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Recent technical developments in the study of ER-associated degradation

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Abstract

Endoplasmic reticulum-associated degradation (ERAD) is a mechanism during which native and misfolded proteins are recognized and retrotranslocated across the ER membrane to the cytosol for degradation by the ubiquitin-proteasome system. Like other cellular pathways, the factors required for ERAD have been analyzed using both conventional genetic and biochemical approaches. More recently, however, an integrated top-down approach has identified a functional network that underlies the ERAD system. In turn, bottom-up reconstitution has become increasingly sophisticated and elucidated the molecular mechanisms underlying substrate recognition, ubiquitylation, retrotranslocation, and degradation. In addition, a live cell imaging technique and a site-specific *in vivo* photo-crosslinking approach have further dissected specific steps during ERAD. These technical developments have revealed an unexpected dynamicity of the membrane-associated ERAD complex. In this article, we will discuss how these technical developments have improved our understanding of the ERAD pathway and have led to new questions.

Introduction

Approximately one-third of newly synthesized proteins are translocated into the endoplasmic reticulum (ER), where they must fold and assemble into their correct conformations. To accommodate these proteins, which exhibit diverse structures, oligomeric states, and folding rates, the ER harbors a high concentration of molecular chaperones that maintain polypeptide solubility, enzymes that posttranslationally modify proteins, and factors that directly assist in protein folding. However, the yield of correctly folded proteins is poor due to intracellular and external stresses, genetic mutations, or stochastic misfolding events [1–4].

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Protein folding is monitored by the ER quality control machinery and terminally misfolded/unassembled proteins are recognized, retrotranslocated (i.e., transported) to the cytosol, and degraded by the ubiquitin-proteasome system. This process is referred to as ER associated degradation (ERAD) [5–12]. Since the discovery that soluble secretory and integral membrane proteins could be degraded in a pre-Golgi compartment, the components that constitute the ERAD pathway have been explored through the use of genetic and biochemical approaches and by examining a relatively limited number of model ERAD substrates. In recent years, considerable progress has been achieved by developing novel genetic and biochemical tools. For example, a top-down approach using mass spectrometry was introduced to systematically analyze physical interactions between components involved in ERAD. In addition, quantitative proteomics such as SILAC (stable isotope labeling using amino acids in cell culture) identified endogenous ERAD substrates. In parallel, bottom-up reconstitutions that recapitulate each step during the ERAD pathway have elucidated the mechanisms that lead to substrate recognition, ubiquitylation, retrotranslocation, and degradation. As a complementary approach to understand ERAD dynamics, a live cell imaging technique and a site-spedific *in vivo* photo-crosslinking technique were introduced.

Physical properties of the membrane-associated ERAD machineries

In the yeast, *Saccharomyces cerevisiae*, the Hrd1 E3 ubiquitin ligase is one of the central components of the membrane-associated ERAD machinery (Figure 1 and Table 1). Hrd1 has six transmembrane segments and harbors a cytosolically exposed RING domain at its C-terminal tail [13,14]. In yeast, the Hrd1 “core” complex comprises Hrd1, Hrd3, Usa1, and Der1. The molecular weight of the complex in digitonin-solubilized lysates was estimated by sucrose density gradient analysis to be >700 kD a [15,16]. In this complex, Hrd1 forms an oligomer and its formation is scaffolded by Usa1 [17–19], which spans the membrane twice. Usa1 also bridges the interaction between Hrd1 and Der1 [15]. A recent report indicated that Der1 forms an oligomer, and its formation is also scaffolded by Usa1 [20]. Another component, Hrd3, binds to and stabilizes Hrd1 and possesses a single transmembrane domain. Because robust ERAD activity was evident when yeast expressed a mutant form of Hrd3 lacking this domain, the lumenal domain of Hrd3 may bind to a Hrd1 lumenal loop [13]. In turn, the large lumenal domain of Hrd3 may directly recognize misfolded proteins [21–23] (Figure 1).

