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Review

Protein dislocation from the ER☆

Katrin Bagola [a], Martin Mehnert [a], Ernst Jarosch [a,*], Thomas Sommer [a,b,*]

[a] Max-Delbrück Center for Molecular Medicine, Robert-Rössle-Str. 10, 13125 Berlin, Germany
[b] Department of Biology, Humboldt University, 10115 Berlin, Germany

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ABSTRACT

Protein folding within the endoplasmic reticulum (ER) of eukaryotic cells is erroneous and often results in the formation of terminally malfolded species. A quality control system retards such molecules in the ER and eventually initiates their dislocation into the cytosol for proteolysis by 26S proteasomes. This process is termed ER associated protein degradation (ERAD). The spatial separation of ER based quality control and cytosolic proteolysis poses the need for a machinery that promotes the extraction of substrates from the ER. Due to the heterogeneous nature of the client proteins this transport system displays several unique features. Selective recognition of ERAD substrates does not involve transferable transport signals in the primary sequence and thus must follow other principles than established for proteins designated for the import into organelles. Moreover, an ER dislcocation system must be capable to ship polypeptides, which may be at least partly folded and are in most cases covalently modified with bulky and hydrophilic glycans, through a membrane without disrupting the integrity of the ER. In this review we present current ideas on the highly dynamic and flexible nature of the dislocation apparatus and speculate on the mechanism that removes aberrant polypeptides from the ER in the course of ERAD. This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

Contents

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* Corresponding authors. Max-Delbrück Center for Molecular Medicine, Robert-Rössle-Str. 10, 13125 Berlin, Germany. Tel.: +49 30 9406 3753, +49 30 9406 3305; fax: +49 30 9406 3363.
E-mail addresses: ejarosch@mdc-berlin.de (E. Jarosch), tsommer@mdc-berlin.de (T. Sommer).

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1. Introduction

Secretory proteins enter the endplasmic reticulum (ER) in an unfolded state through the narrow Sec61 translocation channel [1]. Folding of these polypeptides starts immediately once the nascent chains emerges in the ER. Some proteins adopt their native structure spontaneously within seconds whereas maturation of others requires the action of folding enzymes and may take up to several hours. To facilitate structural biogenesis of newly imported polypeptides the ER is cramped with protein-folding and protein-modifying enzymes. Hsp70- and Hsp90-type chaperones engage hydrophobic patches on unfolded clients, protect them from aggregation and assist in their structural biogenesis. Oligosaccharyltransferase covalently attaches complex glycan structures to most secretory proteins, which assist local folding and increase their hydrophilicity. These moieties undergo rigid processing and serve as dynamic labels for the recruitment of a varying set of chaperone-like lectins like calnexin and calreticulin [2]. Maturation of many secretory proteins also depends on protein disulfide isomerases, which oxidize pairs of cysteine residues for the formation of thioester bonds. Productive folding often requires multiple reshuffling of the disulfide bonds by isomerization or by breaking and reformation in repeated cycles of reduction and oxidation.

Still, folding of polypeptides is error-prone and often produces terminally aberrant conformers. Moreover, defects within the client protein or the folding machinery, environmental stress or the unbalanced synthesis of partner proteins can increase the load of the ER with improperly folded polypeptides. To avoid leakage of such species into neighboring compartments the ER harbors a protein quality control system (PQC) that retards immature proteins until they have adopted their native structure [3,4]. At the same time this system accomplishes the removal of the products of futile folding attempts and thereby prevents congestion of the ER with aberrant molecules. Thus, the ER-PQC has to evaluate the maturation of non-native proteins and eventually distinguish productively folding molecules from polypeptides that ultimately will fail to reach their native conformation. Since both species expose similar properties of unfolding, e.g. hydrophobic patches at their surfaces, additional signals must exist that allow a reliable discrimination.

More than 15 years ago several observations implied that proteolysis of malfolded secretory proteins is mediated by the cytosolic ubiquitin-proteasome system. This process was later termed ER associated protein degradation (ERAD) [5–7]. Central for ERAD are ubiquitin ligases, which form large heteromeric protein complexes in the ER membrane and provide a functional link between the selection of appropriate substrates in the ER and cytosolic protein degradation. These components are conserved in all eukaryotic organisms, which underscores the importance of this process for cellular homeostasis. A simplified view on the steps resulting in the degradation of misfolded proteins from the ER is depicted in Fig. 1. In yeast, two distinct ubiquitin ligase complexes promote degradation of proteins with different topologies. The Hrd1 ligase predominantly mediates degradation of soluble, ER luminal substrates and integral membrane proteins harboring folding defects in either their ER luminal or their transmembrane regions. Integral membrane proteins with lesions in their cytosolic domains are primarily targeted by Doa10. Mammals contain a much larger set of ERAD ligases but the reason for this is unclear (see Table 1).

Although intricate at a first glance, the spatial separation of protein maturation within the ER from the degradation of aberrant species in the cytosol likely contributes to the specificity of the process by the protection of folding intermediates from premature digestion. However, due to this organization proteasomal decomposition of ERAD substrates has to be preceded by their extraction from the ER. Currently, only vague ideas exist on the mechanism by which proteins are removed from the ER in the course of ERAD. Most prominently two major questions await answer: What is the nature of the protein-conducting channel that allows passage of ERAD substrates through the ER membrane, and what provides the driving force that determines the directionality of this transport process. In adaptation to the heterogenous nature of the substrates cells have developed multiple and concurrent pathways for ERAD. In this review we want

