Mechanisms of substrate processing during ER-associated degradation

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Abstract

Maintaining proteome integrity is essential for long-term viability of all organisms and is overseen by intrinsic quality control mechanisms. The secretory pathway of eukaryotes poses a challenge for such quality assurance as proteins destined for secretion enter the endoplasmic reticulum (ER) and become spatially segregated from the cytosolic machinery responsible for disposal of aberrant (misfolded or otherwise damaged) or superfluous polypeptides. The elegant solution provided by evolution is ER membrane-bound ubiquitylation machinery that recognizes misfolded or surplus proteins, or by-products of protein biosynthesis in the ER and delivers them to 26S proteasomes for degradation. Endoplasmic reticulum-associated degradation (ERAD) collectively describes this specialized arm of protein quality control via the ubiquitin-proteasome system (UPS). But rather than providing a single strategy for removing defective or unwanted proteins, ERAD represents a collection of independent processes exhibiting distinct yet overlapping selectivity for wide range of substrates. Not surprisingly, ER membrane-embedded ubiquitin ligases (ER-E3s) act as central

hubs for each of these separate ERAD disposal routes. In these processes, ER-E3s cooperate with a plethora of specialized factors, coordinating recognition, transport, and ubiquitylation of undesirable secretory, membrane, and cytoplasmic proteins. In this Review, we focus on substrate processing during ERAD, highlighting common threads as well as differences between the many routes via ERAD.

[H1] Introduction

Through an assortment of quality control (QC) systems, evolution has equipped cells with a robust toolbox to maintain, adapt and restore the proteome – promoting polypeptide maturation, sensing proteotoxic dangers, facilitating remediation of malfolded forms, and expediting destruction of expendable or unwanted forms. One of the most intricate and important QC systems governs polypeptide maturation within the confines of the eukaryotic secretory pathway, which oversees ~ 30% of all proteins produced in cells [1].

As the entry point for this pathway, the endoplasmic reticulum (ER) is where folding and oligomerization of most secreted and integral membrane proteins takes place to yield mature forms [2,3]. Eliminating any undesired proteins from the ER requires membrane boundaries to be overcome. Non-membranous, secretory substrates with their accompanying secondary structure and post-translational modifications (PTMs) must pass through the hydrophobic phospholipid bilayer while those containing transmembrane domains (TMDs) integrated within the ER membrane must be dislodged and extracted from it. Early indications of a lysosome-independent route for secretory protein destruction were puzzling but eventually confirmed [4,5] and specialized components of the ubiquitin-proteasome system **[G]** (UPS) identified as the machinery facilitating much of the turnover of unwanted proteins from the ER [6–10]. This process would come to be known as endoplasmic reticulum-associated protein degradation or ERAD [10].

ERAD serves two principal physiological roles – 1) protecting against the accumulation of defective, slow-folding or unfolding proteins that might otherwise be toxic and impede secretory flux (protein quality control) and 2) regulating abundance of key enzymes/proteins (ER-resident or cytoplasmic) often conditionally destabilized in response to various metabolic ligands or cellular stimuli (protein quantity control) [11,12]. Consequently, a wide range of cellular and organismal processes are regulated (directly and indirectly) by ERAD (reviewed in [13–15]). Some more recent additions to

this list include - membrane fluidity [16], ferroptosis [17], iron metabolism [18], innate immune signalling [19,20], and sleep [21]. ERAD is coopted for pathophysiology as well. On the one hand, ERAD is responsible for alleviating proteotoxic ER stress[22]. Activation of ERAD can have negative consequences as exemplified by the ΔF508 variant of cystic fibrosis conductance regulator (ΔF508-CFTR) expressed in the majority of individuals suffering from inherited cystic fibrosis. The ΔF508-CFTR mutant results in a fully functional protein once in the plasma membrane, but recognition by ERAD leads to its premature degradation and disease[23]. Furthermore, some components of ERAD are even hijacked by pathogens for their propagation (**Box 1**).

