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Quality control

ER-associated degradation: Protein quality control and beyond

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Even with the assistance of many cellular factors, a significant fraction of newly synthesized proteins ends up misfolded. Cells evolved protein quality control systems to ensure that these potentially toxic species are detected and eliminated. The best characterized of these pathways, the ER-associated protein degradation (ERAD), monitors the folding of membrane and secretory proteins whose biogenesis takes place in the endoplasmic reticulum (ER). There is also increasing evidence that ERAD controls other ER-related functions through regulated degradation of certain folded ER proteins, further highlighting the role of ERAD in cellular homeostasis.

Newly synthesized membrane and secreted proteins enter the ER in an unfolded state through a protein-conducting channel named the translocon (Rapoport, 2007). In the ER, a myriad of chaperones and modifying enzymes assist their membrane integration and folding. In many cases folding involves post-translational modifications, such as glycosylation or disulfide bond formation (Braakman and Hebert, 2013). At this stage many proteins are also assembled into multisubunit complexes with defined stoichiometries. As newly synthesized proteins reach a native conformation, they leave the ER to perform their function elsewhere; either along the secretory pathway or outside of the cell.

Despite all the resources dedicated to protein folding, a significant fraction of newly synthesized polypeptides entering the ER fails to acquire a native conformation (Hartl and Hayer-Hartl, 2009). The degree of misfolding of these proteins varies considerably and can have several causes such as mutations, substoichiometric amounts of a binding partner, or merely a shortage of chaperone availability. In most cases, the misfolded molecules are retained in the ER and eventually become substrates of the ER-associated protein degradation (ERAD), a collection of quality-control mechanisms that clears the ER from these potentially harmful species. Inactivation of ERAD results in the accumulation of misfolded proteins in the lumen and membrane of the ER, a condition known as ER stress that is common to several diseases (Walter and Ron, 2011). For this reason, ERAD plays a key role in ER homeostasis across eukaryotes. Genetic ablation of a number of ERAD components leads to embryonic lethality in mice, also highlighting the importance of this process in cellular and organismal homeostasis (Yagishita et al., 2005; Francisco et al., 2010; Eura et al., 2012). Whether this essential function of ERAD during mouse development is due to its role in the degradation of misfolded proteins remains to be determined.

Certain folded, perfectly active proteins are also targeted by ERAD. However, their degradation is highly regulated and only occurs in the presence of a specific signal. The best-characterized regulated substrate is the 3-hydroxy-3-methylglutaryl acetyl-coenzyme-A reductase (HMGR), a key enzyme in sterol biosynthesis (Gil et al., 1985; Hampton et al., 1996; Bays et al., 2001a; Song et al., 2005). Both in yeast and in mammals, HMGR degradation by ERAD is part of a feedback inhibition system critical for sterol homeostasis. Interestingly, another enzyme of the sterol biosynthetic pathway, squalene monooxygenase (Erg1 in yeast and SQLE in mammals), was recently identified as a regulated ERAD substrate (Foresti et al., 2013). The degradation of Erg1/SQLE by ERAD is again part of a feedback inhibition system to prevent the accumulation of intermediate sterol metabolites, which are toxic for cells (Foresti et al., 2013). Recent evidence shows that regulation of the synthesis of sterols and other sterol-derived metabolites by ERAD is also present in plants (Doblas et al., 2013; Pollier et al., 2013). This evolutionarily conserved role of ERAD in sterol regulation might have been one of its primordial functions.

The ERAD machinery is also exploited by certain viruses to degrade host proteins thereby escaping immune surveillance. Well characterized examples are the degradation of newly synthesized major histocompatibility complex class I (MHC I) heavy

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Abbreviations used in this paper: ERAD, ER-associated degradation; HMGR, 3-hydroxy-3-methylglutaryl acetyl-coenzyme-A reductase; MHC, major histocompatibility complex; SQLE, squalene monooxygenase.

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chain (Wiertz et al., 1996a) or CD4 molecules by the human cytomegalovirus or the immunodeficiency virus (HIV; Fujita et al., 1997; Schubert et al., 1998), respectively. Moreover, some bacterial toxins, such as cholera, and viruses, like simian virus 40 (SV40), travel to the ER retrogradely through the secretory pathway. At the ER these toxins and viruses exploit ERAD components to reach the cytosol, where ultimately they will act (Tsai et al., 2001; Schelhaas et al., 2007; Bernardi et al., 2008).

Finally, ERAD components are also involved in the turnover of several soluble proteins in the cytoplasm and the nucleus of cells (Swanson et al., 2001; Ravid et al., 2006; Yamasaki et al., 2007). Most of these cases, however, involve only a subset of the ERAD steps and components. In sum, although a complete repertoire of substrates is not available, it is clear that misfolded proteins are not the exclusive clients of ERAD.

ERAD, linking ER quality control to cytoplasmic protein degradation

The earliest evidence for protein quality control at the ER came from observations that unassembled subunits of the T cell receptor were rapidly degraded in the cells (Lippincott-Schwartz et al., 1988). This degradation occurred independently of lysosomal proteases, leading to the proposal that the ER itself would house some uncharacterized proteolytic activity toward misfolded proteins. Then a landmark study in yeast showed that the degradation of a short-lived misfolded ER membrane protein was blocked in cells lacking Ubc6, a component of the ubiquitin conjugation machinery (Sommer and Jentsch, 1993). The ubiquitin system mediates the covalent attachment of ubiquitin, a small 76–amino acid protein, to target proteins in the cytoplasm by the sequential action of activating (E1), conjugating (E2), and ligase (E3) enzymes (Pickart, 2001). Ubiquitin-modified proteins are then recognized and degraded by the proteasome. The involvement of the ubiquitin–proteasome system in ER protein quality control was confirmed by studies on the degradation of mutant and wild-type cystic fibrosis transmembrane conductance regulator (CFTR), a large membrane protein with a complicated folding process (Jensen et al., 1995; Ward et al., 1995). Inhibition of proteasome function led to accumulation of CFTR molecules, and interestingly, a significant fraction of these was detected as ubiquitin conjugates (Jensen et al., 1995; Ward et al., 1995). Soon after, it became clear that a similar mechanism could also account for the degradation of luminal misfolded proteins such as CPY*, a mutant version of the yeast vacuolar carboxypeptidase Y and a prototype ERAD substrate (Hiller et al., 1996). Together, these papers demonstrated that aberrant proteins in the lumen and membrane of the ER are degraded in the cytoplasm where the components of the ubiquitin–proteasome system reside.