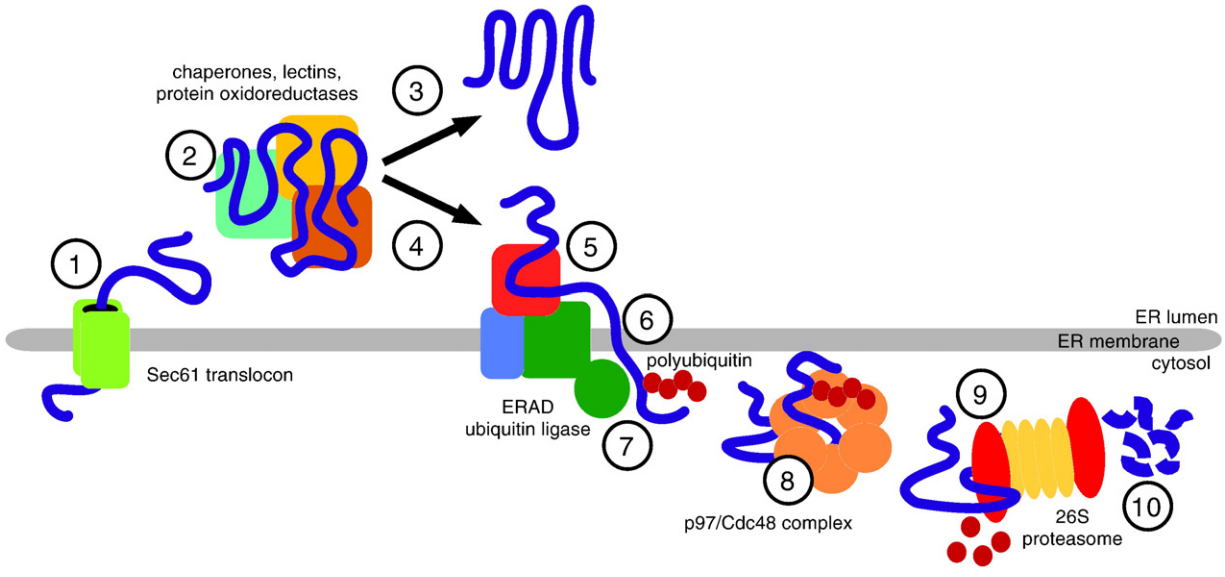

Fig. 1. A simplified model for ERAD. Proteins enter the ER in an unfolded state through the narrow Sec61 translocon (1). During or immediately after their import, these polypeptides are encountered by folding factors like chaperones, glycosylating enzymes or protein oxidoreductases (2). This facilitates structural maturation and retains unfolded polypeptides in the ER. Proteins that have attained their native conformation are released from the folding machinery and are transported to their final destinations (3). A protein quality control system sorts out terminally misfolded species (4) and targets them to substrate-receptors of membrane-bound ubiquitin ligases (5). Subsequently, the misfolded proteins are dislocated into the cytosol by an unknown mechanism (6) where they are polyubiquitylated (7). A complex containing the AAA-ATPase p97/Cdc48 binds to the substrates (8) and promotes the transport to proteasomes (9) where the polypeptides are finally degraded (10).

Table 1
A list of established ERAD factors in yeast and mammals. Most of the mammalian ubiquitin ligases are poorly described and possibly contribute to the degradation of only a limited number of ERAD substrates. In these cases we also lack knowledge on the interacting co-factors that probably determine the specificity of the ligases towards substrates. Please note that components of the ER-PQC and cytosolic factors that are generally involved in ubiquitin-proteasome dependent protein turnover are not included in this list (with the exception of the p97/Cdc48 complex).

Yeast	Mammals	Localization	Function	References
Hrd1	Hrd1/synoviolin	integral ER membrane	ubiquitin ligase	[122-125]
	gp78/AMFR	integral ER membrane	ubiquitin ligase	[126]
Doa10	Teb4/MARCH6	integral ER membrane	ubiquitin ligase	[15, 127]
	TRC8	integral ER membrane	ubiquitin ligase	[128]
	RNF5/RMA1	integral ER membrane	ubiquitin ligase	[16]
	RFP2	integral ER membrane, cytosolic	ubiquitin ligase	[129]
	CHIP	cytosolic	ubiquitin ligase	[16]
	Parkin	cytosolic	ubiquitin ligase	[130, 131]
Hrd3	SEL1L	integral ER membrane	substrate recruitment factor for Hrd1	[29, 30, 32, 132]
Yos9	OS9, XTP3-B	ER lumen; associated with Hrd3/Sel1L	substrate recruitment factor for Hrd1	[133]
Ubc6	Ubc6, Ubc6ep	integral ER membrane	ubiquitin conjugating enzyme	[134, 135]
Ubc7	Ube2g2	cytosolic; associated with ER membrane	ubiquitin conjugating enzyme	[136, 137]
Cue1		integral ER membrane	recruits and activates Ubc7	[138, 139]
Usa1	HERP	integral ER membrane	scaffolding protein for the Hrd1 ligase	[32, 65, 67, 140-142]
Der1	Derlin-1, -2, -3	integral ER membrane	components of a dislocation channel?	[56, 57, 59, 143]
Ubx2	Erasin/UBXD2	integral ER membrane	recruits Cdc48/p97 to the ERAD ligases	[106-108]
Cdc48/Npl4/Ufd1	p97/Npl4/Ufd1	cytosolic	ubiquitin specific AAA-AT Pase that extracts or mobilizes proteins from the ER	[98]

[122–125],[126], [15,127], [128], [16], [129], [16], [130,131], [29,30,32,132], [133], [134,135], [136,137], [138,139], [32,65,67,140–142], [56,57,59,143], [106–108], [98].

to present prevailing ideas on the composition and function of the protein dislocation apparatus that removes defective proteins from the ER for proteasomal degradation.

2. General considerations for an ERAD protein-export machinery

ERAD substrates have to be extracted from the ER to become accessible to the ubiquitin-proteasome system. This transport process comprises several unique features. Proteins destined for the import into organelles often contain defined signal sequences that direct them to the corresponding import machinery [1,8]. ERAD substrates, by contrast, are a heterogeneous mixture of aberrant proteins exhibiting distinct topologies that may expose a variety of different properties. In principal, every secretory protein can be converted to an ERAD substrate by mutations within its coding sequence, environmental stress, exposure to deleterious agents, imbalanced synthesis of binding partners or defects in the folding machinery. Thus, selection of ERAD substrates supposedly involves recognition of global features. Importantly, it is probably the combination of several such properties that triggers export and degradation by the ERAD machinery. Exposure of hydrophobic patches alone is in many cases not sufficient to elicit ERAD probably because such patterns are usually found on aberrant proteins and folding intermediates alike. Current models postulate that additional signals, like the association of certain lectins, allow discrimination between productively folding and terminally aberrant species. These tokens allow binding to receptors, which should reside in close proximity to the dislocation site in the ER membrane and initiate the displacement of the substrate.

Dislocation of single spanning membrane proteins, which only expose small moieties into the ER lumen, may not rely on a dedicated export apparatus but could be accomplished directly by cytosolic factors. By contrast, export of polytopic membrane proteins, which contain multiple hydrophobic transmembrane segments and often form complex structures that are stabilized by intramolecular interactions, most likely depends on a specialized apparatus. These requirements differ again from the demands raised for the dislocation of ER luminal proteins and membrane proteins containing large ER luminal domains. In the case of glycoproteins highly hydrophilic and bulky glycan moieties have to traverse the ER membrane, because removal of the oligosaccharides is exclusively accomplished by peptide N-glycanase, which is confined to the cytosol [9]. Some of the substrates may even have adopted a partial folding, which is not completely dismantled prior to export. Therefore, it is generally believed that the dislocation of ER proteins occurs via proteinacous channels of unknown composition. Such conduits must be capable to accommodate polypeptides that bear complex glycan moieties and that have obtained an at least partly folded state. Hence the size of this pore should be significantly larger than observed for the known protein import channels in the mitochondria or the ER. At the same time an ERAD pore must be efficiently gated to prevent unspecific leakage of small solutes and to preserve the oxidizing environment of the ER. We would also expect the transport apparatus to be in proximity to the site of substrate selection. Most notably the ubiquitin ligase complexes should be closely associated because the export of ER proteins was shown to depend on their polyubiquitylation.

As shown for the import of proteins into organelles, the movement of polypeptides through a membrane depends on a driving force that defines the directionality of the process [1,8]. In these cases, unfolded polypeptides are pushed or pulled through the transport-channels by a mechanical force exerted from energy-consuming protein complexes. A similar requirement is expected for the dislocation of ER-proteins. In principle such transport motors could be localized on either side of the membrane but they should be associated with the export channel and have direct access to the ERAD substrate. Cytosolic factors can only get hold on parts of client proteins that are exposed into this compartment. Therefore, at least ER luminal proteins have to

be partially removed from the ER before they can be engaged by a putative dislocation apparatus in the cytosol.

