/p97 complex in the cytosol.

[5] May trim ubiquitin chains prior to entry into p97/Cdc48.

[6] May also act on ERAD machinery [310].