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Protein quality control in the ER: balancing the ubiquitin chequebook

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Abstract

Protein maturation in the endoplasmic reticulum (ER) is subject to stringent quality control. Terminally misfolded polypeptides are usually ejected into the cytoplasm and targeted for destruction by the proteasome. Ubiquitin conjugation is essential for both extraction and proteolysis. Here, we discuss the role of the ubiquitin conjugation machinery in this pathway and focus on the role of ubiquitin ligase complexes as gatekeepers for membrane passage. We then examine the type of ubiquitin modification applied to the misfolded ER protein and the role of de-ubiquitylating enzymes in the extraction of proteins from the ER.

Where proteins fold

Proteins destined for secretion from the cell or for the endocytic system originate by translation in the cytoplasm, from which they enter the endoplasmic reticulum (ER), typically cotranslationally. The nascent polypeptide enters the ER via the Sec61 translocon and – when the requisite signals are present and can be recognized – engages the glycosylation machinery. Nascent chains encounter chaperones that govern the folding process and allow the introduction of disulfide bonds. For protein complexes composed of multiple subunits, their proper association is an essential criterion for quality control and must not be jeopardized by aggregation. This is all the more remarkable when different subunits of a multi-protein complex are produced from the correspondingly distinct and individually translated mRNAs. Newly synthesized polypeptides thus attain their final conformation – autonomously or in complex with binding partners – while protected from aggregation within the crowded ER environment through transient association with components of the folding machinery (Figure 1for detailed review see [1]).

Nonetheless, protein folding in the ER is inherently imperfect and errors made at any step *en route* to the final product reduce the fraction of proteins that reach their proper conformation. For some proteins, like the cystic fibrosis chloride conductance regulator (CFTR), more than half of the newly synthesized polypeptide may not reach maturity [2]. Any significant accumulation of misfolded proteins inside the ER entails the risk of aggregation, and is likely to compromise ER function. Polypeptides that fail to meet ER quality control and cannot be rescued must be degraded. Indeed, the build-up of misfolded proteins that can occur in either professional secretory cells or in cells treated with

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compounds such as tunicamycin or dithiothreitol (DTT) evokes the unfolded protein response (UPR), a stereotypic transcriptional response that ultimately adjusts the composition – both lipid and protein – of the ER [3]. These changes include upregulation of folding chaperones and quality control machinery, a decrease in protein synthesis and, if the damage is deemed beyond repair, induced cell death (apoptosis). We know of no ER-resident proteases that can deal with the onslaught of terminally misfolded proteins inside the ER lumen of cells exposed to tunicamycin or DTT. Instead, the consensus view is that misfolded proteins are ejected into the cytoplasm – a step we shall refer to as dislocation – where they are targeted for ubiquitin-dependent degradation by the proteasome [4]. The steps that contribute to this means of protein elimination are collectively referred to as ER-associated degradation (ERAD). Although this aspect of ER quality control has received the most attention by far, not all misfolded proteins follow this route; proteins with only slight imperfections may still enter the secretory pathway and eventually be targeted to endolysosomal compartments for degradation, just like proteins that sustain damage at other intracellular locations are delivered to lysosomes to be cannibalized for salvaging of their building blocks. We shall discuss the nature of the misfolded polypeptide and the role of the ubiquitylation machinery in its elimination.

Tracking misfolded proteins

What exactly constitutes a misfolded protein? Structural alterations caused by amino acid replacements, truncations of the polypeptide chain, or non-native disulfide bonds, to name a few examples – while evidently causing alterations in covalent structure – are difficult to characterize in conformational terms. Even more problematic are structural changes that result from a failure to engage the necessary folding assistants without alteration to the covalent structure of the newly synthesized protein itself. None of these products can be obtained in quantities that allow an assessment of their conformation by standard physico-chemical means (crystallography, NMR, CD). Instead, surrogate measures are used to diagnose the misfolded state, such as the failure to enter the secretory pathway and lack of terminal carbohydrate modifications [1], the loss or acquisition of epitopes recognized by antibodies, altered susceptibility to protease digestion and loss of enzymatic or binding activity. There likely exists a continuum of folded and misfolded states, with the tipping point for diagnosis as seriously damaged and terminally misfolded being different for each protein. It has been surprisingly difficult to design mutant versions of endogenous proteins that show drastically altered kinetics of turnover and so serve as substrates to study ER quality control. As a result, relatively few substrates have been analyzed in detail. In addition, such substrates are commonly expressed at high levels in the setting of a transfection experiment. While overexpression allows easy detection of the misfolded product, it has the drawback that such substrates can saturate or even overwhelm the quality control machinery that is the object of study, likely inducing UPR-mediated remodeling of the ER [3].

Dislocation across the ER membrane barrier is proposed to take place through a protein-conducting channel, akin to translocation into the ER. In contrast to the canonical translocon, the composition of which is largely agreed upon [5], no singular or definitive complex has been assigned to perform this task in dislocation (Box 1). Involvement of lipid droplet formation [6] as well as autophagy [7] have been proposed as a possible means to relieve the ER of proteinaceous waste, but so far with little hard evidence in support. At least in yeast, visible lipid droplet formation was dispensable for dislocation of tested substrates [8]. This observation does not formally exclude a role for a mechanism akin to lipid droplet formation in dislocation, and the detection of components of the dislocation machinery (AUP1, UBE2G2) [9,10] on lipid droplets continues to fuel this hypothesis. Autophagy of an ER folding compartment overwhelmed with misfolded proteins (perchance

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UPR-dependent) remains an attractive means to clear misfolded proteins in bulk and deserves further investigation (Figure 1). The fidelity of the ER membrane as a barrier impermeant to proteins unless facilitated by the appropriate channels is perhaps too easily assumed in light of the dynamic nature of the ER and the abundance of fission and fusion events that take place there [11]; perforations, even temporary, of the ER membrane could allow escape of unwanted products.