3. Targeting of ERAD substrates: a tight link of protein folding, quality control and substrate selection

It is currently not known which properties discriminate terminally misfolded proteins from intermediates of the folding process or fully matured species. The identification of such faulty elements probably involves the combination of structural rather than functional criteria. Because unfolded polypeptides are retained in the ER by a PQC the time a polypeptide spends in this compartment may serve to evaluate its maturation status.

3.1. Glycan structures serve as ERAD signals

For glycoproteins a selection process has been elucidated, which is based on the interplay of glycan-processing and the dynamic recruitment of a varying set of lectin-binding proteins to substrates. This system, termed the mannose-timer model, has recently been extensively reviewed [2,7]. During their import, most secretory proteins are covalently modified with complex glycan moieties, which is necessary for their proper folding. Processing by glucosidase I and II removes two terminal glucose residues from this structure and triggers the recruitment of chaperone-like lectins like calnexin and calreticulin to the polypeptide. Glucosidase II eventually cleaves the last remaining glucose from the glycan, which reduces the affinity for calnexin/calreticulin and the polypeptide is released. Proteins that have obtained their native conformation at this stage may leave the ER and get transported to their final destinations. In mammals, UDP-glucose:glycoprotein glucosyltransferase (UGT1) counteracts glucosidase II by the reglucosylation of glycans on unfolded polypeptides and thereby diverts client proteins to calnexin/calreticulin for continuing folding attempts. UGT1 itself has an increased affinity for molten globular structures but binds much less efficient to polypeptides with severe folding defects.

Removal of α(1,2)-bonded mannose-residues on the glycan generates a code that is deciphered by the ERAD machinery: The sequential action of ER mannosidase I (ERManI in mammals and Mns1 in yeast) and EDEMs (Htm1 in yeast) exposes a terminal α(1,6)-bonded mannose on the glycan, which is recognized by ERAD receptors harboring a MRH-domain like OS-9 and XTP3-B (Yos9 in yeast). At the same time the activity of these mannosidases prevents re-glucosylation of the oligosaccharide by UGT1 and therefore irreversibly excludes the polypeptide from the calnexin/calreticulin folding cycle. Processing by ERManI is rather slow and may serve to restrict the time expended for the folding attempts of a polypeptide. EDEM1 associates with the protein oxido-reductase ERdj5 that supposedly dissolves disulfide bonds on ERAD substrates prior to their dislocation. This finding further strengthens the view that EDEMs, and possibly Htm1 in yeast, play a crucial role in the selection of ERAD substrates. However, the α(1,6)-bonded mannose token generated by these enzymes cannot serve as the sole signal to initiate ERAD because native glycoproteins residing in the ER may also expose such moieties but are not degraded.

Not all aberrant secretory proteins are targeted by ERAD. CPY* is a mutant version of the yeast vacuolar carboxypeptidase Y that is retained in the ER and degraded via the Hrd1 ligase complex. This ERAD substrate is glycosylated at four distinct positions. Removal of the most carboxyterminal glycosylation site abrogates recognition and subsequent degradation by the ERAD machinery although the protein is still malfolded and detained in the ER [10,11]. Mutations of the other glycosylation sites does not affect turnover. This finding indicates that not only the structure of glycan moieties but also their positioning within a polypeptide contributes to the selection of ERAD substrates. Indeed, by the introduction of small deletions, which supposedly impair proper folding of the neighboring peptides, non-ERAD glycans could be converted to ERAD targeting signals [12].

Degradation of the type I transmembrane glycoproteins BACE476, a misfolded version of the human-β-secretase BACE, and CD3-δ, an orphaned subunit of the CD3 T-cell receptor, depends on EDEM1 but is unaffected in cells downregulated for OS9 and XTP3-B. Removal of the transmembrane segments from these ERAD substrates detaches them from the membrane but still renders the proteins instable. Importantly, turnover of these glyco-polypeptides now completely depends on OS9 and XTP3-B [13]. This observation implies that the activity of EDEM1 targets membrane-bound and soluble glycoproteins via different mechanisms: de-mannysolation of membrane-bound proteins appears to trigger ERAD predominantly by preventing UGT1 mediated re-glucosylation and association with calnexin/calreticulin. By contrast, processing of soluble glycoproteins requires binding of the substrate receptors OS9 and XTP3-B to oligosaccharide structures generated by EDEM1 and subsequent delivery to the ERAD ligases.

3.2. Targeting of integral membrane proteins

There is not much known, how integral membrane proteins are targeted for ERAD. Supposedly, defects in the folding and assembly of the transmembrane regions also affect the biogenesis of soluble domains, which may then be recognized by the ER-PQC. In yeast membrane proteins with lesions in their cytosolic parts are degraded via the Doa10 ubiquitin ligase [14,15]. Mammalian Teb4/MARCHIV is homologous to Doa10, but also other ubiquitin ligases, like cytosolic CHIP and the membrane-bound RMA1/Rnf5, appear to contribute to this process [16,17]. Substrate recruitment by the Doa10 ligase eventually involves cytosolic Hsp70- and Hsp90-type chaperones, however the detailed mechanisms have to be elucidated here. Moreover, Teb4/Doa10 contains multiple transmembrane segments of unknown function. Since the sequence of some of these domains is highly conserved they may also partake in substrate selection.

BAP31, an integral membrane protein that localizes to the ER and post ER compartments in mammalian cells, participates in the biogenesis of numerous membrane-bound secretory proteins [18–23]. Yeast contains three homologous proteins termed Yet1, 2 and 3 [24]. BAP31 associates with membrane proteins and is required for vesicular trafficking and cell surface expression of several clients. Contrary to this, BAP31 also provokes ER retention of some other polypeptides. It was therefore speculated that BAP31 serves as a component of the ER-PQC for membrane proteins that monitors the structural integrity of targets and routes them either to further transport in the secretory pathway or for degradation by ERAD. Actually, BAP31 was recently shown to be associated with components of the translocon as well as the ERAD apparatus [25]. Moreover, downregulation of BAP31 retards the degradation of CFTRΔF508, a mutant version of the cystic-fibrosis conductance regulator that is retained in the ER and subjected to rapid ERAD. Yet the details of BAP31 function in ERAD are still obscure.

4. Substrate receptors involved in dislocation

Aberrant proteins must be selectively recognized by specific substrate-recruiting factors, which are located at the ER-membrane and should initiate their degradation. SEL1L (Hrd3 in yeast), a protein that is closely associated with the Hrd1 ubiquitin ligase, has been implicated to serve such purpose [26]. This protein comprises a large soluble region exposed into the ER lumen, which contains several TPR-type domains. TPR motives were shown to mediate protein-protein interactions but their function in SEL1L/Hrd3 remains to be elucidated. Yeast Hrd3 regulates the specificity of the Hrd1 ligase. In its absence, Hrd1 becomes instable and is degraded by ERAD [27,28]. Strong overexpression of Hrd1 but not of a catalytically inactive

variant in a *hrd3* deleted strain impairs the growth of yeast cells [29]. Under these conditions Hrd1 mediates the degradation of ER proteins that are not targeted by ERAD in wild type cells. Hrd3 binds unfolded polypeptides in the ER lumen irrespective whether they constitute substrates of the Hrd1 ERAD-ligase [30]. These observations collectively suggest that Hrd3 ascertains specificity of substrate selection by scanning bound proteins for structural defects.