Ubiquitin ligase complexes: The hubs in ERAD

Subsequent genetic and biochemical studies, primarily in budding yeast but also in mammalian cells, identified many ERAD components and led to a general understanding of the organization of the pathway. An important realization was that the "one-size-fits-all" model does not apply to ERAD and that this pathway encompasses multiple branches with distinct specificity for different classes of misfolded proteins (Taxis et al., 2003; Vashist and Ng, 2004; Carvalho et al., 2006; Bernasconi et al., 2010; Christianson et al., 2012). However, irrespective of the branch, the same sequence of events leads to the degradation of all ERAD substrates (Fig. 1 A). The first step is the recognition of a substrate in the crowded ER environment. Then the substrate is transported across the ER lipid bilayer back into the cytoplasm, a step known as retrotranslocation. On the cytosolic side of the ER membrane, the substrate is ubiquitinated by a membrane-associated ubiquitin ligase (or E3 ligase). Subsequently, the ubiquitinated substrate is extracted from the membrane in an ATP-dependent manner and released in the cytoplasm for degradation by the proteasome. The execution of these steps is coordinated by a membrane-embedded protein complex named after the E3 ligase at its core. The canonical E3 ligases involved in ERAD are themselves multispanning membrane proteins, in which the RING (really interesting new gene) domain responsible for the ligase activity is in the cytoplasm. These E3 ligase complexes are best characterized in yeast (Fig. 1 B and Table 1) where Doa10 (Swanson et al., 2001) and Hrd1 (Bordallo et al., 1998; Bays et al., 2001a) assemble into the Doa10 and the Hrd1 complexes, respectively, each responsible for the degradation of a class of ERAD substrates (Carvalho et al., 2006).

Based on the analysis of a few model substrates, the E3 ligase complex specificity appears to be determined by the location of the misfolded lesion on a substrate relative to the ER membrane: proteins with misfolded domains in the cytoplasmic side of the membrane (ERAD-C substrates) are degraded via the Doa10 complex; proteins with luminal (ERAD-L substrates) or intramembrane (ERAD-M substrates) misfolded domains are targeted to the Hrd1 complex (Fig. 1 B and Table 1; Taxis et al., 2003; Vashist and Ng, 2004; Carvalho et al., 2006). Factors involved in substrate recognition are unique to the E3 ligase complex and likely determine the substrate specificity of each ERAD branch. On the other hand, the components that act at late steps of ERAD, such as the Cdc48 ATPase complex (p97 in mammals) required for membrane extraction of ubiquitinated substrates (Bays et al., 2001b; Ye et al., 2001; Jarosch et al., 2002; Rabinovich et al., 2002), are common to both E3 ligase complexes.

In mammalian cells the best-studied E3 ligases are Hrd1 and Gp78 (Table 1). They are both homologous to yeast Hrd1 but assemble distinct E3 ligase complexes that preferably target different substrates (Schulze et al., 2005; Mueller et al., 2008; Bernasconi et al., 2010; Christianson et al., 2012; Burr et al., 2013). Several more E3 ligases have been implicated in ERAD in mammalian cells (such as Rma1/Rnf5, Trc8, Rfp2, Rnf170, and Rnf185) but these are still poorly characterized. Only few substrates are known for each ligase and a preference for particular ERAD substrate classes has been difficult to infer (Claessen et al., 2012).

How are ERAD substrates recognized?

Recognition of misfolded proteins. The commitment to degradation by ERAD occurs at the level of substrate recognition; therefore, this step needs to be tightly controlled. Inefficient detection of misfolded proteins leads to their accumulation,