Ubiquitylation is a general requirement for the dislocation of individual misfolded proteins. Attachment of a poly-ubiquitin chain triggers two important steps: first is the recruitment of the AAA (ATPase associated with a variety of cellular activities) ATPase p97 (VCP; Cdc48 in yeast), thought to provide the mechanical force to extract the misfolded polypeptide from the ER [12]; and, second, ubiquitylation flags the protein for targeting to the proteasome and thus its final demise [4]. The unity of function of poly-ubiquitylation has led to the proposal that dislocation and degradation are tightly coupled. This view requires modification, however; involvement of de-ubiquitylation enzymes (DUBs) in the dislocation reaction [13,14,15], demonstrates an uncoupling of dislocation from degradation [16]. The complexity of the mammalian ubiquitylation machinery, the build and type of ubiquitin linkages themselves, and the association of cytosolic chaperones with dislocation substrates [16], indicate that proteasomal degradation of misfolded ER proteins is more complex than previously considered.

Ubiquitylation drives dislocation

Where examined, the extraction of a misfolded ER glycoprotein requires its ubiquitylation. The poly-ubiquitin chain serves as a recognition handle for p97 through its cofactors UFD1 and NPL4, and recruits it to drive dislocation [12]. Accordingly, obstruction of ubiquitylation causes the misfolded protein to accumulate inside the lumen of the ER. Whether or not the requirement for poly-ubiquitylation applies universally, or whether in select cases even a single ubiquitin (or multiple single ubiquitins) would suffice to engage the dislocation machinery remains to be clarified. Recruitment of substrates to the proteasome is believed to require a minimum of 4 ubiquitin units for a single chain to result in productive engagement of the 19S cap [4], but whether this $(Ub)_4$ rule applies generally is, again, not known. Turnover of at least Pax3 has been reported to rely on only a single ubiquitin moiety [17].

Ubiquitylation of proteins takes place via an E1-E2-E3 cascade [18]. In mammalian cells, two E1s have been identified, about 40 E2s (ubiquitin conjugating enzymes) and possibly as many as 1000 E3s (ubiquitin ligases), although for the vast majority of E3s their enzymatic activity remains to be verified experimentally. Notwithstanding striking homologies, their function as enzymatically active E3s may not simply be assumed, as proteins with near-identical folds may serve very different functions. Nonetheless, this hierarchy would obviously allow a great deal of specificity. Variables that control the operation of the ubiquitin system include the identity of the substrate and the E3 ligase that modifies it, the amino acid to which ubiquitin is conjugated, and the type of oligo- or poly-ubiquitin linkage made. The possibility of mono-, multi-, or poly-ubiquitylation adds yet further complexity. The fact that certain proteins can be ubiquitylated at multiple distinct acceptor residues implies that different E3-type activities target these substrates, or that a single E3 activity is capable of sequential and/or processive engagement of one and the same substrate. The relevance of this point will become clear when we discuss sequential rounds of ubiquitin addition and removal as a requirement for destruction of misfolded ER proteins.

In the case of poly-ubiquitylation, there is the variety in linkage to consider. Ubiquitin offers seven lysine residues to which a following ubiquitin molecule can be attached. Individual

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linkages are named for the position of the accepting lysine residue (e.g. K48 for attachment of ubiquitin to the lysine at position 48). Each type of linkage results in a characteristic and separate spatial structure. Ubiquitin is conjugated via its C-terminal di-glycine motif in thio-ester bond to the relevant E2 and is then transferred to an acceptor residue in the substrate polypeptide. Lysine is the preferred, but by no means the only, ubiquitin acceptor; ubiquitin conjugation to cysteine, threonine, serine or a protein's N-terminus has been reported [19]. The genesis of the various types of poly-ubiquitin chains has been extensively reviewed [19].

A physical interaction between the dislocation substrate in the ER and the E3 of choice must occur to enable ubiquitin conjugation, as we discussed for the Hrd1p ligase in yeast (see Box 1) [20,21]. How a soluble ER protein crosses the membrane barrier to reach the enzymatic domain of the E3 ligase is unclear. The polypeptide would at least have to protrude partially from a putative dislocon to allow ubiquitylation. Therefore transfer of a part of the polypeptide to be destroyed must already have progressed to the point where a residue suitable for ubiquitylation is exposed and accessible to the E3. The force needed to extract a polypeptide from the ER is provided by cytosolic p97, which engages the substrate only after poly-ubiquitin attachment. In much the same way as yeast Kar2p (yeast BiP ortholog) ratchets proteins into the ER in the course of translation, a similar mechanism might be employed by p97 for extraction of proteins from the ER in dislocation. If we extend this parallel, there might even exist the need for a force that pushes the protein out of the ER, akin to the translating ribosome for protein import. If indeed there were such an ER-resident first mover, its identity is yet to be discovered.