SEL1L/Hrd3 interacts with the *bona fide* substrate receptors XTP3-B and OS9/Yos9 and recruits them to the ERAD ligases [26,31–33]. Yeast Yos9 was shown to bind glycans via its MRH-domain with a strong preference for molecules exposing terminal α(1,6)-bonded mannoses [34]. Therefore, this protein supposedly detects aberrant glycoproteins processed by Mns1 and Htm1 and routes them to ERAD. Indeed, deletion of Yos9 delays the turnover of glycosylated ERAD substrates, whereas degradation of non-glycosylated proteins is not affected [35–38]. However, Yos9 and its mammalian homologues bind malfolded proteins even when their MRH-domains are mutated and eventually also interact with non-glycosylated ERAD substrates. Thus, selection by these lectins probably relies on the combination of certain features within the glycan as well as the peptide part of a substrate. In some cases EDEM1 may also directly deliver glycoproteins to the ERAD ligases [39].

5. A protein-conducting channel for ERAD

5.1. The role of the Sec61 translocon

Before ERAD substrates can be encountered by the proteasome, they have to be extracted from the ER and get translocated into the cytosol. This process involves most likely a proteinaceous conduit in the ER membrane. An attractive candidate for such a dislocation channel is the Sec61 translocon, which mediates the import of proteins into the ER. Central component of this complex is Sec61α (Sec61 in yeast), an integral protein of the ER membrane that forms an aqueous pore. Due to extensive biochemical studies the function of Sec61 in protein transport is well documented [1]. Because no other ER protein with the obvious ability to convey proteins across a membrane was identified so far it is reasonable to speculate that the Sec61 channel may promote both the import and the dislocation of secretory proteins from the ER. Indeed, intense research over the previous years provided some evidence for such a function.

In mammals the cytomegalovirus (CMV) co–opts ERAD and eliminates major histocompatibilty complex class I molecules (MHC I) from infected cells to increase the infection rate. Two viral proteins, US2 and US11 individually bind to MHC I and trigger their polyubiquitylation and degradation. Interestingly, upon inhibition of the proteasome an interaction of Sec61α with MHC I in US2-expressing cells was observed [40]. Similarly, yeast Sec61 could be chemically crosslinked with a misfolded version of pre-pro α-factor but not with a fully folded form [41]. Very recently, Schäfer *et al.* demonstrated that Sec61 binds a soluble and short-lived ER protein that was fully glycosylated and thus has apparently been completely imported into the ER [42]. Noteworthy, components of the HRD-ligase were not required to establish this association implying a putative function of Sec61 upstream of this enzyme complex. Moreover, Sec61 can be co-purified with Hrd3, the substrate receptor of the HRD-ligase.

Expression of Sec61-2, a mutant variant of the translocon, in yeast causes the accumulation of a fully glycosylated ERAD substrate whereas the amount of the cytosolic precursor is not increased [43]. These data imply that Sec61-2 specifically affects the export of this protein from the ER while remaining functional for import. Another mutant, Sec61-3, also abolishes the degradation of soluble and membrane bound ER proteins although translocation of these molecules into the ER is not disturbed [44]. Still, impaired ERAD observed in Sec61 mutant strains could also result from a mild delay in the import of substrates or some limiting ERAD factors.

Furthermore, Sec61 was implicated to serve as a transporter for the export of cholera toxin from the ER of mammalian cells [45]. This AB-type toxin enters the cell by endocytosis and reaches the ER via vesicular transport, where the catalytic A-subunit is separated from the regulatory B-polypeptide by the protein oxidoreductase PDI. The A-subunit is subsequently activated to the A1 form (CTA1) that is transported across the ER membrane into the cytosol where it constitutively activates a chloride channel. In an *in vitro* reconstituted system CTA1 was imported into isolated microsomes and could be re-exported in an ATP dependent manner. CTA1 specifically co-purified with Sec61α in these experiments. Moreover, incubation of CTA1-loaded microsomes with saturating amounts of an import substrate blocked the release of the toxin. However, in this study CTA1 was first imported into the microsomes via Sec61, which complicates the interpretation of the results.

Convincing data argue against a general involvement of the Sec61 translocon in protein retrotranslocation. It is currently unclear whether aberrant proteins completely dissociate from the translocon after their import into the ER. Structural maturation of newly imported polypeptides, such as glycosylation, is accomplished co-translocationally during their import. At this early stage of protein biogenesis irreversible structural aberrations may have already been manifested within the polypeptide and attracted the ERAD machinery. Additionally, misfolding of secretory proteins may eventually cause prolonged association with the translocation complex. Therefore, binding of ERAD substrates to Sec61 may result from delayed import rather than an active retargeting to this channel.

In mammalian cells a chimeric protein of MHC I and green flourescent protein (GFP) that is exposed into the ER lumen is exported from the ER and degraded by the proteasome in the presence of the CMV proteins US2 or US11 [46]. Retention of GFP fluorescence throughout the export process implies that a complete unfolding of the substrate is not required for efficient dislocation. However, this experimental setup does not exclude partial unfolding during transport followed by fast and spontaneous refolding in the cytosol. Intriguingly, dihydrofolatreductase (DHFR) exposed to the ER lumen by its fusion to MHC I can be efficiently degraded by ERAD, even when the globular structure of DHFR was arrested by the binding of the folate analogue trimetrexate [47]. Thus, folding of DHFR does not affect its extraction from the ER while under similar conditions its import into the ER is blocked [48]. This suggests different mechanistic principles and/or the implication of distinct factors for either transport process.

The crystal structure of the bacterial SecYEG translocon implies that also the highly homologous Sec61 channel adopts an hourglass shape in the ER membrane with two funnels facing the cytosol and the ER lumen, respectively [49]. Both funnels are connected by a central hydrophilic passage with a maximal diameter of 20 Å that can accommodate only unfolded or loosely structured polypeptides [1]. The ER-luminal side of this pore is filled by a short helix, termed the plug, which supposedly serves as a gatekeeper to maintain the closed state of the channel [50]. Current models suggest that the Sec61 conduit is opened by displacement of the plug once the signal sequence of import substrates is inserted from the cytosolic side. Conversely, insertion of proteins from the ER luminal side should arrest the plug within the pore and thereby jam the channel for protein transport. These findings conflict with a putative role of the translocon in the shipment of partly folded and glycosylated proteins from the ER into the cytosol.

Dislocation and degradation of at least some ER proteins occurs independently of the Sec61 translocon. Yeast Ubc6 constitutes a tail-anchored protein, which is integrated into the ER membrane in a Sec61 independent manner [51]. Ubc6 is naturally short-lived and constitutively degraded in a ubiquitin-proteasome dependent manner via the Doa10 ligase [15]. Mutations affecting the function of Sec61 do not delay Ubc6 turnover, even under condition where Sec61 mediated protein import is substantially impaired [52].