The Hrd1 core complex also associates with a number of peripheral components in the cytoplasm and in the ER lumen. These components include Yos9 (a lumenal substrate recognition lectin) [24–28], Ubx2 (a UBX-UBA domain containing protein) [29,30], and Cdc48/p97-Npl4-Ufd1 complex (a AAA-ATPase complex, which drives the retrotranslocation of ubiquitylated substrates) [31–35]. The association of the peripheral components with the Hrd1 core complex seems to be weaker and/or transient because they do not co-migrate with the core complex in a density gradient but are co-immunoprecipitated with Hrd1 [15,36]. Together, the Hrd1 core complex links events that include substrate recognition, ubiquitylation, and retrotranslocation [16,36] (Figure 1).

Another E3 ligase involved in ERAD is Doa10, which contains fourteen transmembrane segments with both termini facing the cytoplasm [37] (Figure 1 and Table 1). Doa10 was initially found as an E3 ligase that functions in the degradation of a soluble substrate, the Matα2 transcriptional repressor [38,39]. It subsequently turned out that Doa10 is responsible for the ubiquitylation of ER membrane proteins with misfolded cytosolic domains (the "ERAD-C pathway"), whereas Hrd1 recognizes and ubiquitylates misfolded substrates containing intra-membrane or ER-lumenal lesions (the "ERAD-M" and "ERAD-L" pathways, respectively) [15,40,41]. For both Hrd1 and Doa10, two E2 ubiquitin conjugating enzymes, Ubc6 and Ubc7, are required for efficient substrate ubiquitylation [38,41–44]. Ubc6 is bound to the ER membrane via a C-terminal transmembrane anchor [42], whereas the soluble Ubc7 enzyme localizes to the ER by binding the transmembrane Cue1 receptor protein, which also allosterically activates the E2 enzyme [45–47] (Figure 1 and Table 1).

In mammals, as many as 16 E3 ligases have been implicated in ERAD [11], including Hrd1 [48], gp78 (also called AMFR or RNF45) [49] and TEB4 (which is a Doa10 homolog and also called MARCH-VI) [37,50]. Of these, Hrd1 complex assembly has been best documented. Constituents of the Hrd1 complex in mammals are similar to those in yeast. These include the adaptor protein SEL1L (which is similar to Hrd3) [51], the rhomboid pseudoprotease Derlin-1 (a homolog of Der1) [52–54], the scaffold protein HERP (which may be functionally related to Usa1) [15,55,56], and a series of membrane-embedded, lumenal and cytosolic peripheral factors [57]. The molecular weight of the Hrd1 core complex in CHAPS-solubilized lysate was estimated by sucrose density gradient analysis and was substantially larger than that found in yeast (>700 kDa, see above). The mammalian Hrd1 complex exhibited two peaks, and the larger one was >2000 kDa. This complex was retrotranslocation-competent and included HERP, SEL1L, and Derlin-1 [58] (see below).

The membrane-associated ERAD machinery is dynamic

In recent years, several lines of evidence suggested a dynamic behavior of the Hrd1 complex. Substrates in the ER lumen are targeted to the lumenal side of the Hrd1 complex and should re-emerge on the cytosolic face of the complex for ubiquitylation [13,43]. The potential role of the Hrd1 complex in facilitating the retrotranslocation of lumenal substrates is being investigated in many laboratories. However, because membrane protein complexes that facilitate the translocation of proteins into the ER, mitochondria, and chloroplast are known to be dynamic [59], it is not unreasonable to assume that the Hrd1 complex is also dynamic and its conformation may significantly change during substrate recognition and retrotranslocation. However, given the lack of an *in vitro* reconstitution system with purified membranous components and in the absence of structural data, definitive evidence for this model is lacking. Recently, however, an analysis of the Hrd1 complex's catalytic cycle through the application of *in vivo* site-specific photocross-linking was reported. This approach provides protein-protein interaction data at amino acid resolution [60,61]. The details of this technique will be mentioned in a later section, but germane to this section we note that direct interactions between lumenal substrates and Hrd3, Hrd1, and Der1 were observed [62]. Within this complex, Der1 appears to facilitate the movement of misfolded lumenal proteins through the Hrd1 complex [20].