Substrate ubiquitylation is more easily understood for proteins that span the ER membrane and so may provide a naturally exposed handle for ubiquitylation. For some substrates, cytosolic – not membrane-bound – E3 ligases participate in their removal; the Hsc70/CHIP E3 ligase complex is recruited to the ER to target CFTRΔF508 for degradation [22] and the HIV encoded protein Vpu recruits the cytosolic ligase complex SCFbeta-TrCP to target CD4 for dislocation and proteasomal degradation [23]. The number of mammalian E3 ligases implicated in ER quality control continues to expand, including both integral membrane as well as cytosolic E3 ligases (Tables 1 and 2). Propelled by studies of yeast Hrd1p, most of the current mechanistic information has been acquired for its mammalian ortholog HRD1, which may or may not represent all avenues open to dislocation.

It is common practice to test the involvement of any particular E3 ligase in turnover of known dislocation substrates and thus implicate the ligase in ER quality control. These experiments rely on the limited set of often artificial substrates that the E3 can be tested against. These tools provide a skewed view of substrate turnover at endogenous protein expression levels, as overexpression of the artificial substrate can modulate the landscape of the ER through activation of the UPR. Examples that approach more physiological levels of turnover include endogenous Class I MHC heavy chains [24,25], the turnover of immunoglobulin subunits [26] or degradation of proteins that undergo extensive processing in the ER such as the Hedgehog protein, which matures by autocatalytic cleavage, separating into an N-terminal signaling molecule and a C-terminal fragment that undergoes dislocation [27].

Connecting a particular E3 ligase complex with the turnover of specific ER-resident proteins tackles only one type of variability of ubiquitin-conjugation. Ubiquitin-conjugation to amino acid side chains other than lysine has been described for ER dislocation-induced ubiquitylation of TCRα [28], Class I MHC [29], as well as the NS-1 non-secreted immunoglobulin light chain [26]. Degradation of these substrates required the HRD1 ligase for ubiquitin-conjugation to serine, threonine and lysine residues. One can argue whether the

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identity of the acceptor amino acid plays a specific role, such as conferring susceptibility to hydrolysis of the linkage produced (amide versus thioester or hydroxy ester) or whether this is simply determined by its accessibility, caused by partial unfolding of the substrate protein. Attachment of ubiquitin may further modify the physical properties of the substrate [30], and help start it to unfold, which in return could free up preferred acceptor amino acids, if any.

Ubiquitylation is required for the dislocation reaction, but the exact role of ubiquitylation in this context remains ill-defined. Does it serve as a direct handle for extraction of the misfolded protein? Does it enable the recruitment of additional factors that continue and complete the dislocation reaction after its initial engagement? It is not clear whether p97 recruitment depends on mono-, poly- or multi-ubiquitylation of a substrate, nor do we know whether there is a single or several preferred ubiquitin-linkages. A possible complicating factor in interpretation of such experiments is the application of proteasome inhibitors, commonly used to visualize the presence of poly-ubiquitin adducts. While it is likely that poly-ubiquitin chains are present also in cells not treated with proteasome inhibitors, neither the extent nor dynamics of poly-ubiquitylation are immediately obvious in the untreated control setting. A K48-linked poly-ubiquitin tag was initially thought to determine proteasomal targeting, but a more complex picture emerged with the demonstrated involvement of all lysine linked ubiquitin-linkages in proteasomal targeting [31,32]. There may be a prominent role for K11-linked ubiquitin in ER quality control [31]. Furthermore, there is no evidence that poly-ubiquitin chains *in vivo* are homogeneous in linkage, leaving open the possibility of heterogeneity within a single or between multiple chains conjugated to a given substrate, thus rephrasing the question as linkage-dominant instead of linkage-specific.

Ubiquitin-linkage specificity is largely determined by the E2 enzyme; it was recently demonstrated how the E2 UBE2S specifically builds K11-linked ubiquitin chains [33]. Mammalian E2s currently implicated in dislocation, such as UBE2J1, UBE2J2 and UBE2G2 remain to be examined from the perspective of linkage type and the E3s they serve. Monoclonal antibodies that recognize specific ubiquitin linkages will be of considerable help [34,35]. The *in vivo* pairing of E2s with E3s remains enigmatic, and thus resolving the extent to which the promiscuity for E2–E3 pairings observed *in vitro* translates to the *in vivo* situation is a technical challenge.

Substrate extraction and deubiquitylation

Modification of a substrate with ubiquitin can recruit either of two multiprotein complexes that can extract the protein from the ER: p97 with its associated co-factors [12], and the 19S cap of the proteasome [36]. Although different in composition, the core of each complex consists of a ring-shaped, hexameric ATPase of the AAA family that can unfold polypeptides at the expense of ATP hydrolysis [4,12] (Figure2).

Two different types of ubiquitin receptors allow substrate engagement by the proteasome, either integrated into the structure of the proteasome itself, such as Rpn10 and Rpn13, or in the form of shuttling factors such as Rad23, Dsk2 and Ddi1 (in yeast) [4]. Upon engagement, a de-ubiquitylating activity (Rpn11) associated with the cap of the proteasome removes ubiquitin from the substrate. This allows recycling of ubiquitin and ensures compliance with size constraints of the access portal to the proteasome, which can accommodate looped polypeptides but not those with a conjugated complex ubiquitin ensemble.