5.2. Derlin-1, a component of the ERAD ligases involved in substrate transport

Other ER transmembrane proteins have been implicated to serve as transport channels for ERAD (Fig. 2). Yeast Der1 is a small protein that associates with the Hrd1 ERAD ligase. Deletion of *der1* abolishes the turnover of proteins exposing large ER luminal domains, whereas degradation of the majority of membrane-bound polypeptides is not affected [53,54]. Der1 is integrated into the ER by four transmembrane segments and exposes a small carboxyterminal region into the cytosol [55]. Mammals express at least three homologous proteins termed Derlin-1, -2, -3. Whereas Derlin-1 and -2/-3 show a sequence similarity of about 30%, Derlin-2 and Derlin-3 seem to be more evolutionaly related being ~70% identical [56–58]. Like Der1 the Derlins have also been found in association with ERAD ligases but are involved in the turnover of soluble as well as integral membrane proteins. Derlin-1, -2, -3 seem to be required for distinct degradation pathways. US11 mediated MHC I degradation exclusively involves Derlin-1 whereas Derlin-2 and -3 are specifically required for the turnover of the null Hong Kong (NHK) mutant of α1-antitrypsin [57,59]. Interestingly, Derlin-1 was shown to bind substrates before and after their extraction from the ER. Immunoprecipitation experiments from lysates of cells treated with proteasomal inhibitor reveal a preferred association of Derlin-1 with glycosylated MHC I, which is still integrated in the ER membrane. Noteworthy, deglycosylated and thus cytosolic forms of MHC I can also be purified with Derlin-1 [57]. Although it was not determined whether Derlin-1 directly interacted with MHC I under the described conditions these observations suggest that Derlin-1 is in close proximity to ERAD substrates during the entire export process.

Such an assumption is supported by *in vitro* studies on the carriage of pre-pro α-factor and cholera toxin through microsomal membranes. Isolated microsomes were loaded with fluorescently labelled pre-pro α-factor and subsequently incubated with reticulocyte lysate and ATP [60]. Under these conditions a considerable amount of fluorescence is released from the microsomes indicative for the export of the pre-pro α-factor. Antibodies against Derlin-1 abolished export in this system whereas Sec61α specific antibodies had no effect. Furthermore, pre-pro α-factor could be chemically cross-linked to Derlin-1 but not to Sec61α. In a similar experimental setup, cholera toxin can be liberated from microsomes in semi-permeabilized cells [61]. Expression of a dominant negative Derlin-1 variant substantially delays toxin release. These findings have been taken as an indication that the Derlin proteins partake in the formation of an export channel. In line with this, Derlin-1 as well as yeast Der1 was shown to form oligomeric assemblies in the ER membrane [62,63].

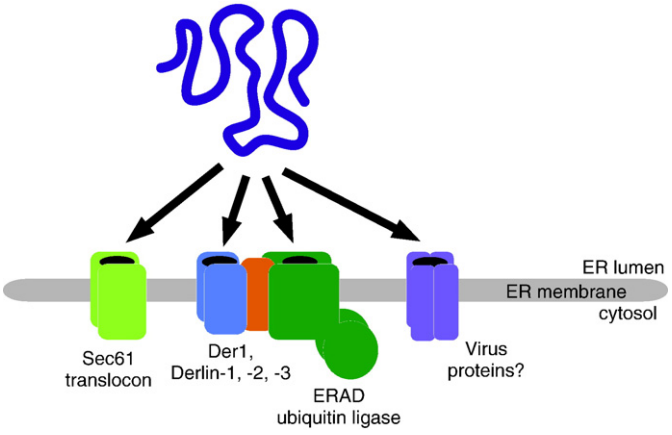

Fig. 2. Candidates for a protein-conducting channel for ERAD. The export of aberrant proteins from the ER most likely depends on a proteinaceous pore that promotes traversal of hydrophilic polypeptides through the ER membrane. Early observations favored an involvement of the Sec61 translocon in this process, although the properties of this channel deduced from its crystal structure are in conflict with a general function in ERAD. Instead, components of the ERAD apparatus, like Der1/Derlin-1, -2, -3 or the transmembrane segments found in the ERAD ubiquitin ligases may form highly dynamic oligomeric assemblies that comprise transient pores for the export of proteins. Although highly attractive, this model is only speculative and awaits proof. In some cases, specialized factors like viral proteins may bind to particular target proteins in the ER and thereby promote their extraction from the membrane.

5.3. ERAD ligases may contribute to pore formation

Most ERAD ligases contain multiple transmembrane segments that anchor them in the ER-membrane. Although they comprise a large portion of the enzymes, no specific function has been assigned to these parts. Of note, the transmembrane segments contain polar and hydrophilic amino acids at conserved positions. Changing some of these residues of yeast Hrd1 to a hydrophobic one specifically affects the activity of the ligase towards membrane-bound proteins [64]. Binding of substrates to the ligase is not impaired in these mutants. Intriguingly, some of these Hrd1 variants are defective for the turnover of a specific substrate while being functional for the processing of others. Studies using gel filtration and sucrose gradient centrifugation revealed an apparent molecular weight of the yeast Hrd1 ERAD ligase complex of about 800 kDa [32,65]. In line with this, Hrd1 was shown to form oligomers via its interaction with the membrane protein Usa1 [65]. This oligomeric assembly seems to be required for the degradation of membrane proteins but dispensable for the turnover of luminal substrates. A dimeric organization has also been proposed for mammalian Hrd1 and gp78, respectively, but the functional consequences on protein dislocation have not been investigated [66,67]. Still, these observations imply that the transmembrane segments of Hrd1 and possibly also of other ERAD ligases are important elements involved in the selection and/or the export of membrane-bound proteins.

Extraction of certain ER proteins may rely on a highly specialized membrane-bound machinery. Expression of the HIV protein Vpu abolishes surface expression of the CD4 receptor. Vpu binds to newly synthesized CD4 in the ER and recruits the cytosolic ubiquitin ligase complex SCF-βTRCP, which polyubiquitylates CD4 and thereby initiates its dislocation from the ER and proteasomal degradation [68]. This process relies on p97 function but does not seem to require any of the previously discussed channel candidates [69]. Notably, the membrane protein Vpu has been shown to oligomerize and form ion-conducting channels, which may also partake in the export of CD4 from the ER [70].

6. ER proteins may be exported by lipid droplets

It is currently under debate whether a channel composed solely by proteins can adopt the very large pore size required for the export of ERAD substrates. Alternatively, removal of misfolded proteins from the ER could be accomplished by the formation of lipid droplets [71]. Lipid droplets are found in nearly all organisms and were primarily identified as lipid storage devices. They contain a core of neutral lipids, which are surrounded by a monolayer of phospholipids [72]. Lipid droplets bud and form an independent organelle upon the synthesis of neutral lipids between the leaflets of the ER membrane. Due to their high content of proteins like enzymes for cholesterol synthesis, Rab proteins and also chaperones it is assumed that lipid droplets are involved in diverse metabolic and trafficking pathways [73]. Recent studies have shown that lipid droplets partake in the degradation of the secretory protein ApoB [73,74]. During its turnover ubiquitylated ApoB accumulates around lipid droplets and is thereby exposed to the proteasomal and autophagic degradation system, respectively. Additionally, ER stress increases the formation of lipid droplets, which encourages the clearance of accumulating misfolded proteins from the ER [75]. It remains to be determined, whether clearance of

secretory proteins via lipid droplets constitutes a regular cellular degradation pathway or rather represents a specialized stress induced mechanism in certain cell types.