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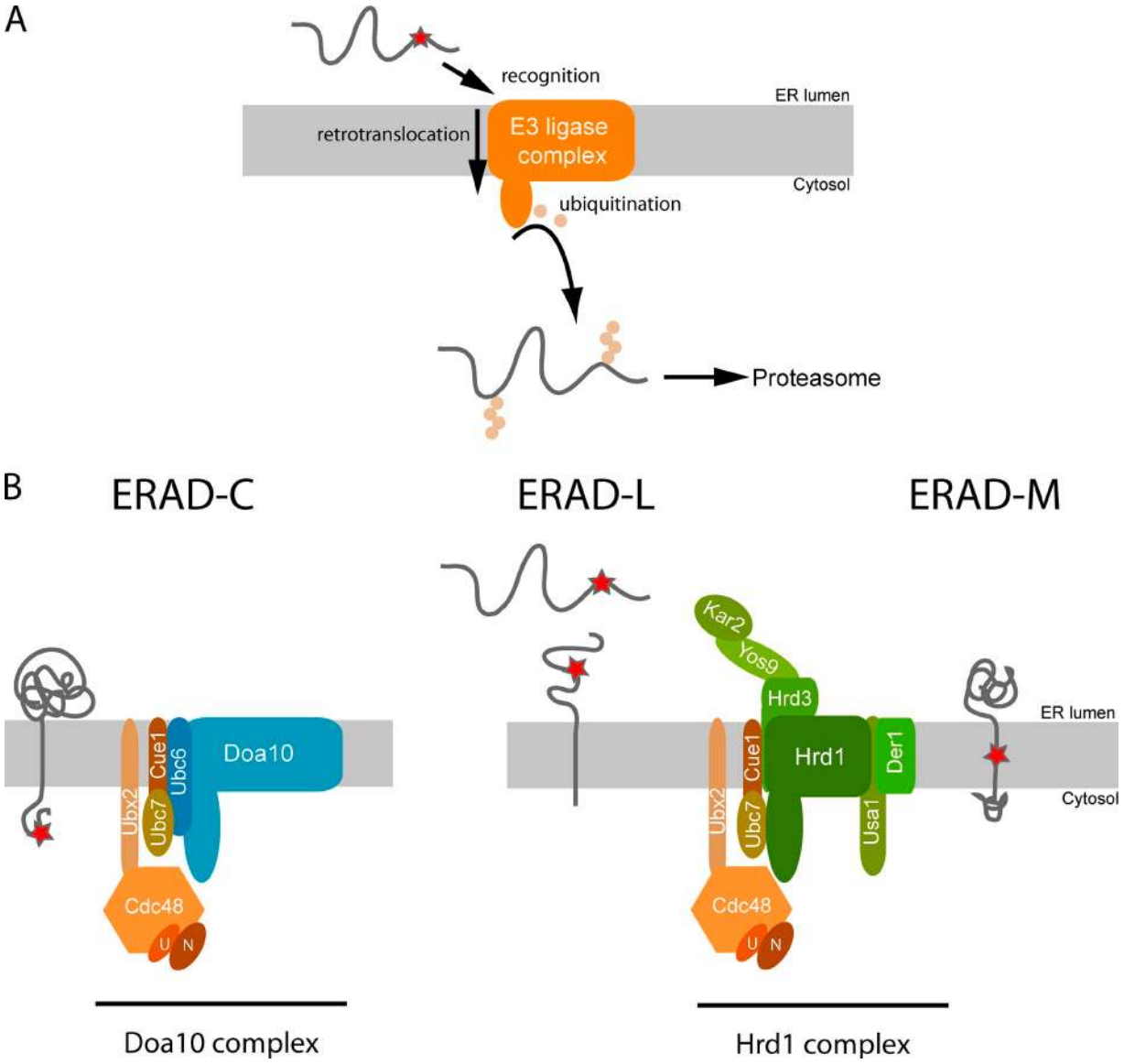

Figure 1. **The different steps and branches in ERAD.** (A) The events defining the ERAD of a generic luminal substrate with a misfolded domain (red star). Substrate recognition, retrotranslocation, and ubiquitination are coordinated by a membrane-embedded E3 ligase complex. Ubiquitin is depicted as small circles. (B) The E3 ligase complexes involved in ERAD in *S. cerevisiae* and their substrate specificities. ER proteins with a misfolded domain in the cytoplasm (ERAD-C substrates) are degraded via the Doa10 complex. Proteins with luminal (ERAD-L) or intramembrane (ERAD-M) misfolded domains are degraded via the Hrd1 complex. Misfolded domains on proteins are indicated by a red star. The Cdc48 cofactors Npl4 and Ufd1 are depicted as N and U, respectively.

ultimately affecting cell function (Travers et al., 2000; Jonikas et al., 2009). On the other hand, overactive ERAD would likely have its cost, with the degradation of significant amounts of folding intermediates. For this reason, substrate recognition by ERAD has to be finely balanced. This is a complex task considering the ER environment, in which a complete spectrum of protein species coexist, from newly synthesized unfolded molecules to fully folded proteins.

Folding intermediates and terminally misfolded proteins share structural similarities, for example the exposure of hydrophobic patches that are normally hidden once proteins acquire the native structure. These molecules are kept in a soluble state by binding to chaperones such as the ER Hsp70 (Kar2 in yeast and BiP in mammals), which are essential for the folding of newly synthesized polypeptides as well as for the disposal of misfolded proteins. However, chaperones on their own do not appear to determine the fate of their clients. Instead, recognition factors that are part of the E3 ligase complexes play a major role in ERAD substrate selection. For example, the recognition of ERAD-L substrates requires the luminal components of the Hrd1 complex Hrd3, Kar2 and, in the case of glycosylated substrates, the lectin Yos9 (Plemper et al., 1997, 1999; Bhamidipati et al., 2005; Kim et al., 2005; Szathmary et al., 2005; Carvalho et al., 2006; Denic et al., 2006; Gauss et al., 2006a). The yeast derlin Der1, a membrane protein of the Hrd1 complex, might also be involved in ERAD-L substrate recognition (Gauss et al., 2006b; Stanley et al., 2011). Moreover, certain ERAD-M substrates appear to be recognized directly by the E3 ligase Hrd1 (Sato et al., 2009). However,

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Table 1. **Components of the yeast E3 ligase complexes and their mammalian counterparts**

Component	Function	Mammalian homologue
Hrd1 complex		
Hrd1	E3 ligase activity/retrotranslocation?	HRD1, gp78
Hrd3	Substrate recognition, Hrd1 stability	SEL1
Yos9	Substrate recognition	OS9, XTP3-B
Kar2	Chaperone activity, substrate recognition	Bip
Usa1	Hrd1 and Der1 oligomerization	HERP
Der1	Recognition/transfer of substrate to Hrd1/retrotranslocation?	Derlin-1, -2, -3
Doa10 complex		
Doa10	E3 ligase activity	TEB4
Ubc6	E2 ubiquitin-conjugating activity	Ubc6, Ubc6e
Common to Hrd1 and Doa10 complexes		
Ubc7	E2 ubiquitin-conjugating activity	UBE2G1, UBE2G2
Cue1	Recruitment and activation of Ubc7	
Ubx2	Membrane-recruiting factor for Cdc48	UBXD8
Cdc48	Substrate retrotranslocation and membrane extraction	p97/VCP
Npl4	Cdc48 cofactor	NPL4
Ufd1	Cdc48 cofactor	UFD1

the features recognized on the misfolded proteins by these ERAD factors are largely unknown.