The 19S cap has been reported to associate with the Sec61 translocon. Moreover, the purified cap complex supports dislocation *in vitro* [36, 37, 38]. The proposed structure of the

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Sec61 translocon [39] includes a narrow pore that can accommodate only unstructured polypeptides (the folding status of dislocated proteins is discussed in Box 3), and therefore the functional relevance of this physical link requires further experimental support. Whereas one can see how the short alpha-helical 'plug' [39] in the Sec61 pore is displaced from the ER luminal side of the translocon upon protein translocation, it would presumably occlude the pore upon reverse passage. More appealing is the proposal that the proteasome can directly engage membrane proteins tagged with poly-ubiquitin[40], as has been suggested for turnover of Ubc6 in yeast. When proteasome function was selectively impaired at one of the chymotryptic sites (pre1-1), a distinct breakdown intermediate remained associated with the ER membrane, thought to represent the trans-membrane domain of Ubc6 severed from its digested cytosolic domain [41].

p97 nucleates distinct multiprotein complexes implicated in diverse functions in the cell, ranging from dislocation and proteasomal targeting to cell cycle control and vesicular trafficking [12]. Adaptor proteins engage p97 and can either recruit or adapt it to a specific function. p97 engages ubiquitylated proteins via a dimeric adaptor consisting of NPL4 and UFD1. This dimer can engage ubiquitin and associates with the N-terminal domain of p97. Membrane-anchored auxiliaries such as VIMP [42], UBXD2 [43,44] and UBXD8 [45] may recruit this dislocation-competent complex to the ER membrane via their p97-interacting UBX-domain. p97 can directly interact with the ligase as shown for gp78 [40]. A hierarchy in p97 cofactors was described, such that FAF1 and UBXD7 only bind to p97 when in complex with the NPL4/UFD1 dimer, but neither engages p97 alone [44]. Of note, a p97-driven dislocation reaction has been described independently of NPL4/UFD1 [46]. It will be important to determine whether a binding hierarchy exists for the p97 complex implicated in dislocation, as this can provide insight into the timing of the dislocation reaction (Table 3 lists p97 cofactors involved in dislocation). Modulation of p97 co-factors can be regulated by phosphorylation or acetylation of p97 itself [47,48].

Akin to the proteasome, the hexameric pore of p97 can accommodate a polypeptide modified with ubiquitin [49]. There is some debate as to how p97 engages the polypeptide. One model suggests the protein enters via the D1 ring, and is threaded through the structure to exit via the D2 ring (Figure 2). This is contrasted with models where the polypeptide loops into the D2 ring alone, or where the hexamer could even dissociate to release the substrate [12]. Either model suggests similar space constraints, which is most relevant for the current discussion. It is unlikely that p97 can engage a protein bearing a poly-ubiquitin chain, exactly the type of chain that is thought to facilitate transfer to the proteasome with the help of shuttling factors. Indeed, DUBs participate in the mammalian dislocation reaction, in agreement with their function at the 19S cap of the proteasome. Expression of a catalytically inactive form of the ER-membrane anchored USP19 hampered dislocation of several substrates [14]. Also, the de-ubiquitylating enzyme Ataxin 3 associates with p97 and expression of a catalytically inactive mutant causes accumulation of poly-ubiquitylated substrates in association with p97 [15]. In a rather unconventional proposal, such poly-ubiquitin chains were deemed shielded from Ataxin 3 engagement by the p97 adaptor protein SAKS1, which could thus negatively regulate dislocation [50]. Furthermore, the de-ubiquitylating enzyme YOD1 is recruited to p97 via its UBX domain. Tampering with its function results in a near-complete blockade of dislocation [13]. Interference with dislocation/p97-associated de-ubiquitylating activity causes accumulation of misfolded proteins at a step prior to membrane extraction [13]. If de-ubiquitylating activity indeed were required to complete p97-catalyzed extraction, then such a blockade should be overcome by expression of a de-ubiquitylating enzyme that can engage these stalled poly-ubiquitinated dislocation intermediates. Exactly this was shown by co-expression of the catalytic domain of the Epstein Barr Virus large tegument protein BPFL1 (EBV DUB). Completion of the dislocation reaction thus relies on a de-ubiquitylating activity [16].

As proteasomal targeting requires ubiquitylation, it follows that substrates that undergo p97-mediated extraction, once de-ubiquitylated, require a second round of ubiquitin modification. Do p97-associated DUBs merely trim ubiquitin-chains or do they remove them completely? Polypeptides modified with mono-ubiquitin might pass through the central pore of p97 and then engage the proteasome directly, or do so after ubiquitin chain extension by either an E-3-mediated ligase reaction or via an E4-like activity (enzymes thought to engage and extend existing ubiquitin conjugates). The E4 Ufd2 has been linked to dislocation via Cdc48 in yeast [51]. In fact, soluble (dislocated) Ste6(p)* was observed in a system deprived of Ufd2 [52], where Ufd2 was suggested to increase the level of poly-ubiquitylation and thus facilitate proteasomal turnover, a suggestion that corroborates the model described above (Figure 2).

De-ubiquitylating activity at p97 opens the exciting possibility to modify the type of ubiquitin-linkage utilized, where one type of ubiquitin-build could induce extraction, followed by a switch to target the polypeptide to the proteasome.