Recent studies have suggested that the assembly and disassembly of the Hrd1 complex is triggered by misfolded proteins. When retrotranslocation is blocked in yeast expressing a thermosensitive allele of *CDC48*, ubiquitylated substrates accumulate on the Hrd1 complex and nucleate the assembly of a supramolecular complex that includes the Hrd1 complex as well as Ubx2, Yos9, and the 19S proteasome [16]. These results suggest that the Hrd1 complex transiently forms a larger complex that links lumenal and cytosolic events during retrotranslocation [16]. Several lines of evidence hinted that the integrity of the Hrd1 complex in mammalian cells is also regulated by misfolded proteins. Hrd1 and HERP, which are normally short lived proteins [63–65], are stabilized when the concentration of misfolded proteins rises [58]. The stabilized Hrd1 complex is retrotranslocation competent and is >2000 kDa. These data imply that misfolded proteins may directly enhance the activity of the ERAD machinery, a concept referred to as “ERAD tuning” [58,66,67].

Components of the Hrd1 complex are transcriptionally upregulated by the unfolded protein response (UPR) [68], which is triggered in response to an increase in the concentration of non-native proteins in the ER [69,70]. Therefore, one might assume that the architecture of the Hrd1 complex is altered and its activity might be enhanced by the UPR. The response of the Hrd1 complex upon UPR activation could be different from and slower than ERAD tuning because the UPR requires transcriptional and translational upregulation of ERAD components [66]. One intriguing observation is that the members of the Hrd1 complex are induced to widely varying degrees (between 1.5- and 4-fold) [16]. Moreover, UPR signaling not only upregulates genes involved in the ERAD pathway but many other genes, including those encoding molecular chaperones and those required for protein translocation into the ER, glycosylation, vesicle transport, and lipid metabolism [68]. In addition, accumulation of misfolded proteins in the ER results in significant morphological changes of the ER membrane [71] and induces autophagy [72–78], which alleviate the ER stress. Given these many effects, it remains mysterious how the assembly of the Hrd1 complex is regulated by the UPR and how the rate-limiting step(s) during ERAD are modulated by ER stress.

Systematic approaches have expanded the genetic and physical linkages of the membrane-associated ERAD machineries

Until recently, the ERAD pathway and an analysis of how ERAD-requiring complexes interact have primarily been measured in yeast. In contrast, Kopito and colleagues performed a mass spectrometry analysis of proteins that co-precipitated with 25 “bait” proteins corresponding to different ERAD components in mammalian cells [57]. Over 170 unique interacting proteins were identified, including 71 proteins that were not previously associated with ERAD. Many of the genes encoding these proteins are upregulated by the UPR, suggesting a role in ER protein quality control. Consistent with previous observations [79–85], the lumenal recognition factors OS-9 (a homolog of yeast Yos9) and XTP3-B were associated with Hrd1 and SEL1L, whereas components involved in retrotranslocation such as Derlin and UBXD8 (a UBX domain containing protein) were connected to both Hrd1 and to gp78. Gp78 is another E3 ubiquitin ligase that plays a vital role in the modification and degradation of specific ERAD substrates [49]. The study also uncovered FAM8A1, which is a potential regulator of Hrd1; UBAC2, a ubiquitin binding protein associated with gp78; and

the uncharacterized ER-membrane complex (EMC), which has been connected to various ERAD-related phenomena. For example, six out of 10 of the yeast EMC subunits were identified in a study that systematically identified genes contributing to ER protein folding [86].