ERAD is active in all eukaryotes where it functions similarly to other well characterized ubiquitylation reactions – covalently attaching ubiquitin polymers to recruited protein substrates resulting in their degradation by 26S proteasomes. These reactions employ an enzymatic cascade of an E1 enzyme **[G]** that primes and activates ubiquitin, an E2 enzyme **[G]** that transfers ubiquitin, and an E3 enzyme **[G]** (ubiquitin ligase) that recognizes and coordinates substrate position for the ubiquitin modification (reviewed in [24]). The majority of E3s involved in ERAD are of the Really Interesting New Gene (RING) finger domain **[G]** variety, which promote ubiquitin attachment to substrates by recruiting activated E2s rather than controlling direct ubiquitin transfer [25]. Most ERAD E3s also contain membrane-spanning TMDs - organizing and tethering the initial ubiquitylation reactions to the cytoplasmic face of the ER membrane and necessitating substrate proximity to this interface. The ingenuity of ERAD is that different solutions - operating as parallel routes for disposal and incorporating specialized recognition and protein transport strategies to the membrane-adjacent sites of ubiquitylation - have evolved within the assemblies of E3s and associated factors (**Table 1**) to accommodate the variety of substrates requiring removal. Lower eukaryotes such as yeast manage with three ERAD routes (one of which patrols the inner nuclear membrane contiguous with the ER), while higher eukaryotes may have as many as 20 more that involve a variety of distinct cofactors [26]. This difference could be explained

by the fact that while yeast can use asymmetric inheritance of damaged material to daughter cells upon cell division to circumvent processing of problematic ERAD clients [27], this strategy is not an option for multicellular organisms. Equally, proteome expansion and the evolution of specialized factors for a metazoan lifestyle requiring regulation, might also rationalize this diversification.

To understand the different disposal routes used for ERAD, we must address: 1) how they recognize and differentiate substrates, 2) how they facilitate substrate movement to the cytoplasm, and 3) how these processes are spatiotemporally coordinated to ensure efficient ubiquitylation. In this Review, we summarize our knowledge of the diversity of ERAD routes and the underlying ER-E3s-based protein complexes, discussing how this allows them to recognize and degrade topologically distinct substrates. We focus on molecular mechanisms elucidated from both yeast and mammalian systems. For discussion about physiological implications of ERAD we refer the reader to other Reviews [13–15]. To close, we highlight several challenges that lie ahead for further understanding of this specialized branch of the UPS.

[H1] Fundamental steps in the ERAD pathway

The integrated activities for each route encompassed by the term ERAD include the following steps (**Figure 1**): 1) the presentation of a feature/s atypical for folded proteins to recognition factors that discriminate and encourage proximity to E3s; 2) the retrotranslocation **[G]** of polypeptides' soluble domains and/or the dislocation **[G]** of TMDs from the ER membrane to reach the cytoplasm that is directly coupled to; 3) modification with primary polyubiquitin adducts by proximal membrane-associated ubiquitylation machineries; 4) unfolding and threading through the central cavity of the type II AAA ATPase **[G]** (ATPase associated with diverse cellular activities) Cdc48 (yeast) or VCP (also known as p97; mammals) to facilitate ER extraction; 5) remodeling of emerging polyubiquitin adducts through secondary extension and diversification of ubiquitin chains by soluble E3 complexes upon exiting the ATPase

assembly; 6) delivery to 26S proteasomes for degradation. While the initial targeting steps of each ERAD route (1-3) are executed through specialized complexes formed from ER-E3s and their cofactors and dictated by the individual properties of substrates and their topologies, the latter delivery steps (4-6) converge to use more generalized machineries, which will only be briefly discussed here.

ER-E3s form multi-subunit protein complexes underlying each ERAD disposal route by assembling with exclusive and shared cofactors that enable spatiotemporal coordination and implementation of its discrete steps. Each ER-E3 complex at the center of an ERAD route has a unique organization with a different membrane-spanning topology – envisioned to endow it with capacity to recognize and process different substrates through elements distinguishing one or more common features. To do so, each complex must necessarily contain fundamental elements: 1) a recognition element/s to differentiate and engage substrates: 2) a TMD-containing element able to form a passage of sufficient dimensions and hydrophilicity to permit lumenal segments, PTMs, and TMDs of substrates to traverse the ER membrane (if required); 3) an element/s that coordinates substrate position as well as recruits and activates appointed E2 enzymes for primary substrate ubiquitylation; and 4) an element that coordinates recruitment of cytoplasmic machineries to extract substrates from the ER (**Figure 1**).