Soluble in the cytosol

How the cell avoids aggregation of (partly) unfolded ER glycoproteins discharged into the cytosol is poorly understood. Hydrophobic protein domains find themselves exposed to an aqueous environment upon escape from the ER. As an example, when proteasomal proteolysis is blocked, Class I MHC products with their transmembrane segment fully intact occur as soluble intermediates in the cytoplasm of cells that express viral immunoevasins [24]. The cytoplasm houses an extensive chaperone network involved in the quality control of cytosolic proteins, with Hsp70 and Hsp90 its most famous family members [53]. Both have been proposed to triage the folding of complex membrane proteins, but there is limited direct evidence that ties them to quality control/dislocation of lumenal ER proteins.

One can visualize dislocated ER proteins in the cytosol by blocking proteasomal degradation, either via chemical (as just discussed for Class I MHC) or enzymatic means [16]. Expression of the catalytic domain of the large tegument-embedded ubiquitin-specific protease domain taken from Epstein-Barr virus, EBV-DUB, impairs proteasomal degradation by preemptively removing the ubiquitin-tag from substrates. In cells that express this EBV-DUB, dislocation of ER proteins continues, albeit at a reduced rate (as expected, if ubiquitylation is a prerequisite for the first step(s) in the dislocation pathway) and the misfolded ER-derived glycoprotein accumulates as a deglycosylated product in association with the cytosolic chaperone BAG6 (BAT3) [16]. In addition, an interaction with the TRICC/CCT complex was detected. BAG6 shuttles defective translation products for degradation to the proteasome [54,55], tying it to unfolded proteins. BAG6 does not merely associate with dislocation substrates when degradation is blocked, but is required to complete the dislocation reaction itself [56].

Engagement by chaperones, combined with a de-ubiquitylation step as an ER-resident protein exits from the ER, opens a window where substrates could deviate from the path to the proteasome. Such escape from proteolysis and the possibility of chaperone-mediated refolding could explain a set of observations where proteins (such as cholera toxin), formerly localized to the ER, escape to the cytoplasm and acquire their active conformation. Bacterial toxins may utilize dislocation machinery to reach the cytosol, be released from cytosolic chaperones – if they interact with them at all – and be allowed to reach their target for covalent modification (ADP-ribosylation, proteolysis, etc.).

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Concluding remarks

Misfolded ER proteins are modified with ubiquitin, first, to complete their extraction and second, to mark them for destruction. The need for de-ubiquitylation ezymes manifests itself in p97-mediated extraction, and immediately prior to degradation by the proteasome, presumably to allow the unfolded polypeptide to access the pore of p97 or the proteasome. A clear understanding of this process will depend on detailed knowledge of the nature of the ubiquitin chains as they are constructed or trimmed (see Box3 for outstanding questions).

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Box 1: Identity of a dislocon

To achieve delivery to the proteasome, misfolded ER glycoproteins must be discharged across the ER membrane into the cytoplasm. This passage likely requires a proteinaceous channel, and several promising 'dislocon' candidates have been proposed.

Sec61: The first candidate is the translocon itself. This dual function was proposed on the basis of the rapid dislocation of Class I MHC products by the immuno-evasin US2, and the occurrence of a diagnostic deglycosylated dislocation intermediate in association with Sec61β [57]. Sec61 has since been found to engage in complex formation with different members of the dislocation machinery [58].

Derlins: In mammals, the family of Derlin proteins (whose name derives from its founding member, the yeast Der1p protein involved in degradation of misfolded CPY* [59]) consists of three members (Derlin1–3) that have all been implicated in ER quality control [40]. They bear no obvious functional domains, carry multiple transmembrane domains and form both homo-and heterodimers, giving rise to the hypothesis that they could form (part of) a protein-conducting channel.

Ubiquitin ligases: A sizeable group of E3 ubiquitin ligases is directly anchored to, or associates with, the ER membrane and a growing number are implicated in ER quality control (Table 2). Sizeable proteins, they often include multiple transmembrane domains that do not obviously contribute to their enzymatic function and are more likely important for their intracellular positioning. The strongest case for ubiquitin ligase-mediated transport has been made for Hrd1p in yeast, which forms oligomers with the aid of Usa1 [60], associates with Der1p [60], and has been site-specifically photo-crosslinked to a dislocation substrate at residues in the Hrd1p transmembrane domains [20,21].

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Box2: Folding status of dislocation substrates

Translocation of proteins, as well as their threading into the central channel of the proteasome, presumably requires complete unfolding of the polypeptide chain. Whether this is also required for a misfolded protein to pass the ER membrane in the course of dislocation is still unclear. A substrate-GFP fusion protein shows no obvious loss of fluorescence while the protein undergoes dislocation [61], although this observation could be attributed to the β-barrel of GFP snapping back into shape once it reaches the cytosol. Also, DHFR-substrate fusion proteins are readily dislocated even in the presence of cell-permeable analogs of methotrexate that stabilize the DHFR moiety [62], albeit with slower kinetics [63]. These observations, while by no means conclusive, raise the possibility of a dislocon that can accommodate partially folded proteins, a suggestion not easily reconciled with the structures proposed for the Sec61 channel [39]. It is important to keep in mind that multi-domain proteins deemed misfolded in quality control may still have acquired fully folded domains. Furthermore, no protein with unfoldase activity (e.g. p97, 19S cap) has been detected on the luminal side of the ER, although a candidate in the form of the AAA ATPase TorsinA has been implicated in ER quality control [64]. The folding status of a protein undergoing dislocation across the ER membrane will remain a thorny issue until the identity of (a) putative channel(s) has been firmly established.