Exploring the endogenous substrates highlights the importance of ERAD under normal physiological conditions

ERAD is often examined using artificial model substrates or disease relevant mutant proteins, which are usually overexpressed relative to their native levels *in vivo*. A notable exception is HMG CoA reductase-2 (Hmg2 in yeast), which catalyzes the rate-limiting step in sterol biosynthesis and whose stability is controlled by ERAD in a sterol intermediate-dependent manner [87–90]. Nevertheless, due to the presence of a small number of endogenous substrates, the importance of ERAD function under normal physiological conditions is poorly understood. Recently, however, the identities of endogenous ERAD substrates have been uncovered by proteomic methods and by taking advantage of specific cell lines. For example, squalene monooxygenase, which is encoded by *ERG1* in yeast, was identified as a Doa10 substrate by SILAC and mass spectrometry [91]. The Doa10-mediated degradation of Erg1 is stimulated by lanosterol, which is a downstream product of this pathway. Depletion of Doa10 led to the accumulation of lanosterol and sterol esters, indicating feed-back regulation of Erg1 [91]. In mammalian cells, the Hedgehog precursor molecule was identified as an endogenous ERAD substrate [92]. This protein is processed in the ER by self-cleavage into the N-terminal Hedgehog ligand and the C-terminal fragment. The C-terminal fragment is a lumenal glycoprotein, which is constitutively degraded through the SEL1L-HRD1 pathway. Another endogenous substrate in mammalian cells is the core-glycosylated version of a protein, CD147, which was identified as a factor that co-precipitated with OS-9 and is degraded via the OS-9/SEL1L/Hrd1 pathway [93].

Finally, the integral membrane UPR transducer, ATF6, is degraded in a SEL1L-dependent manner, a result that was substantiated with stable knock-out SEL1L DT 40 cells [94]. Continued work in this area is sure to uncover other examples in which the ERAD pathway contributes to cellular homeostasis.

In vitro reconstitution of ERAD

Most cell free ERAD systems are substrate specific and require the addition of crude materials (e.g., microsome fractions and cytosol). Typically, substrates are translated *in vitro*, mixed with ER-derived microsomes, and then incubated with cytosol and/or purified components, which facilitate substrate ubiquitylation, retrotranslocation, and/or degradation. In some studies, especially when polytopic integral membrane proteins are examined, microsomes are prepared from cells expressing the substrate. In either case, retrotranslocation is typically assessed by detection of substrates in a cytosolic fraction after centrifugation. Alternatively, deglycosylation of *N*-glycosylated substrates, which occurs in the cytosol, is an indicator of retrotranslocation. Model substrates used for *in vitro* systems include ΔgpαF (an immature version of the yeast α-factor mating pheromone) [95–99], apolipoprotein B [100], cholera toxin [101,102], the cystic fibrosis transmembrane

conductance regulator [103–106], Hmg2 (in yeast, see above) [107], HMGCoA-reductase (in mammalian cells) [108], Ste6* (a mutated form of a yeast plasma membrane transporter) [109], the cell surface CD4 receptor [110], and the MHC class I heavy chain [111,112].