An informative exception is the recognition of luminal misfolded N-linked glycoproteins in *Saccharomyces cerevisiae* (Fig. 2 A). As they enter the ER lumen, proteins are often modified at asparagine residues (in the context of the N-X-S/T sequence) with a well-defined, branched glycan moiety made up of three glucose, nine mannose, and two *N*-acetylglucosamine residues, Glc3–Man9–GlcNAc2 (Fig. 2 A; Braakman and Hebert, 2013). This N-linked glycan is subsequently trimmed by several enzymes. Early glycan-processing enzymes such as glucosidases lead to the binding of lectins that facilitate the folding of the newly synthesized proteins. In contrast, late acting enzymes, such as the mannosidase Htm1, trigger the binding of a different lectin that engages the protein in ERAD (Jakob et al., 2001; Quan et al., 2008; Clerc et al., 2009). This difference in the kinetics of the glycan-trimming enzymes provides an opportunity for newly synthesized proteins to acquire the native conformation and traffic beyond the ER (Fig. 2 A). A long ER residency, indicative of folding problems, results in the processing of the misfolded glycoproteins by Htm1, which generates a biochemical mark (α1,6-linked mannose) decoded by the lectin Yos9, an ERAD substrate recognition factor (Quan et al., 2008; Clerc et al., 2009).

Both yeast Htm1 and its mammalian counterpart EDEM are in complex with oxidoreductases (Pdi1 in yeast, Erdj5 in mammals), required for the stability of Htm1 and also for reducing disulfide bonds in misfolded proteins, which might affect subsequent ERAD steps (Ushioda et al., 2008; Clerc et al., 2009). The binding of Yos9 to the α1,6-linked mannose is not sufficient to trigger the degradation of the misfolded protein. This processed glycan must be located in an unstructured polypeptide segment that is bound by Hrd3 (Xie et al., 2009). The delivery of substrates to Hrd3 might be facilitated by the luminal chaperone Kar2 that is essential for ERAD (Plemper et al., 1997; Denic et al., 2006; Xie et al., 2009). The dual recognition of a specific N-linked glycan by Yos9 and an unstructured segment by Hrd3 likely enhances the stringency of ERAD substrate recognition, perhaps by a kinetic proofreading mechanism (Fig. 2 A; Denic et al., 2006).

The recognition mechanism of misfolded luminal N-linked glycoproteins is likely similar in mammalian cells because the yeast components are largely conserved in higher eukaryotes (Table 1). However, the situation might be more complicated. For example, OS-9 and XTP3-B, the mammalian homologues of Yos9, use their glycan-binding domain not only to interact with glycans in the misfolded protein but also with Sel1, the mammalian version of Hrd3, which is itself a glycoprotein (Christianson et al., 2008). Whether the interactions with Sel1 and the substrates occur sequentially or correspond to different pools of OS-9/XTP3-B is unclear. In either case, using the same domain to interact with a component and a substrate offers OS-9/XTP3-B an additional mechanism to regulate recognition of N-glycosylated ERAD substrates.

Recent work in yeast suggests that a different type of glycosylation, O-mannosylation, plays an important role in removing certain luminal proteins from futile folding cycles and thus favoring their degradation by ERAD after prolonged residency in the ER (Goder and Melero, 2011; Xu et al., 2013). The enzymes involved in protein O-mannosylation physically associate with ERAD machinery, but how O-mannosylated proteins are captured by the ERAD components is still unclear (Goder and Melero, 2011). Therefore, O-mannosylation is another appealing mechanism for generating an irreversible biochemical mark on proteins displaying folding problems.

A common feature between ERAD substrate recognition by N-glycan trimming and O-mannosylating enzymes is that both appear to be slow processes, requiring substrates to stay for relatively prolonged periods in the ER. Whether other mechanisms involved in recognition of misfolded proteins by ERAD also

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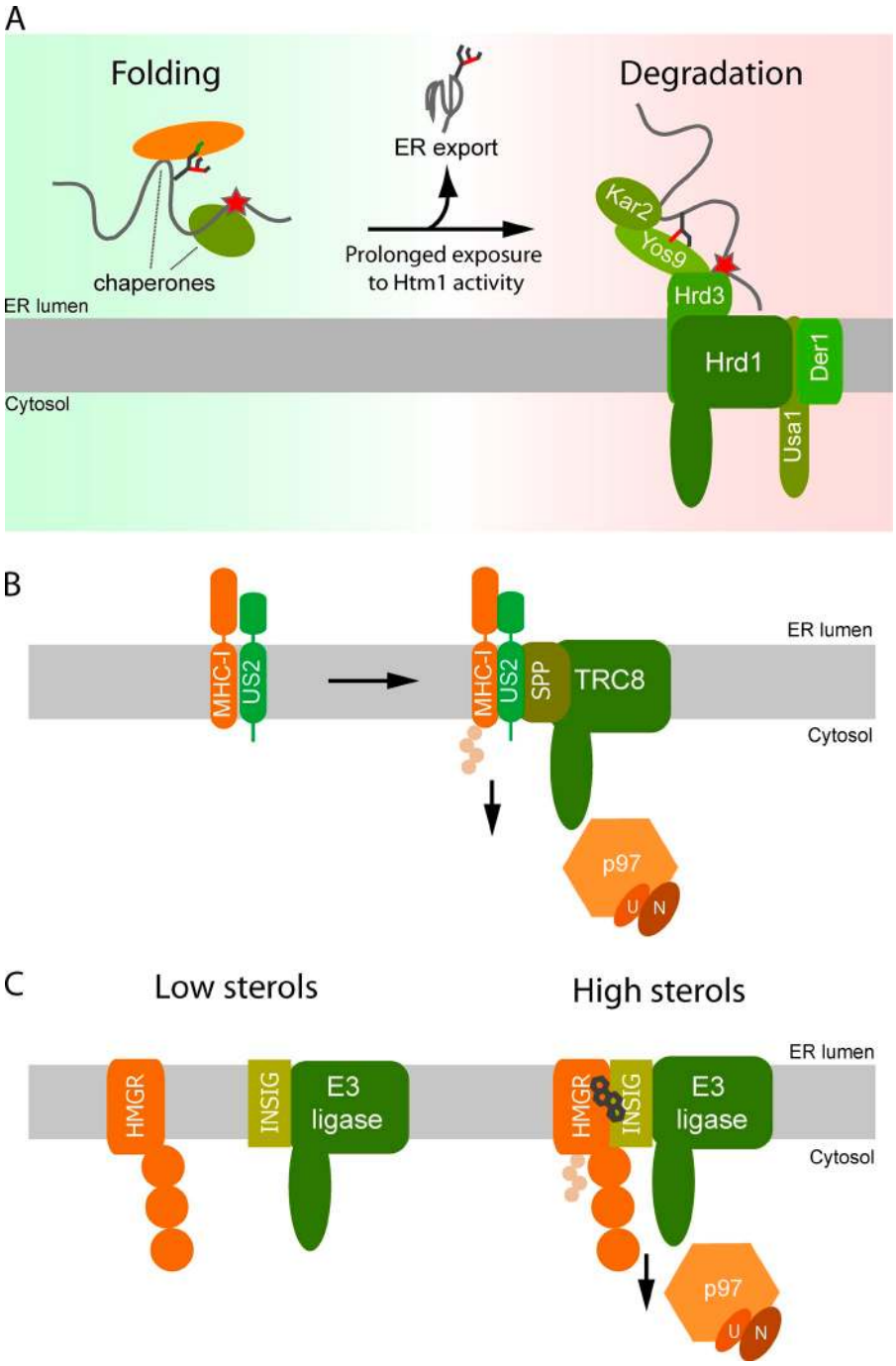