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Box3: Outstanding questions

- Identity of dislocation channel(s): exclusively proteinaceous? Membrane discontinuities?
- Which E2-E3 pairs are formed to facilitate dislocation and how promiscuous is such pairing (redundancy)?
- Does dislocation require a preferred type of ubiquitin-linkage?
- What is the folding status of misfolded ER proteins in the course of dislocation?
- What is the role of cytosolic chaperones in dislocation, and which chaperones are involved?
- Are there ER sub-compartments set aside for co-translational folding and modification reactions, physically and compositionally distinct from areas where quality control and dislocation occur?

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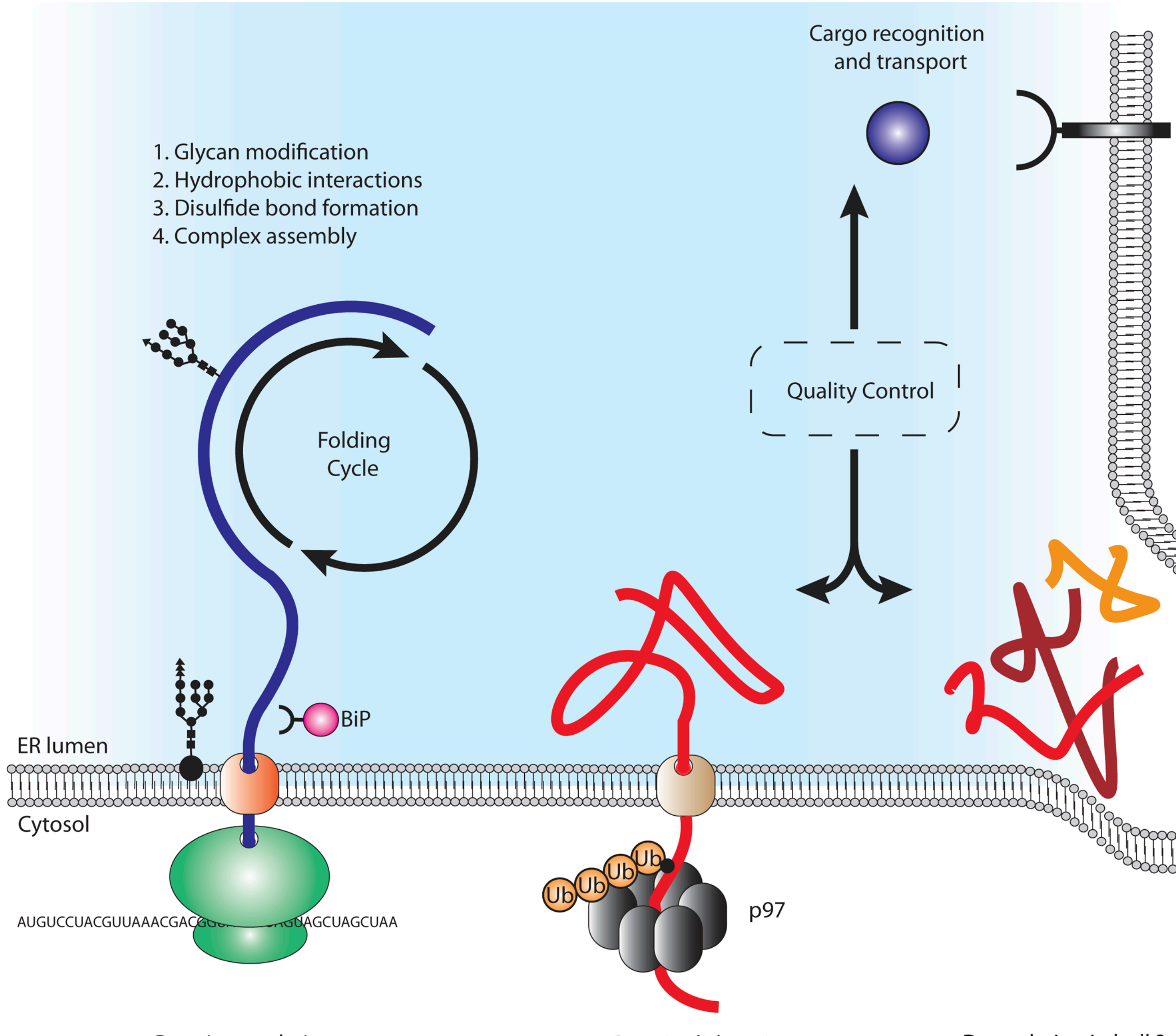

Figure 1. Protein folding in the ER

Schematic overview of a nascent polypeptide entering the ER lumen co-translationally, where it engages folding machinery to obtain its final conformation (folding cycle). Quality control check-point(s) establish the folding status of the poly-peptide which then either proceeds to its final destination or is selectively degraded, either via the dislocation pathway or via a bulk degradation mechanism (e.g. autophagy or lipid droplet formation).