In cell-free systems, the cytosol can be replaced with or added to purified components such as the proteasome or proteasome subparticles (i.e., the 19S “cap” and the 20S core) [98], the Cdc48 complex (see above; also known as p97 in mammalian cells), and/or other regulatory factors [31]. Alternatively, specific components can be immune-depleted from the cytosol to assess their function [106]. When ERAD is examined using components derived from yeast, membranes and cytosol from cells containing specific mutant alleles can be used [96,107,109]. However, replacement or just removal of lumenal and membrane-associated components presents a challenge when the goal is to reconstitute the ERAD pathway in mammalian cells. One way to replace lumenal components with a defined component is to use alkaline extraction. For example, Johnson and colleagues reconstituted rough mammalian microsomes with defined lumenal contents and measured the retrotranslocation and degradation of ΔgpαF, a model ERAD substrate in yeast that was used in the first reconstituted ERAD system. The ΔgpαF was covalently modified with a fluorescence dye, which permitted measurements of substrate retrotranslocation and degradation. In this assay, addition of antibodies against Derlin-1 blocked the ERAD of ΔgpαF but only after a short lag time, suggesting that Derlin-1 contributes to cycling the retrotranslocation complex for multiple rounds of retrotranslocation. This study also demonstrated that protein disulfide isomerase is necessary and sufficient for ΔgpαF retrotranslocation, at least in the mammalian ER [99]. A more recent study recapitulated the ubiquitylation of a single-spanning membrane protein embedded in a liposome with purified components. An HIV-encoded membrane protein, Vpu, bridges the interaction between the substrate, CD4, and the $SCF^{\beta TrcP}$ E3 ubiquitin ligase complex in the ER membrane. Surprisingly, the extent of ubiquitylation of wild-type CD4 (which is degraded) and a stable mutant (which is defective for Vpu binding) was similar. However, the addition of cytosol led to significantly greater levels of ubiquitin conjugation to the degraded versus the stable substrate. Ultimately, the activity of a deubiquitylating enzyme was found to mediate substrate discrimination. These data indicate that an ERAD substrate undergoes multiple rounds of ubiquitylation/deubiquitylation, an event that is required for the proteasome to engage only a *bona fide* substrate but not a wild type protein [110].

Fluorescent reporters of retrotranslocation

The ultimate goal of ERAD reconstitution is to recapitulate the entirety of the reaction with a minimal set of purified components and chemically defined lipids. Given the large number of potential ERAD regulators and parallel/redundant pathways that route a protein for ERAD, this goal has not been achieved. In contrast, the development of a live cell assay could significantly bolster our understanding of specific steps in the ERAD pathway, particularly with the development of powerful genetic tools that are available in yeast and increasingly in mammalian cells. One recent study took advantage of the split-GFP system [113], where the bulk of GFP (the N-terminal 10 β-strands, designated S1–10) was expressed in the cytosol and the other part (a C-terminal β -strand, designated as S11) was fused with model substrates. When S11-tagged ERAD substrates retrotranslocated back to

the cytosol, S11 reassembled with S1–10, resulting in a fluorescent signal that was detected in the microscope [114]. Another study utilized variants of Venus fluorescent protein, which fluoresce after they are first glycosylated in the ER and subsequently deglycosylated by the cytosolic peptide-*N*-glycanase (PNGase) after retrotranslocation [115]. Both studies demonstrated the value of the split and/or mutant fluorescent protein system and supported the notion that Hrd1, HERP, Sel1L, and the p97/Cdc48 complex play a central role in ERAD. As evidenced in prior studies, this work also demonstrated that integral membrane proteins can be liberated into the cytosol and remain soluble before degradation [107,109]. It is important to bear in mind that the expression of GFP in the ER lumen can result in an unexpected post-translational modification and may be degraded by the proteasome, perhaps due to its slow rate of folding [116]. Nonetheless, the continued development of fluorescence-based live cell assays for ERAD will continue to be of great value [117].

In vivo site-specific crosslinking

Conventional biochemical methods to detect protein-protein interactions, such as co-immunoprecipitation, chemical crosslinking, and density gradient centrifugation, provide important information regarding component identities, the nature of their interactions, and the sizes of protein complexes. In principle, a systematic mapping of the direct interactions at the amino acid level between a substrate and an ERAD component with high resolution would provide a “snap-shot” of substrate retrotranslocation, a process that remains enigmatic. In the absence of x-ray cystallographic data, an alternate approach is to adopt *in vivo* site-specific photocrosslinking [60].