[H1] Substrate engagement

Proteins become targets for ERAD because they misfold, unfold, or fold too slowly – resulting in persistent forms that are not conducive to optimal functionality (Figure 2). The origins of these defects can be traced to reduced transcriptional/translational fidelity, germline/acquired mutations, failure of timely assembly into complexes, a suboptimal folding environment, or ligand-induced destabilization that conditionally mimics an aberrant conformation (also known as mallostery) [28,29]. The resulting "lesions" effectively produce a degradation signal or "degron **[G]** " within one (or more)

specific domain/region of the polypeptide. Degron presentation for ERAD could occur within the ER lumen, lipid bilayer, or cytoplasm. Thus, each of these compartments is anticipated to harbour a suitable repertoire of recognition elements provided by different ER-E3 complexes able to initiate ERAD. With a myriad of aberrant protein forms possible, the challenge for ERAD lies in how recognition elements can effectively differentiate and separate degron-containing, unsalvageable forms from the normal folding intermediates. Experimental evidence supports a system of parallel (and sometimes redundant) ERAD strategies and ER-E3 complexes able to engage and process degron-presenting substrates with different topologies [30,31]. A "division of labour" among these ER-E3s is undoubtedly present, but the complete picture governing how and where they select substrates, their exclusivity, and prioritization parameters has not yet come into focus. This is particularly evident amongst mammalian membrane protein substrates for which co-dependency on more than one ER-E3 and consequently the ERAD disposal routes has been observed [32,33]. The diversity of engagement paradigms here could be attributable to the presence of one degron commonly recognized by different ER-E3s or by multiple degrons presented to an established ER-E3 hierarchy. Moreover, there may be participating E3s which have not yet been identified. It is important to note that not all proteins misfolding in the ER are cleared via ERAD. Removal of aberrant proteins via autophagy of the ER (ER-phagy) serves a complementary role to ERAD [34]. In addition, some aggregation-prone forms that are ERAD-resistant can instead be removed through the alternative ER-lysosomal degradation (ERLAD) pathway **[G]** [35]. Maturation-incompetent GPI-linked proteins do not rely on ERAD but instead are exported and degrade via the lysosomal/vacuolar pathway, potentially involving microautophagy **[G]** [36,37].

[H2] Lumenal protein recognition

The principal agents shaping our view of ERAD to date have been misfolded, soluble glycoproteins. The current understanding of ERAD for these substrates is based on

the “mannose timer”. According to this model, unproductive folding cycles (or remediation attempts) prolong immature polypeptide residency in the ER lumen - increasing N-linked glycan exposure to resident mannosidases and glucosidases that trim substrate oligosaccharides making them shorter (**Figure 2a**). The primary enzymes involved in this process are ER-resident lectin **[G]** like proteins including Htm1 (yeast) and EDEM1/2/3 (mammals) [38,39]. Redox factors including Pdi1 in yeast and PDI/P4HB, TXNDC11), ERp46 and ERdj5 in mammals form complexes with these lectins (e.g. Htm1-Pdi1, EDEM1/2-TXNDC11, EDEM1-ERdj5, EDEM1/2-PDI, EDEM3-ERp46) through intermolecular disulfide bonds, which enable the catalytic mannosidase activity [40–45]. The disulfide reductase activities of TXNDC11 and ERdj5 also ensure efficient ERAD of soluble substrates [46,47], suggesting that reducing substrate disulfide bonds facilitates subsequent processing events. Reducing disulfides and irreparably trimming N-linked glycans ($GlcNAc_2Man_9Glc_3$ → $GlcNAc_2Man_{5\text{-}6;}$ **Figure 2a**) yields glycoproteins unable to reengage with the ER chaperones calnexin and calreticulin and consequently, remediating folding cycles that could return them to the maturation-competent pool (reviewed in [48,49]). This results in what is effectively a “glycodegron” – a degradation signature dependent upon oligosaccharide structure.