Figure 2. **Mechanisms of substrate recognition in ERAD.** (A) Recognition of misfolded luminal glycoproteins in yeast. Newly synthesized glycoproteins are bound by lectins and other chaperones which facilitate their folding. If properly folded, the proteins leave the ER. Prolonged residency in the ER, indicative of a persistent misfolded domain (red star), leads to Htm1-dependent exposure of an α-1,6–linked mannose residue (red bar). Together, the misfolded domain and the terminal α-1,6 mannose form the degradation signal recognized by Hrd3/Yos9. (B) Recognition of native MHC I heavy chain by the cytomegalovirus -encoded US2 adaptor in infected cells. US2 binds to folded MHC I in the ER membrane and delivers it to an E3 ligase complex containing the E3 Trc8 and the signal-peptide peptidase SPP resulting in MHC I degradation by ERAD. (C) Sterol-dependent recognition of HMGR by Insigs in mammalian cells. Under low sterol levels, HMGR is a stable protein at the ER membrane. High sterol levels, in particular the accumulation of 24,25-dihydrolanosterol (four-ringed structure in gray), cause Insig to bind to HMGR and to deliver it to an E3 ligase complex that promotes HMGR degradation by ERAD. The p97 cofactors Npl4 and Ufd1 are depicted as N and U, respectively. Ubiquitin is depicted as small circles.

require a lag period in the ER is not known. Nevertheless, it is curious that newly synthesized proteins were shown to be protected from degradation for a period of time, even under conditions that favor their misfolding (Vabulas and Hartl, 2005). Therefore, recognition of misfolded proteins might have evolved as an intrinsically slow process, perhaps to spare some folding intermediates from prematurely engaging in ERAD.

Adaptor-mediated substrate recognition. The degradation of specific folded proteins by ERAD is mediated by the same general machinery, but the recognition of these substrates involves distinct factors. A simple mechanism to target a native protein to ERAD is by a substrate-specific adaptor. For example, the human cytomegalovirus encodes ER membrane adaptor proteins, US2 and US11, which bind independently to newly synthesized MHC I molecules and deliver them to ERAD components for degradation. As a consequence, infected cells display less MHC I complex at their surface and escape detection by the immune system (Wiertz et al., 1996a). Despite this common outcome, the two viral proteins interact differently with ERAD components. US11 uses its transmembrane domain to recruit MHC I into a complex which contains Derlin-1 as well as Sel1L, the p97 ATPase complex and its membrane adaptor UBXD8 (Lilley and Ploegh, 2004; Ye et al., 2004; Mueller et al., 2008). Intriguingly, the E3 ligase required for US11-mediated MHC I degradation is not known and both Hrd1 and Gp78 E3 ligases, which are found in complex with Derlin-1, appear to be dispensable.

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US2, on the other hand, delivers its substrate to the ERAD ligase complex containing the E3 Trc8 and SPP, the signal peptide peptidase (Fig. 2 B; Loureiro et al., 2006; Stagg et al., 2009). The precise function of SPP in ERAD is not known and it is not even clear whether it involves its proteolytic activity. Despite these differences, both US2 and US11 act as adaptors to deliver a specific ERAD substrate, MHC I heavy chain, to the E3 ligase complexes that promote its degradation.

A similar mechanism targets CD4 for degradation in cells expressing the HIV-encoded ER membrane protein Vpu. In this case, Vpu works not only as the substrate adaptor for CD4 but also as a scaffold to recruit a cytosolic E3 ligase complex, $SCF^{\beta TrCP}$, required for CD4 ubiquitination (Fujita et al., 1997; Schubert et al., 1998). In vitro reconstitution of Vpu-mediated CD4 ubiquitination revealed that the specificity of adaptor-mediated substrate selection can be further increased at the level of substrate ubiquitination, which can be counteracted by the activity of de-ubiquitinating enzymes (Zhang et al., 2013). The balance between these activities helps discriminating small differences in adaptor–substrate affinity. Whether this mechanism aids the selection of other ERAD substrates is not known yet.

The strategy of using a substrate-specific adaptor is not exclusive to viral encoded proteins. Both in *Drosophila* and in mammalian cells, the derlin-related iRhom proteins function as adaptors in the ERAD-mediated degradation of EGFR ligands as they traffic through the ER (Zettl et al., 2011). Elegant genetic experiments in flies showed that this mode of regulated ERAD was important to control sleeping behavior that requires EGFR signaling (Zettl et al., 2011). It is likely that more substrate-specific adaptors will be identified as our knowledge of the mechanisms of regulated ERAD expands.