To assess the interactions between a lumenal substrate and the Hrd1 complex, the gene encoding CPY* (a model ERAD substrate) that contains an amber codon at several positions was expressed in yeast. The cells also expressed an orthogonal pair consisting of an amber suppressor tRNA and a cognate aminoacyl-tRNA synthetase specific for DL-2-amino-3-(p-benzoylphenyl)pentanoic acid (BPA), which is photoactive. Yeast cells were cultured in media containing BPA, which was incorporated into CPY* at the position specified by the amber codon, and UV irradiation resulted in the BPA-dependent crosslinking of CPY* to adjacent proteins. CPY* bound directly to Hrd1, Der1, and Hrd3, consistent with these proteins playing a central role during substrate recognition and retrotranslocation [62]. A more recent analysis employing the same method established the sequence of events during retrotranslocation: Misfolded lumenal substrates are first bound by Hrd3 and Yos9 and are then transferred to Der1, which initiates their insertion into the membrane. Next, the substrates are transported to the cytosolic face of Hrd1. Although the pore through which the substrate was threaded is still not completely clear, it may have been formed by the transmembrane regions of Der1 and Hrd1 [20]. An important next goal will be to trap a retrotranslocating substrate in the membrane in order to view this critical but undefined step in the ERAD pathway.

Conclusions

Significant progresses in our understanding of the ERAD pathway has been made by numerous technical developments, only some of which we were able to discuss here (Figure

2). These advances have uncovered key features of this pathway, but many questions remained answered. Specific events during retrotranslocation and the energetics that initially drive a protein across the membrane, before ubiquitylation and Cdc48/p97 engagement, remain unknown. It is also unknown which constituents are required to regulate the ERAD machinery in specific tissues and under conditions of stress or changes in nutrient availability. The continued development of techniques for *in vitro* reconstitution, for live cell imaging, for monitoring the interactions of components and substrates at the amino acid level, for the development of specific chemical inhibitors, and for structural analyses will provide important mechanistic insights relevant to multiple aspects of the ERAD pathway and to cellular function.

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• Of special interest

• • Of outstanding interest

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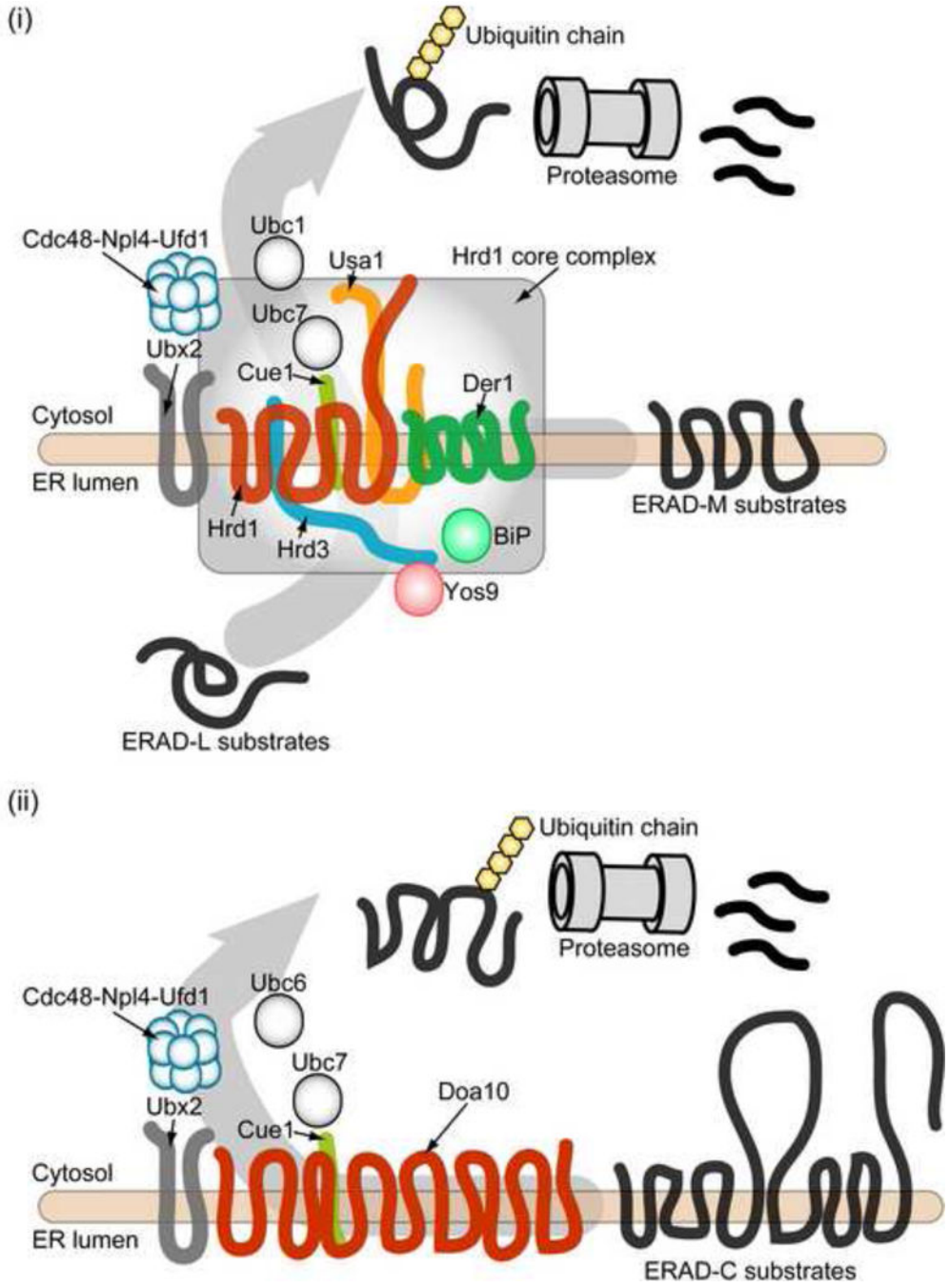