Glycodegrons decorating substrates are preferentially recognized for ERAD by ER lectins Yos9 in yeast and both OS-9 and XTP3-B in mammals through their mannose 6-phosphate receptor homology (MRH) domains **[G]**. These lectins also bind the Hsp70-type chaperones of the ER Kar2 in yeast or BiP (also known as HSPA5) in mammals to engage surface-exposed hydrophobic polypeptide segments on substrates [50–58]. Thus, these ER lectins employ this “bipartite” recognition strategy (recognizing both glycodegrons and polypeptide segments) that together with the membrane-bound adaptor proteins Hrd3 (yeast) and SEL1L (mammals) form a lumenal surveillance complex able to capture and deliver ERAD substrates to the E3 HRD1 [59–62]. In mammals, employing two different lectins (OS-9 and XTP3-B) to

207 balance the recognition of trimmed N-linked glycans and polypeptide sections fine
208 tunes detection by an ERAD receptor and contributes to the specificity and fidelity of
209 substrate processing [55].
210 Lumenal substrates without N-linked oligosaccharides and consequently no
211 glycodegrons, still rely on HRD1 and Hrd3/SEL1L but do not require ER lectin MRH
212 domains [63,64]. Instead, aberrantly exposed hydrophobic segments form the degron/s -
213 recognized by Kar2 (through Yos9 and Hrd3) and required for ERAD in yeast [62]. In
214 mammals, BiP, ERdj5, EDEM1 and HERP1 are associated with delivery to HRD1-
215 SEL1L [42,47,65,66] (**Figure 2b)**. There is evidence that ERdj5-BiP incorporation into HRD1
216 complexes for ERAD may be mutually exclusive to those assembling with ER lectins
217 that engage trimmed oligosaccharides[67], suggesting that HRD1 complexes are
218 heterogeneous in their constituencies.
219 Overall, by linking to elements able to recognize both polypeptide-derived degrons
220 and/or glycodegrons, HRD1 is able to function as the principal executioner of ERAD
221 for most lumenal substrates (although some aggregation prone substrates may use an
222 alternative path [68]). This versatility is a satisfactory explanation for why HRD1 and its
223 cofactors have evolved to be regulated by the unfolded protein response **[G]** (UPR),
224 which senses ER stress caused by misfolding in the ER lumen [22,26,69–72].
225
226 *[H2] Membrane protein recognition*
227 Topologies that span the lipid bilayer means degrons hallmarking integral membrane
228 substrates can present in the ER lumen, membrane or cytoplasm. The nature and
229 position of these degrons relative to the ER membrane is are factors likely to determine
230 through which ERAD disposal route/s they are processed. The molecular strategies
231 used by elements within ER-E3s to recognize different degrons may have evolved
232 independently and are just beginning to be defined. Lumenally exposed domains of
233 many transmembrane proteins contain N-linked glycans and so can present the same
234 glycodegrons as the soluble, mis-/unfolded proteins (see above). In the case of these

transmembrane substrates, engaging ER lectins and an ERAD route through HRD1 inherently implies that its TMD/s are able to diffuse laterally within the ER membrane to engage with and access HRD1 (discussed below). Single pass membrane proteins (Type I) with extensive lumenal domains exemplify these candidates (e.g., unassembled MHC Class I; major histocompatibility class I protein, CD147) and many examples exhibit such HRD1 dependency [73–75].

Degrons present within TMDs or cytoplasmic domains of integral membrane proteins could be recognized by the HRD1 complex independently of ER lectins but may be equivalently or preferentially suitable for other ER-E3 complexes and ERAD routes. For example, ERAD of destabilized forms of the polytopic membrane protein (Type IV) HMGCR 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) is dependent on Hrd1 in yeast [8]. Mammalian HMGCR however, exhibits only moderate dependence on HRD1, instead preferentially relying on the E3s RNF145, TRC8 (also known as RNF139) and gp78 (also known as AMFR), recruited for ERAD through regulatory proteins of lipid metabolism INSIG1 and INSIG2 [32,76,77] (**Figure 2c**). Tail anchored membrane proteins (Type III), often with little exposure to the ER lumen might also be less likely to be engaged by the HRD1 complex. In fact, the single TMD domain of the yeast orthologue of Sec61β — a subunit of the Sec61 translocon complex **[G]**—Sbh2 constitutes an intramembrane degron recognized by the E3 Doa10, although details on the properties of this degron and its recognition are not known (**Figure 2d**) [78]. The Doa10 homologue MARCH6 (also known as TEB4; mammals), along with TRC8, degrade the tail-anchored protein hemeoxygenase-1 (HO-1) after its TMD is clipped by the signal peptide peptidase SPP (discussed below) [79]. Notably, the length and hydrophobicity of helices/TMDs present in the ER membrane also appear to be important determinants differentiating substrate dependence between these two ER-E3s. Still, degradation (and possibly recognition) of most Doa10 substrates in yeast rely on cytoplasmic chaperone components that may assist their recruitment to Doa10 (**Figure 2d**) [80,81]. Doa10 contains a TMD as well as a C-terminal element (CTE) in the