Signal-mediated substrate recognition. A substrate-specific adaptor also functions in the regulated ERAD of HMGR, a key enzyme of the sterol biosynthetic pathway. In this case the adaptor, either Insig-1 or Insig-2, does not bind constitutively to HMGR (Song et al., 2005). Instead, the interaction only occurs in the presence of 24,25-dihydrolanosterol, an intermediate metabolite in sterol biosynthesis. Under low sterols levels HMGR is a stable protein, actively producing sterol precursors (Fig. 2 C). On the other hand, high sterol synthesis leads to a rise in 24,25-dihydrolanosterol concentration, which favors the binding of HMGR to one of the Insig proteins, its delivery to an E3 ligase complex, and consequently its degradation by the proteasome (Fig. 2 C). Whereas Gp78 and the Trc8 were originally implicated in HMGR regulated ERAD, recent data suggest that additional E3 ligases might also be involved (Song et al., 2005; Lee et al., 2010; Jo et al., 2011; Tsai et al., 2012). Degradation of HMGR by ERAD results in reduced flux through the sterol biosynthetic pathway and reestablishment of membrane lipid homeostasis.

Interestingly, Insig-1 (but not Insig-2) is itself subjected to reciprocal sterol-regulated ERAD (Lee et al., 2006). Depletion of cellular sterols stimulates Insig-1 ubiquitination by the E3 Gp78 ligase complex. Conversely, if sterol levels are high Insig-1 binds to SCAP, another key ER membrane protein required for sterol homeostasis, leading to a much longer Insig-1 half-life. These data illustrate the complex interplay between the opposing effects of sterols on the stability of the HMGR enzyme and one of its adaptors for regulated degradation by ERAD.

In yeast, sterol homeostasis also involves negative feedback of an HMGR homologue, Hmg2 (Hampton et al., 1996). Like in mammals, degradation of yeast Hmg2 is controlled by the Insig-like proteins Nsg1 and Nsg2 and requires the E3 ligase Hrd1 (Bays et al., 2001a; Gardner et al., 2001; Flury et al., 2005). In fact, Hrd1 was originally identified in a genetic screen for mutants defective in HMGR degradation (HRD genes; Hampton et al., 1996). The binding of Hmg2 to Nsg1 is also modulated by sterol levels (Theesfeld and Hampton, 2013). However, in contrast to mammalian cells, the binding of Nsg1 promotes Hmg2 stability, indicating that the recognition of this substrate is mechanistically different in the two systems (Flury et al., 2005; Theesfeld and Hampton, 2013). Based on limited proteolysis experiments, it has been proposed that Nsg1 and Nsg2 work as Hmg2-specific chaperones and that in their absence Hmg2 presents sufficient conformation instability to engage in ERAD as a misfolded protein (Flury et al., 2005; Shearer and Hampton, 2005). This degradation is further accelerated by high concentrations of an early sterol-intermediate metabolite, geranylgeranyl pyrophosphate (Theesfeld and Hampton, 2013). Therefore, a change in the affinity to a binding partner is another strategy to target a protein for ERAD in a signal-dependent manner.

Squalene monooxygenase (SQLE), another enzyme of the sterol biosynthetic pathway, is also targeted by regulated ERAD both in yeast and in mammals (Foresti et al., 2013). SQLE degradation requires the yeast Doa10 E3 ligase complex or its mammalian homologue Teb4, indicating that two independent branches of ERAD control distinct steps in sterol biosynthesis (Foresti et al., 2013). Although the mechanism for the recognition of SQLE by ERAD machinery is still not known, it is clear that Insigs are dispensable for this recognition, both in mammals and in yeast (Gill et al., 2011; Foresti et al., 2013). In mammals, the N-terminal domain of SQLE is necessary and sufficient for cholesterol-dependent degradation (Gill et al., 2011). Whether this domain binds directly to cholesterol or interacts with an ERAD-specific adaptor is not known. The mechanism for SQLE recognition by ERAD in yeast is likely to be different because this N-terminal domain is only conserved among certain animals.

Based on these few examples, it is clear that signal-mediated ERAD depends on the ability of cells to sense the concentration of some lipids in their membranes and on specific adaptors to selectively degrade key enzymes. Our knowledge on the mechanisms by which other classes of regulated ERAD substrates are recognized will grow as more of these are identified.

Shipping out the trash: Substrate retrotranslocation and cytoplasmic events in ERAD

After being selected, ERAD substrates are retrotranslocated across the ER membrane back into the cytoplasm. In the case of misfolded luminal proteins the complete polypeptide needs to be retrotranslocated, whereas for membrane substrates this step requires the transport of only certain domains. As a consequence, ubiquitination of luminal substrates only occurs at late stages of retrotranslocation, once a portion of the substrate has been

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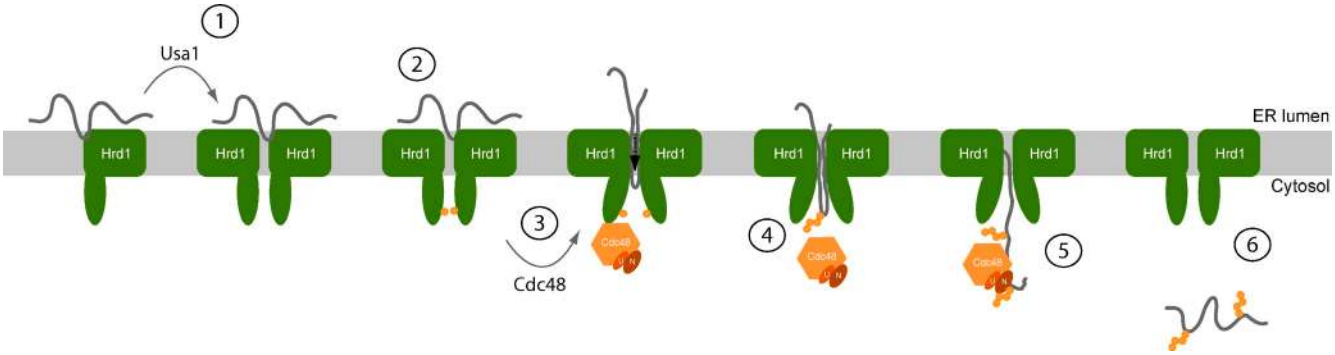