Figure 1.

The membrane-associated E3 ligase complexes in yeast. (i) The Hrd1 "core" complex contains Hrd1, Hrd3, Usa1, and Der1. ERAD-L substrates are recognized by a lumenal Hsp70 (BiP; Kar2 in yeast), Yos9, and the lumenal domain of Hrd3. ERAD-M substrates may be directly recognized by the transmembrane domain of Hrd1. Recognized substrates are retrotranslocated to the cytosol and ubiquitylated by Ubc7 and Hrd1 before proteasomal degradation. (ii) Doa10 (TEB4 in mammals) ubiquitylates membrane substrates that have a

misfolded domain facing the cytosol. Ubiquitylated membrane proteins may also be extracted to the cytosol and degraded by the proteasome.

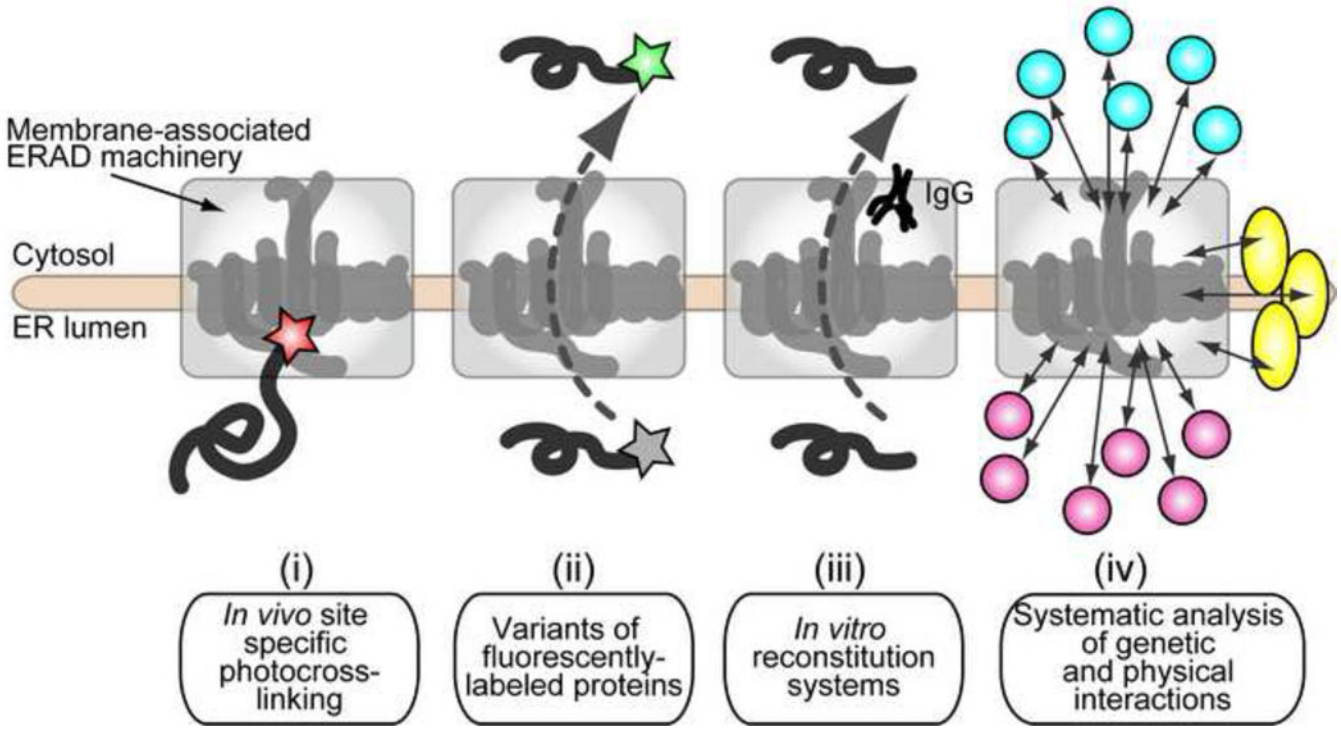

Figure 2.

Select technologies that have improved our understanding of the ERAD pathway. (i) *In vivo* site specific photocross-linking revealed the direct interaction of substrates with components of the Hrd1 complex. (ii) Variants of fluorescent proteins were adopted to analyze retrotranslocation *in vivo*. (iii) Bottom-up reconstitutions that recapitulate each step during the ERAD pathway have elucidated the mechanisms that lead to substrate recognition, ubiquitylation, retrotranslocation, and degradation. (iv) Top-down and systematic genetic and biochemical approaches have identified novel genetic and physical interactions with membrane-associated ERAD machineries.