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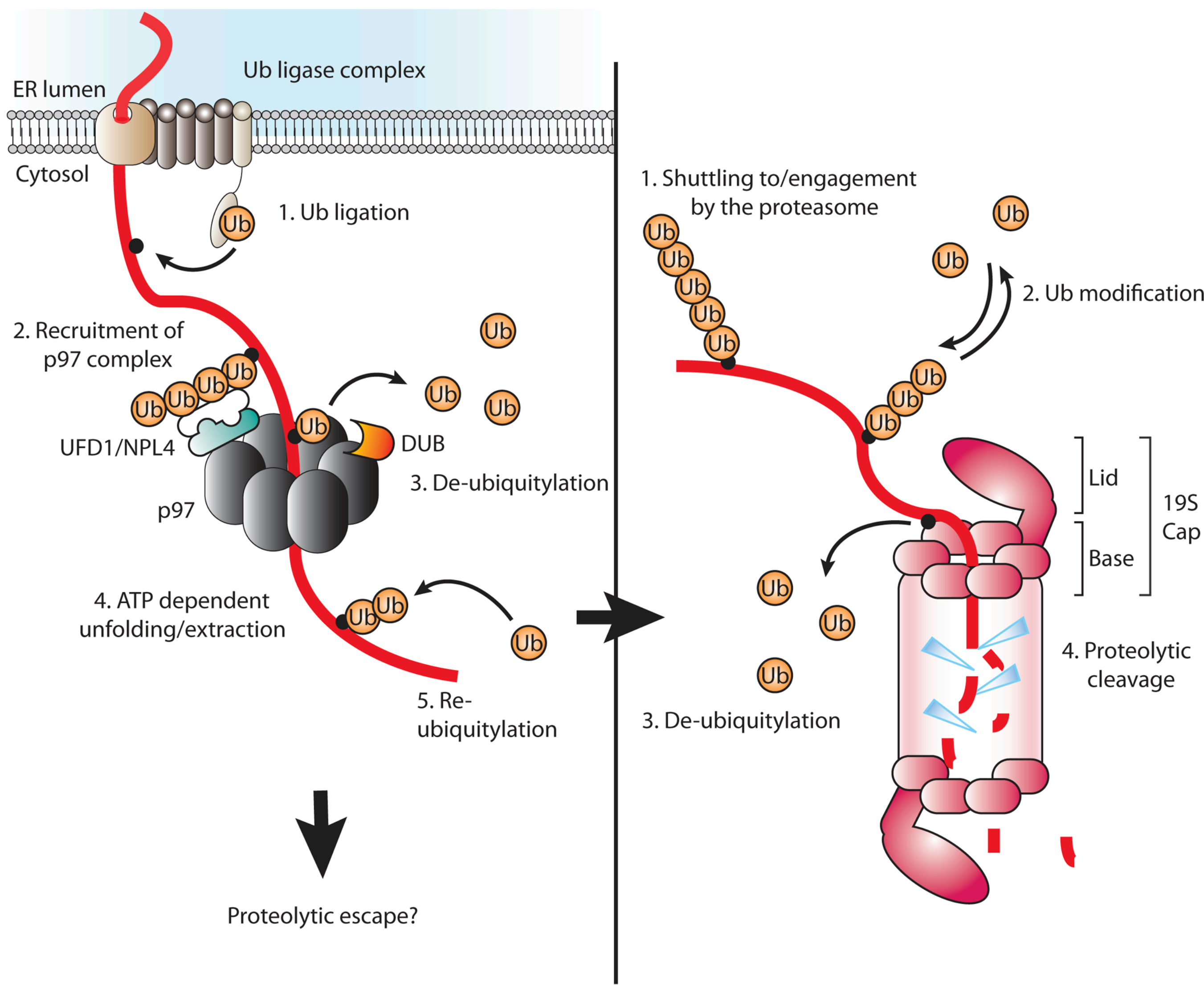

Figure 2. Protein dislocation and/or degradation

Left panel: A proposed model of a dislocated protein that is ubiquitylated at the ER membrane and consequently engaged by p97 via NFD1/NPL4. A de-ubiquitylating enzyme cleaves ubiquitin to allow threading of the polypeptide through the central pore of p97. A hypothesized re-ubiquitylation step post-p97 then facilitates proteasomal targeting. Right panel: A poly-ubiquitin tag targets the protein for proteasomal degradation. The poly-ubiquitin chain is probably modified before it is finally removed to allow threading of the polypeptide through the central pore of the base of the 19S cap and into the proteolytic chamber of the 20S proteasome core particle.

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Table 1

Mammalian and yeast ubiquitin conjugating enzymes and interacting ubiquitin ligases involved in ER dislocation

UBC (ubiquitin conjugating domain); UBA (ubiquitin-associated domain). The ER membrane is represented by a shaded bar with the ER lumen in the upper right corner.

Mammalian ubiquitin conjugating enzymes	**Topology/Functional Domains**	**Interacting mammalian ubiquitin ligases**
UBE2J1 (UBC6E, NCUBE1) [65]	C UBC N	HRD1 [45] RMA1 [22]
UBE2J2 (UBC6, NCUBE2) [65]	C UBC N	Parkin [66]
UBE2G2 (UBC7) [65]	UBC	Gp78 [65] HRD1 [67] Parkin [65]
Yeast ubiquitin conjugating enzymes		**Interacting yeast ubiquitin ligases**
Ubc1 [65]	UBC UBA	Hrd1p [65]
Ubc6 [65]	C UBC N	Doa10 [65]
Ubc7 [65]	UBC	Hrd1p [65] Doa10 [65]

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Table 2

Mammalian and yeast ubiquitin ligases involved in ER dislocation and corresponding in vivo substrates

The ER membrane is represented by a shaded bar with the ER lumen in the upper right corner. Except for TEB4/Doa10, the indicated membrane topologies of polytopic ubiquitin ligases are predicted based on the sequence but not experimentally confirmed. Polytopic membrane substrates are in blue, single-pass membrane substrates are in green, ER luminal substrates are in red, and tail-anchored substrates are indicated in brown color. RING (really interesting new gene); PA (protease-associated domain); TPR (tetratricopeptide repeat domain); IBR (in-between RING domain); FBA (F-box-associated domain); HECT (homologous to the E6-AP carboxyl terminus).