Figure 3. **A working model for Hrd1-mediated retrotranslocation of a luminal misfolded glycoprotein.** Upon recognition (not depicted), the misfolded protein (gray) is transferred to Hrd1. The binding can occur either to Hrd1 monomers or to Usa1-mediated Hrd1 dimers (1). Substrate-bound Hrd1 dimer self-ubiquitinates (2), which leads to the recruitment of the Cdc48 ATPase complex. ATP hydrolysis by Cdc48 induces a conformational change in Hrd1 dimer that facilitates the early stages of substrate retrotranslocation (3). Once exposed to the cytoplasm, the substrate is ubiquitinated by Hrd1 and recognized by the Cdc48 complex (4), which uses its ATPase activity to complete substrate retrotranslocation (5). After retrotranslocation, the ubiquitinated substrate is released in the cytosol for degradation by the proteasome (6). The Cdc48 cofactors Npl4 and Ufd1 are depicted as N and U, respectively. Ubiquitin is depicted as small circles.

exposed to the cytoplasm. In contrast, ubiquitination of most, but not all (Burr et al., 2013), membrane substrates is coupled to their retrotranslocation.

In analogy to the transport of newly synthesized proteins into the ER or mitochondria, it has been postulated that retrotranslocation occurs through a protein-conducting channel. However, the identity of the retrotranslocation channel has been at the center of an intense debate that is almost as old as the research in this field. Over the years, several channel candidates have been proposed but it has been difficult to gather definitive evidence in support of any of them. The Sec61 translocon used for protein import into the ER was the first proposed retrotranslocation channel (Wiertz et al., 1996b). Sec61 was found to interact with ERAD substrates both in yeast and in mammalian cells as well as with the yeast proteasome (Wiertz et al., 1996b; Kalies et al., 2005; Scott and Schekman, 2008). However, the significance of these associations is not clear. Recent work showed that proteins engaging the Sec61 translocon aberrantly or persistently in their way into the ER become substrates of the Hrd1 ligase complex, which might explain the interaction between Sec61 and some ERAD substrates (Rubenstein et al., 2012). In addition, certain yeast *sec61* mutants displayed defects in degrading model ERAD substrates, even under conditions in which general "forward" translocation appeared not to be dramatically affected (Pilon et al., 1997; Plemper et al., 1997; Willer et al., 2008). It remains to be determined whether this phenotype is caused by a specific impairment in retrotranslocation.

The E3 ligase complexes interact with ERAD substrates immediately before and after their retrotranslocation, indicating this step occurs in their immediate vicinity. Therefore, multispanning membrane proteins within the E3 ligase complexes have also been seen as good candidates to mediate retrotranslocation (Ye et al., 2001; Lilley and Ploegh, 2004; Kreft et al., 2006; Horn et al., 2009; Carvalho et al., 2010; Mehnert et al., 2014). These include the E3 ligases themselves as well as proteins of the Derlin family (Der1 in yeast), which are essential for the degradation of all luminal ERAD substrates but whose function has remained elusive. In vitro experiments using mammalian-derived microsomes loaded with the yeast ERAD substrate mutant pro-α-factor indicate that Derlin might be involved in retrotranslocation (Wahlman et al., 2007). In this simplified system, in which synthesis and retrotranslocation were uncoupled, the substrate cross-linked to Derlin but not to Sec61. Moreover, its retrotranslocation was blocked by anti-Derlin antibodies whereas antibodies directed to Sec61 had no effect (Wahlman et al., 2007). It should be noted that mutant pro-α-factor is a noncanonical substrate because its retrotranslocation does not require ubiquitination. Interactions between the yeast Der1 and the prototype ERAD-L substrate CPY* were also detected by site-specific photocrosslinking in yeast (Carvalho et al., 2010; Stanley et al., 2011; Mehnert et al., 2014). These cross-links were seen even in cells lacking the substrate recognition factors Hrd3 and Yos9, and were increased if conserved polar residues in the membrane domain of Der1 were mutated. An interpretation of these results is that Der1 mediates the transfer of substrates from the recognition factors Hrd3/Yos9 to the Hrd1 ligase inside the membrane (Mehnert et al., 2014).

A compelling piece of evidence for a function during substrate retrotranslocation was reported for the E3 ligase Hrd1 (Fig. 3; Carvalho et al., 2010). Although commonly working in the context of the Hrd1 complex, overexpressed Hrd1 can mediate the degradation of ERAD-L substrates even in the absence of its membrane partners Hrd3, Der1, and Usa1 (Plemper et al., 1999; Denic et al., 2006; Carvalho et al., 2010). Under these conditions Hrd1 selectivity for misfolded proteins is lost, suggesting that the other subunits of the complex are critical to control Hrd1 activity and substrate specificity (Denic et al., 2006). Hrd1 interacts with a sizeable region of a modified version of CPY* during the early stages of retrotranslocation, as assayed by site-specific photocrosslinking (Carvalho et al., 2010). Importantly, the interaction likely occurs inside of the ER bilayer because it is lost if substrate recognition is blocked or if the transmembrane segments of Hrd1 are mutated. Hrd1 contains only six transmembrane segments; therefore, ERAD-L substrate retrotranslocation requires Hrd1 oligomerization, which normally is facilitated by Usa1 but can occur spontaneously upon Hrd1 overexpression (Horn et al., 2009; Carvalho et al.,

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2010). All these data make a strong case for a direct role of Hrd1 in the retrotranslocation of ERAD-L substrates, but the possibility that it works with some partner(s), such as Der1, Sec61, or other unknown factors cannot be excluded at this point. Moreover, it is not known whether this is a unique feature of Hrd1 or whether the membrane domains of other E3 ligases also participate in the retrotranslocation of other classes of ERAD substrates.