7. ATP-hydrolysis provides the energy for substrate re-translocation

7.1. Involvement of Hsp70-type chaperones

As determined in a reconstituted *in vitro* system, export of proteins from the ER relies on ATP hydrolysis [76]. This energy requirement may in part contribute to the unfolding of client proteins, which could precede their dislocation. Indeed, the ER luminal Hsp70-type chaperone BiP/Kar2p and its Hsp40-type cofactors Jem1 and Scj1 have been found in association with ERAD ligases where they supposedly prevent the aggregation of substrates [77]. It is still unclear, however, whether complete unfolding of all substrate molecules is a prerequisite for their transport into the cytosol [46,47]. Hsp70-type chaperones have also been shown to contribute to the selection and recruitment of substrates to the ERAD ligases. In analogy to organellar import systems, conformational changes by these proteins that are coupled to ATP hydrolysis may also directly contribute to the driving force for protein dislocation. Intriguingly, though, there is no indication for such a function of Hsp70s in ERAD [78].

7.2. Polyubiquitylation is required for protein export

Attachment of ubiquitin, a 76 residue polypeptide that is highly conserved in all eukaryotes, modulates protein function in numerous cellular processes like cell cycle progression, DNA repair, endocytosis and cell signalling [79,80]. By formation of an isopeptide bond this modifier is covalently linked via its carboxyterminus usually to lysine or an aminoterminal methionine residue within target proteins. Ubiquitin can also be conjugated to one of the seven lysines within other ubiquitin molecules that are already attached to substrates. Depending on the lysine used the resulting polyubiquitin chains display individual structural properties and fulfill different cellular functions [81]. Most prominently, lysine 48-linked (K48) polyubiquitin chains target proteins for proteasomal degradation.

Protein ubiquitylation depends on the sequential action of an enzymatic cascade. In a first step a cysteine residue within the catalytic active center of the ubiquitin-activating enzyme forms a thioester with the carboxyterminal glycine of ubiquitin. By a transesterification reaction ubiquiuitin is then delivered to a cysteine in the catalytic active centre of highly conserved ubiquitin-conjugating enzymes. The final attachment to client proteins relies in most cases on the activity of ubiquitin ligases, which also account for the specificity of this process. Thus, a particular combination of ubiquitin-conjugating enzymes and ubiquitin ligases usually targets a defined set of client proteins in a spatially and temporally tightly regulated manner. For ERAD, several pathways have evolved that are each defined by a specialized ubiquitin ligase. Yeast ERAD comprises two different ligase complexes for the degradation of secretory proteins with distinct topologies [32,54,82]. Mammals contain multiple ERAD pathways with partly overlapping functions [6]. Interestingly, the activity of certain ligases may be regulated by proteolysis but the reason for this is unknown [83–85].

Polyubiquitylation of ERAD substrates does not only label these polypeptides for proteasomal degradation but is also crucial for their dislocation from the ER. Expression of mutant ubiquitin that cannot form K48-linked chains blocks the export of ribophorin A, unassembled TCRα and of MHC I in the presence of viral US11 [86–88]. Yeast cells lacking the ubiquitin-conjugating enzymes Ubc1 and Ubc7 accumulate the ERAD substrate CPY* in a proteinase K resistant form at the ER [89]. The function of polyubiquitylation in the export step is not completely clarified. Most likely the attachment of multiple ubiquitin moieties serves as a handle for the association of ubiquitin-binding factors that facilitate the extraction of the polypeptides from the ER [90].

7.3. Involvement of proteasomes in dislocation

The 26S proteasome is a multi-enzyme protease that is built up by two large sub-complexes [91,92]. A 20S core particle (CP) consists of four heteroheptameric rings that are organized in a barrel shaped sructure. The inner pair of the rings is assembled by the β-subunits, three of which comprise peptidase activity, whereas the outer rings solely contain structural components termed the α-subunits. Inside this assembly resides a narrow cavity, which holds the proteolytically active domains of the protease. Since the cavity can only be accessed via narrow openings at either end of the barrel the peptidases are sealed from the environment. These entry sites are each gated by a 19S regulatory complex (RC) that binds to the CP. The RC is composed of a basal ring containing six AAA-ATPases and an associated structure termed the lid. Selectivity of proteasomal degradation is assured by substrate-recruiting factors within the lid, which preferentially bind K48-linked polyubiquitylated proteins [81]. Subsequently, ubiquitin is removed from these clients by deubiquitylating enzymes associated with the lid. Proteolytic processing of substrates depends on their unfolding by the AAA-ATPases of the RC-base, which also funnel the polypeptides into the cavity of the 20S core.

Perturbation of the proteasomal activity by the treatment with chemical drugs or the use of specific loss-of-function mutations generally results in the accumulation of polyubiquitylated ERAD substrates. These species are found in the cytosol or at the cytosolic side of the ER membrane demonstrating that they were exported from the ER. It is therefore assumed that the dislocation of most ERAD substrates occurs independently of proteasomal activity. Still there may be exceptions of the rule. The degradation of some membrane-bound proteins seems to be coupled to their extraction from the ER. Deg1-Sec62-ProtA is an artificial ERAD reporter that was constructed by the fusion of a short amino acid stretch conferring fast turnover to the yeast Mat-α factor with transmembrane segments derived from the ER protein Sec62 [93]. The resulting polypeptide is integrated into the ER membrane of yeast cells but rapidly degraded in a ubiquitin-proteasome dependent manner. Conditional mutants that cause a partial inhibition of the proteasome resulted in the accumulation of degradation intermediates, which contained the transmembrane segments of the substrate but lacked the soluble cytosolic domain. Proteasomal inhibition also leads to the accumulation of the transmembrane part of Ubc6, a naturally short-lived tail anchored ER protein [52]. These data led to the speculation that proteasomes are directly involved in the extraction of some proteins from the ER. The partial obstruction of proteasomal activity may primarily impair export and decomposition of the transmembrane regions of the substrate whereas proteolysis of the cytosolic parts is affected to a much lesser extent. Such a scenario is schematically displayed in Fig. 3B and C. Still, efficient turnover of at least Ubc6 depends on another cytosolic AAA-ATPase termed the Cdc48/Npl4/Ufd1 complex (see Section 7.4).