Mammalian ER membrane-anchored ubiquitin ligases	Topology/Functional Domains	Corresponding *in vivo* dislocation substrates

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HRD1 (Synoviolin) [67]	N RING-H2 C	T-cell receptor subunits TCR-α and CD3-δ [67] HMG CoA reductase [67] null Hong Kong variant of apha-1-antitrypsin (premature stop codon) [68] Z variant of alpha-1-antitrypsin (E342K mutation) [69] Ig κ light chain [70] Ig μ heavy chain [70] Class I MHC heavy chain [45,70] Ribophorin$_{332}$ (deletion of transmembrane domain) [70] Hedgehog precursor and C-terminal fragment [71] Neuroserpin mutants (various point mutants) [72] Gp78 [73] Pael (Parkin-associated endothelin receptor-like) receptor [65]
Gp78 (autocrine motility factor receptor, AMFR, RNF45) [65]	N RING Cue C	T-cell receptor subunits TCR-α and CD3-δ [65] Z variant of alpha-1-antitrypsin (E342K mutation) [65] CFTR mutant (F508 deletion in cytosolic domain) [74] Apolipoprotein B100 [65] HMG-CoA reductase [65] Insig-1 [65] KAI1 [70] Cytochrome P450 3A [75] Neuroserpin mutants (various point

		mutants) [72]
RMA1 (RNF5) [70]		CFTR and mutant CFTR (F508 deletion in cytosolic domain) [22]
TEB4 (MARCH-VI) [65,76]		Type 2 iodothyronine deiodinase [77]
RFP2 (Leu5, Trim13) [78]		CD3-δ [78] Cav1.2 channels [79]
TRC8 (translocation in renal carcinoma, chromosome 8 gene, RNF139) [80]		Class I MHC (cytomegalovirus US2-mediated) [80] Insig-1 [81] Sterol regulatory element binding protein precursor [81]
Kf-1 (RNF103) [82]		None
RNF170 [83]		Inositol 1,4,5-trisphosphate (IP_3)[3] receptor [83]

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Nixin/ZNRF4 [84]	N PA RING C	Calnexin [84]
Mammalian cytosolic ubiquitin ligases		
CHIP (C-teminus of Hsc70 interacting protein) [65]	TPR U-box	CFTR and mutant CFTR (F508 deletion in cytosolic domain) [22] Cytochrome P450 3A [75]
Parkin [65]	Ub-like domain RING IBR RING	Pael (Parkin-associated endothelin receptor-like) receptor [66] Glucocerebrosidase mutants (various point mutations) [85]
SCF$^{\beta\text{-TrCP}}$ [23]	F-box 7x WD40 β-TrCP F-box protein Skp1 cullin RBX SCF complex	CD4 (HIV-1 Vpu-mediated) [23] Tetherin (HIV-1 Vpu-mediated) [86]
SCF$^{\text{Fbs1}}$ (NFB42, Fbx2) [70]	F-box FBA Fbs1	Pre-integrin β1 [87] CFTR mutant (F508 deletion in cytosolic domain) [87] TCR-α [87] **N**-methyl-D-aspartate-type glutamate receptor NR1 subunit [88] β-secretase (BACE1) [89]

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SCF$^{\mathbf{Fbs2}}$ (Fbx6b, FBG2) [70]	F-box FBA Fbs2	TCR-α [90]
Smurf1 (Smad ubiquitination regulatory factor 1) [91]	C2 WW1 WW2 HECT	Wolfram syndrome protein [91]
Nrdp1 (FLRF) [92]	RING Zinc fingers coiled coil domain	ErbB3 [92]
Yeast ubiquitin ligases		
Hrd1p [65]	N RING-H2 C	Carboxypeptidase Y mutant (CPY*) (G255R mutation) [65] Sec61-2p [65] Pdr5 mutant (Pdr5*) (C1427Y mutation) [65] Hmg2p [65] Proteinase A mutant (PrA*) (glycosylation and deletion mutants) [93] Epithelial sodium channel (EnaC) α-, β-, γ-subunits [94] Sterol regulatory element-binding protein precursor [95]

Doa10 [65,76]	C RING N	Ubc6 [96] Ste6–166 mutant (Ste*) (premature stop codon) [97] Pma1 mutant (D378N) [98] Mps2-1 mutant [99] Epithelial sodium channel (EnaC) α-, β-, γ-subunits [94]

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Table 3

p97 co-factors involved in mammalian dislocation

Protein	Function	Interaction Domain
UFD1/NPL4 [12]	Facilitates engagement of poly-ubiquitin proteins	UBX
UBXD2 (Erasin) [43]	p97 recruitment	UBX
UBXD8 [45]	p97 recruitment	UBX
YOD1 [13]	De-ubiquitylating activity at p97	UBX
VIMP [42]	Recruits p97 to the ER membrane	Unknown
Peptide: N-glycosidase [100]	Enzymatic removal of N-linked glycans	PUB
Ataxin3 [15]	De-ubiquitylating activity at p97	VCP binding motif (VBM)
SAKS1 [50]	Mediates ubiquitin interactions	UBX