In most of these cross-linking experiments the retrotranslocation of CPY* was dampened by fusing it to a very tightly folded domain, indicating that ERAD-L substrates need to be unfolded before this step (Bhamidipati et al., 2005; Carvalho et al., 2010). Whether unfolding is also a prerequisite for the retrotranslocation of other classes of ERAD substrates is not settled yet (Fiebiger et al., 2002; Tirosh et al., 2003).

At the cytoplasmic side of the ER membrane, substrates are ubiquitinated, a modification that allows their recognition by the Cdc48/p97 ATPase complex composed of a homohexamer of Cdc48/p97 and by the cofactors Ufd1 and Npl4 (Bays et al., 2001b; Ye et al., 2001, 2003; Jarosch et al., 2002; Rabinovich et al., 2002). The recruitment of this ATPase complex to the ER membrane is facilitated by ubiquitin regulatory X (UBX) domain–containing proteins, Ubx2 in yeast and UBXD8 in mammals (Neuber et al., 2005; Schuberth and Buchberger, 2005; Mueller et al., 2008). The ATPase activity of the Cdc48/p97 complex provides the driving force to move ubiquitinated substrates out of the membrane into the cytosol (Ye et al., 2003). Although the role of Cdc48/p97 in this process is well established, the mechanism that couples ATP hydrolysis to membrane extraction of the substrate is still not understood. In addition to the Cdc48/p97 complex, the ATPase subunits of the proteasome regulatory particle were also shown to play a role in retrotranslocation of some ERAD substrates (Lipson et al., 2008). The driving force for the retrotranslocation of the few non-ubiquitinated substrates, like the cholera toxin or pro-α-factor, is not known (Kothe et al., 2005; Moore et al., 2013).

The Cdc48/p97 complex also serves as a platform for other ubiquitin-modifying enzymes such as de-ubiquitinating enzymes (DUBs; Rumpf and Jentsch, 2006; Jentsch and Rumpf, 2007; Ernst et al., 2009). Although the role of some of these Cdc48/p97-binding factors is not clear yet, it was shown that interfering with the DUBs YOD1 and ataxin-3 affected substrate retrotranslocation (Wang et al., 2006; Ernst et al., 2009). The requirement for DUB activity during retrotranslocation suggests that the process involves cycles of ubiquitination and de-ubiquitination. In some cases these cycles might be important to increase the specificity of substrate recognition, as was shown for the Vpu-mediated degradation of CD4 (Zhang et al., 2013). Interestingly, the retrotranslocation of some noncanonical substrates like cholera toxin, which are not ubiquitinated, is also affected by manipulation of both E3 ligase and DUB activities (Hassink et al., 2006; Bernardi et al., 2013). These results suggest that retrotranslocation requires the ubiquitination of some factors other than the substrates. Future studies should test some obvious candidates such as the components of the E3 ligase complexes themselves.

There is some recent evidence that the yeast Cdc48 complex might be acting also much earlier, during the initial stages of retrotranslocation of an ERAD-L substrate, before it is exposed to the cytoplasm (Fig. 3). Using a cross-linking strategy, it was shown that the interaction between Hrd1 and an early retrotranslocation intermediate was lost in mutants of the Cdc48 complex (Carvalho et al., 2010). Interestingly, a similar defect was detected in Hrd1 mutants impaired for E3 ligase activity (Carvalho et al., 2010). Based on these observations it was proposed that in early stages of ERAD-L substrate retrotranslocation, Hrd1 induces the ubiquitination of an ERAD component, perhaps Hrd1 itself. This ubiquitination signals the recruitment of the Cdc48 complex, which upon ATP hydrolysis would induce changes in the conformation or in the oligomeric status of Hrd1, resulting in substrate retrotranslocation. This model is consistent with the well-established role of Cdc48/p97 in the disassembly and remodeling of protein complexes (Jentsch and Rumpf, 2007). Once the substrate emerges on the cytosolic side and is ubiquitinated by Hrd1, the ATPase complex binds to it and promotes the final stages of its retrotranslocation. Although many aspects of this model still wait for experimental support, it would provide a unifying role for the Cdc48/p97 ATPase as the driving force for substrate retrotranslocation in ERAD (Fig. 3).

After being released from the membrane, substrates are kept soluble and transferred to the proteasome by cytosolic chaperones such as the BAG6 complex (Claessen and Ploegh, 2011; Wang et al., 2011; Xu et al., 2012) or shuttle factors like Rad23 and Dsk2 (Medicherla et al., 2004). The long journey of ERAD substrates ends with their degradation by the proteasome.

Conclusions and future perspectives

In recent years there has been tremendous progress in understanding ERAD. The identification of most of the components involved in this process and how these are pieced together and organized in the different ERAD branches were important achievements. A major challenge for the future is to reveal the mechanistic aspects of the pathway. Such developments should, for example, help in discerning the basis by which misfolded proteins are recognized in each of the different ERAD branches.

The mechanisms of substrate retrotranslocation and the roles played by the different components, such as the E3 ligases and the Cdc48/p97 complex, will certainly be another interesting area to follow. However, progress on these topics might require the development of new approaches, such as in vitro systems with purified components recapitulating individual ERAD steps.

In early days, ERAD was perceived as a process mainly dedicated to ER protein quality control. The picture now emerging places ERAD closer to other ubiquitination systems in the cytoplasm and nucleus, which control the turnover of specific proteins to achieve a certain physiological state. Therefore, another major challenge for the coming years is the detailed characterization of the roles of ERAD beyond quality control. Although the central role of ERAD in sterol homeostasis is

unequivocal, it will be important to clarify whether ERAD has a more general role in the regulated degradation of folded ER proteins and in that way modulates other ER-related functions. A systematic and rigorous identification of regulated ERAD substrates should help in addressing these issues. Uncovering the intersections of ERAD with other cellular pathways will provide important insights into the mechanisms of ER and cellular homeostasis.

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