cytoplasmic portion conserved with MARCH6 that are important for ERAD [82,83]. For squalene monooxygenase (SM), an N-terminal amphipathic helix attaching to the ER membrane when cholesterol is excessive, results in dissociation from the membrane, exposing a once-masked hydrophobic patch between amino acids 62 and 73 and allowing recognition of SM by MARCH6 for ERAD [84]. Curiously, yeast SM does not contain the N-terminal extension but is still degraded via Doa10 [85]. The TMDs of the Lanosterol 14α-demethylase CYP51A1 and its yeast ortholog Erg11, found differential dependence on two ERAD routes defined by the E3s RNF185 and its cofactor TMEM259 (also known as membralin) and MARCH6, respectively [86]. This study constructed chimeras of CYP51A1 and Erg11 and found that MARCH6 recognizes substrates primarily via specific, albeit ill-defined attributes within their TMDs, whereas RNF185-Membralin relied on presumed degrons in both the ER lumen and within the TM region.

Another example of a transmembrane target of ERAD is IP_3R1 (inositol 1,4,5-trisphosphate receptor 1) — and ER-located calcium channel, which is routed to degradation following its activation by inositol-tri-phosphate (IP_3) and Ca^{2+}. IP_3R1 destined for degradation is recognized by the cofactors Erlin1 and Erlin2, which engage and link the $IP_3R1$to the E3 RNF170 [87,88] (**Figure 2f**). Erlin2 also routes the WNT receptor protein Evi (also known as WLS and Gpr177) to the E3 CGRRF1 [89,90]. More recently, Erlin1 and Erlin2 have been shown to recognize an intralumenal loop of the IP_3R1 and not a TMD or cytoplasmic domain to initiate ERAD [91], highlighting the need to consider that E3 cofactors may recognize degrons in the ER lumen of membrane bound substrates even when they are not glycosylated. Some of these cofactors may be highly selective and ‘tailored’ to just one (or a few) clients. One example is the limb region 1-like (LMBR1L) protein that binds and delivers Frizzled-6, a receptor of the WNT pathway, to the E3 gp78 for degradation thereby reducing WNT signalling [92].

With only a limited number of examples, it is still not clear whether different intramembrane and/or cytoplasmic lesions also produce degrons with common features that could be used to consolidate targeting towards one (or a limited set of) ER-E3 complexes. Efforts are now underway to better define these degrons and the recognition elements within ER-E3s complexes as this will aid in establishing how and under which context they engage substrates. From this, the relationships between degrons arising within membrane proteins and cognate recognition factors for each ERAD disposal route, will gradually come into greater focus.

[H1] Translocating substrates for ubiquitylation

The lumenal domains of proteins targeted for ERAD are structurally disrupted and often decorated with residual N-linked oligosaccharide/s that must somehow pass through the ER membrane to reach ubiquitylation machinery and proteasomes. Equally, the hydrophobic TMDs of the membrane-bound ERAD substrates must be extracted from the stable surroundings provided by that lipid bilayer. These actions are energetically unfavourable, require extensive coordination, and are fundamentally complex, necessitating elaborate solutions to be employed, the details of which are described below.

[H2] Retrotranslocation of lumenal substrates

Lumenal substrates pass back through the ER membrane using the process referred to as “retrotranslocation”. Our best model for this has emerged recently from high resolution single particle cryo-electron microscopy (cryo-EM) structures of yeast Hrd1 [93] and complementary cysteine-crosslinking assays [94]. After being recruited by Yos9 and Hrd3, soluble substrates move towards a complex formed by the TMDs of Hrd1 and its cofactor Der1. A groove in Hrd3 appears to help present a substrate loop to the lumenal vestibule formed by Hrd1-Der1 TMDs [93,94]. Der1 is a member of the rhomboid pseudoprotease family that comprises proteolytically inactive variants of rhomboid

intramembrane proteases [G] [95]. Conventionally, rhomboid proteases are multi-spanning, integral membrane proteins that cleave TMD-containing proteins at sites in/near the lipid bilayer. It is noteworthy that enrichment of specific phospholipids in their surroundings causes a distortion and local thinning of the lipid bilayer, which stimulates the proteolytic activity of the rhomboids, accelerates their lateral diffusion within a membrane, and may lower the energy barrier for the extraction of cleavage products (reviewed in [96–98]). A similar thinning of the membrane was observed around the Hrd1 and Der1 TMDs in the cryo-EM structure [93] and there are recent indications that ER membrane lipid composition contributes to the efficiency of ERAD [99].