In vitro studies revealed a direct interaction of the Sec61 translocon with the 19S regulatory particle base in the ATP-bound conformation [94]. A mutant variant of Sec61α that displays defects in the degradation of ERAD substrates shows reduced affinity for the 19S base in this assay [95]. Interestingly, proteasomes compete with ribosomes for the interaction with the translocon, although either complex seems to bind to a different site at Sec61α. These results indicate that Sec61-bound proteasomes can directly extract proteins from the ER. Support for such a scenario comes from *in vitro* studies on the dislocation of yeast pre-pro α-factor. This substrate can be exported from isolated microsomes upon the addition of purified RC in an ATP dependent manner [96]. Subsequent apposition of 20S core

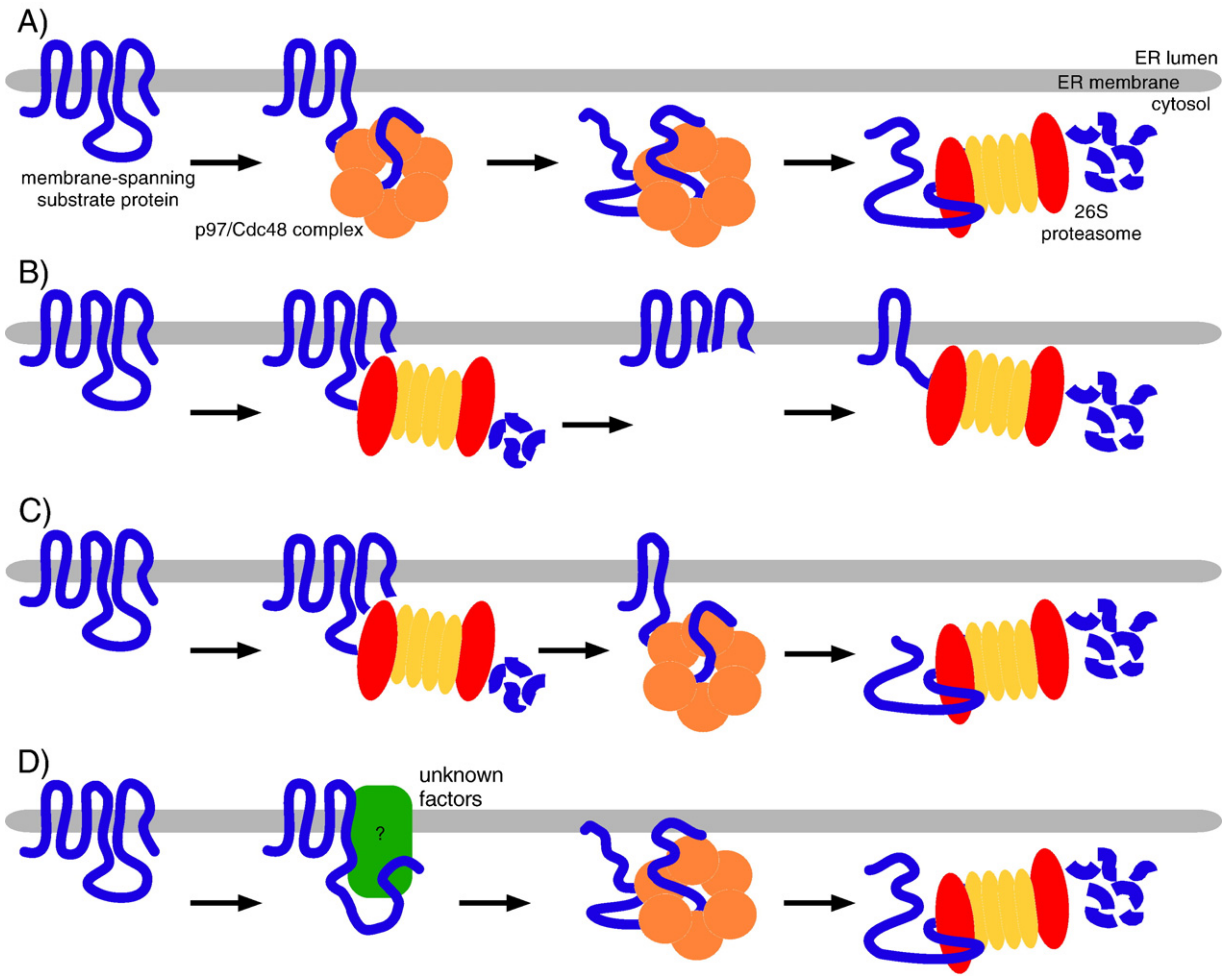

Fig. 3. Models on the extraction of ER proteins by the p97/Cdc48 complex and proteasomes. A) Polyubiquitylated proteins bind to p97/Cdc48 and are extracted from the ER upon conformational transitions within this AAA-ATPase induced by ATP hydrolysis. Subsequently, the substrates are delivered to the proteasomes where they are degraded. B) In some cases proteasomes may process membrane-bound polypeptides after their polyubiquitylation and also directly promote their extraction from the ER presumably by the activity of the AAA-ATPases within the 19 S regulatory particle. C) Membrane-bound proteins may also be encountered by proteasomes that remove loosely folded, cytosolically exposed regions. Extraction of the transmembrane segments may rely on the activity of p97/Cdc48, which then delivers these parts to proteasomes. D) Alternatively, dislocation of the substrates may be accomplished by a so far unknown machinery. The predominant function of p97/Cdc48 in this process may be the mobilization of already exported polypeptides from the cytosolic surface of the ER and their transport to proteasomes.

initiates degradation of pre-pro α-factor. However, ERAD of pre-pro α-factor is peculiar because polyubiquitylation appears to be dispensable for its degradation *in vivo*.

7.4. A cytosolic AAA-ATPase involved in substrate export

In the cytosol the ERAD ligases team up with a multimeric enzyme complex containing the AAA-ATPase p97/VCP (Cdc48 in yeast). p97/Cdc48 forms homohexameric assemblies that are involved in many cellular processes like homeotypic membrane fusion, cell cycle progression, transcription factor processing and the degradation of proteins by the ubiquitin-proteasome system [97,98]. Adaptation to these different functions is most likely mediated by a large number of interacting factors, which bind to p97/Cdc48 in an exclusive manner and modulate its activities towards specific client proteins. For its function in proteasomal protein degradation p97 associates with the ubiquitin-binding proteins Ufd1 and Npl4, the ubiquitin chain-elongation factor Ufd2, several deubiquitylating enzymes and other, less well characterized proteins. Each monomer of p97 contains two ATPase domains (D1 and D2) that catalyze ATP binding and hydrolysis. The so-called N-domain of p97 directly binds ubiquitin with a preference for multiubiquitin chains [99–101]. Structural analysis indicates a substantial re-arrangement within p97 upon nucleotide binding and ATP hydrolysis [102]. These transformations are thought to be conferred to bound substrates thereby loosening the structure of the client molecules [103].

The majority of p97/Cdc48 localizes to the cytosol and the nucleus [104,105]. Still, a significant amount of this protein is found at the ER and in association with the ERAD ligases. In yeast, the integral membrane protein Ubx2 recruits Cdc48 via its UBX domain to the Hrd1 and Doa10 ubiquitin ligases [106,107]. Remarkably, the association of Cdc48 to the yeast ERAD ligases also depends on their catalytic activity and may thus rely on the availability of substrates [30,106]. Mammals contain a functional homologue of Ubx2 termed erasin, which seems to be required for degradation of at least some ERAD substrates [108,109]. The gp78 ERAD ligase directly interacts with p97 by means of a discrete binding site at its very carboxy-terminus [110].