Table 1

Select, conserved components required for ubiquitylation and retrotranslocation during ERAD

Yeast	Mammal	Function
	Hrd1 complex	
Hrd1	HRD1, gp78	E3 ubiquitin ligase
Hrd3	SEL1	Substrate recognition, Hrd1 stability
Usa1	HERP	Hrd1 and Der1 oligomerization
Der1	Derlin-1, -2, -3	Substrate recognition, transfer of substrate to the Hrd1 complex
Yos9	OS9, XTP3-B	Substrate recognition
Kar2	BiP	Chaperone activity, substrate recognition
	Doa10 complex	
Doa10	TEB4 (also known as MARCH-VI)	E3 ubiquitin ligase
Ubc6	Ubc6, Ubc6e	E2 ubiquitin-conjugating enzyme
	Common to the Hrd1 and Doa10 complexes	
Ubc7	UBE2G1 (also known as UBCH7 or UBC7), UBE2G2	E2 ubiquitin-conjugating enzyme
Cue1		Recruitment and activation of Ubc7
Ubx2	UBXD8	Membrane-recruiting factor for Cdc48
Cdc48	p97/VCP	Substrate retrotranslocation and membrane extraction
Npl4	NPL4	Cdc48 cofactor
Ufd1	UFD1	Cdc48 cofactor

Components in the Hrd1 and Doa10 E3 ligase complexes shown in Figure 1 and their mammalian counterparts are listed. In both yeast and mammals, other ancillary factors including chaperones, E3 ubiquitin ligase enzymes, UBL-UBA domain-containing proteins, deglycosylating enzymes, and deubiquitylating enzymes facilitate ERAD.