In cells defective for p97/Cdc48 function degradation of most ERAD substrates is impaired. Similarly, ERAD is abrogated in cells lacking functional Ufd1 or Npl4. Under these conditions ERAD substrates appear to have been at least partly dislocated into the cytosol as indicated by their polyubiquitylation and susceptibility to proteinase treatment but stay associated with the ER membrane

[89,111]. However, some polypeptides accumulate in a non-ubiquitylated form inside the ER under comparable conditions [112,113]. Thus, the p97/Cdc48 complex may serve varying purposes in the degradation of individual proteins: On the one hand it facilitates the export of at least some ERAD substrates from the ER, possibly by generating the driving force for their dislocation (Fig. 3A). The polyubiquitylation of substrates most likely provides handles for the association of the p97/Cdc48 complex here [90]. On the other hand, this complex may also promote the mobilization of already dislocated substrates from the ERAD ligases. Support for such a function arises from the analysis of the Cdc48 dependent activation of the yeast transcription factor Spt23 [100]. In its inactive form Spt23 forms a homodimer that is integrated into the ER membrane. Limited proteolytic processing by the proteasome removes the transmembrane segment of one subunit, thereby generating a 90 kDa protein that is still bound to the ER by its tight association with the full length partner. The Cdc48/Npl4/Ufd1 complex separates the heterodimer thereby liberating the cleaved polypeptide from the membrane and triggering Spt23 dependent transcription. Analogously, the Cdc48 complex may dissolve tight protein-protein interactions of ERAD substrates and promote their release from the membrane [114], (see Fig. 3D).

Finally, the activity of p97/Cdc48 is thought to prevent the aggregation of ERAD substrates in the cytosol and to transport them to the proteasome. In yeast, the U-box containing protein Ufd2 associates with p97/Cdc48 and extends the size of polyubiquitin chains on client proteins [115]. Cells lacking Ufd2 exhibit a defect in proteasomal degradation, possibly because the transport of substrates from the Cdc48 complex to the proteasome is disturbed. The activity of Ufd2 is counteracted by the deubiquitylating enzyme Otu1, which binds to Cdc48 and restricts the size of polyubiquitin chains on substrates [116]. This may adapt the chain length for the binding of downstream acting ubiquitin binding factors. A direct requirement of Otu1 for ERAD has not been shown, though. In mammalian cells the deubiquitylating enzyme YOD1 binds to the p97 complex [117]. Overexpression of catalytically inactive YOD1 blocks the degradation of the canonical ERAD substrates RI322, TCRα and α1-antitrypsin. Strikingly, glycosylated as well as deglycosylated forms of RI322 accumulate under these conditions, indicating that protein dislocation from the ER was at least partly compromised. The functional implications of protein deubiquitylation in ERAD remain unclear at this point.

8. A model for the dynamic organization of the dislocation apparatus

The vast diversity of ERAD substrates and their individual structural and topological properties necessitates a varying array of factors for their recognition, ubiquitylation and transport to the proteasomes. It is conceivable to speculate that also the dislocation of such molecules from the ER requires the dynamic assembly of multiple components that adapts these machineries for the processing of a broad range of clients. Mammals contain numerous ERAD ligases, which might reflect an adaptation for the processing of peculiar substrates. As it has been implicated from the work on the yeast Hrd1 ligase the transmembrane segments of these enzymes may contribute to the dislocation of client proteins. We may assume that an oligomerization of these domains forms a protein-conducting channel for the transport of substrates. The addition of ancillary proteins like the Derlins or Der1 in yeast could extend the channel's properties to handle substrates with particular features like folded ER luminal domains. Alternatively, the Derlins and Der1 may form pores on their own that are tightly associated with and controlled by the ERAD ligases. Gating of such conduits is most likely accomplished by substrate receptors like yeast Hrd3 or mammalian SEL1L, which expose large domains into the ER lumen. In addition, these channels may be regulated by the reversible assembly of membrane-bound ERAD components that will dissociate upon completion of transport. Thus, the dimension of the conduits could be dynamically adjusted to meet the size of the cargo and allow passage of partly folded and glycosylated polypeptides. Such a model matches with the observation that substrate dislocation is highly dependent on polyubiquitylation because it unites these processes in a single entity. Moreover, this organization of the dislocation apparatus places substrate selection in the ER lumen and the activity of the cytosolic p97/Cdc48 complex in close spatial proximity, which correlates to their tight functional connection. Yet, this model is only speculative at the moment and needs to be substantiated by experimental work.

Recent work implies that the import of proteins into peroxisomes involves a dynamically organized importomer. In isolated microsomes the peroxisomal import receptor Pex5 and its membrane-docking factor Pex14 can form variable pores with a diameter of up to 9 nm [118]. Such channels fit the notion that unlike other organelles peroxisomes are capable to import even preassembled protein complexes from the cytosol. Strikingly, also the recycling of the import receptor Pex5 into the cytosol displays mechanistic similarities to ERAD. Pex5 binds import substrates in the cytosol and delivers them to peroxisomes by insertion into the peroxisomal membrane. After cargo release Pex5 is ubiquitylated by the ubiquitin ligases Pex2 and Pex12 [119]. This modification is a prerequisite for the extraction from the peroxisomal membrane by the AAA-ATPases Pex1 and Pex6 [120,121]. Polyubiquitylated Pex5 is degraded by proteasomes whereas the monoubiquitylated form is recycled and may participate in another round of protein import. Thus, the extraction of Pex5 from peroxisomes parallels the current ideas on how the p97/Cdc48 complex dislocates ubiquitylated proteins from the ER membrane.

9. Concluding remarks

Intense research of the last two decades aimed to identify a general ERAD apparatus for the degradation of aberrant secretory proteins. Given the diversity of ERAD substrates it is of little surprise that these efforts resulted in the identification of multiple pathways that exhibit discrete yet overlapping specificity towards client proteins. While yeast gets along with two ubiquitin ligase complexes for the processing of most ERAD substrates the number in mammalian cells is considerably larger. The recruitment of ancillary factors further expands the functional properties of the ligases although we currently lack a complete inventory of the components involved in these individual ERAD routes. This variety may also result in mechanistic differences for dislocation step that tribute to structural peculiarities of some of the substrates. While the AAA-ATPase p97/Cdc48 appears to procure the driving force in most of these pathways, the nature of a dislocation channel remains obscure. A growing number of observations supports the idea that components of the ERAD ligases form highly dynamic and adaptive pores capable of transporting glycosylated and folded proteins through the ER membrane. Still, this assumption awaits proof. Because some mammalian ERAD ligases contain few or no transmembrane segments a putative dislocation channel must be formed by additional factors in these pathways. In the past studies on protein transport through biological membranes strongly benefited from potent *in vitro* systems that allowed the characterization of defined operational sequences. We are looking forward to see the development of such a powerful tool for the ERAD field, which will boost our knowledge on the mechanisms of this highly complex and dynamic process.

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