According to the cryo-EM data, the TMDs of Der1 establish a shallow, hydrophilic pit at the luminal side of the ER membrane (**Figure 3a**). TMDs 2 and 5 line a laterally open groove that is juxtaposed by a hydrophilic cleft in Hrd1 built by TMDs 3 and 8. These half- (or hemi-) channels align to form a proteinaceous conduit (**Figure 3a**). TMDs 3 and 8 of Hrd1 (possibly in conjunction with TMD2 in Der1 and parts of another cofactor, Usa1) also form a hydrophilic cavity at the cytoplasmic side of the ER membrane, which substantially reduces the thickness of the membrane at this site and thus may decrease the energy barrier for dislocation. Based on crosslinking experiments, a peptide loop of the substrate advancing from Hrd3–Yos9 first inserts into an aperture formed from Der1 and is then threaded through the Der1–Hrd1 channel. Having traversed the lipid bilayer, substrate polypeptides emerge into the cytoplasmically-facing Hrd1 vestibule [93]. It remains to be determined, whether contact sites within the Der1–Hrd1 conduit identified in the crosslinking experiments also reflect key points necessary to engage substrates and orienting them for subsequent ubiquitylation [93,100–102]. In mammals, Derlin2 and Derlin3 are both orthologues of Der1 and interact with mammalian HRD1 for ERAD [59,103]. While a functionally conserved retrotranslocation mechanism is envisioned, structural insight from mammalian HRD1 complex has not yet been possible.

[H2] Dislocation of membrane-bound substrates

Membrane proteins with a variety of topologies reportedly utilize HRD1 for ERAD [104], implying that a channel formed by HRD1 is somehow accessible to TMDs. Indeed, Hrd1-Der1 cryo-EM structures would seem to support entry of TMDs through a lateral gateway in the lipid bilayer [93]. Still, dislocation of membrane-bound substrates in yeast mostly occurs in a Der1-independent manner [105]. An alternative path to the cytoplasm could come in the form of a "universal dislocon" – available to and recruited by different ER-E3s and commonly accessible to substrates. The most promising candidate for this is another rhomboid pseudo-protease Dfm1 (yeast)/Derlin1 (metazoans), which exhibits some compelling evidence of this kind of activity [105,106]. Yeast cells lacking Dfm1 accumulate ubiquitylated TMD-containing substrates indicating a contribution to substrate dislocation rather than recognition [105]. Its mammalian orthologue Derlin1 oligomerizes into a pore-like structure [107], and either co-purifies or is functionally associated with different mammalian ER-E3s participating in ERAD including RNF5 [108], gp78 [109], and TMEM129 [110]. Co-assembly with ER-E3s could facilitate lateral access for substrate TMDs, or alternatively recruitment might enable Dfm1/Derlin1 to assemble around them (**Figure 3b**). With more rhomboid pseudoproteases identified in the ER of higher eukaryotes, the number of potential components of the ERAD dislocation apparatus is growing [96]. Mammalian UBAC2 associates with gp78 and the VCP adapter protein, UBXD8, and while its function is not fully understood in detail, it may contribute to different type of channel forming in the ER membrane alongside this ER-E3 [30,111].

Curiously, requirement of either Der1 or Dfm1 for dislocation of soluble or membrane-bound substrates can be bypassed by over-expressing Hrd1 [101,105]. This raises possibilities of additional polypeptide conduits formed among the Hrd1-containing complexes, perhaps by Hrd1 homo-oligomerization [100,112] and further increases the complexity and versatility of the dislocation machineries engaged in ERAD. While

convincingly demonstrated for yeast, it is not clear that such circumvention can be recapitulated by mammalian HRD1.

Dislocation of selected membrane-bound substrates may occur via conduits that don't include rhomboid pseudoproteases or HRD1. ER-E3s like yeast Doa10 or mammalian MARCH6, RNF145, TRC8, and gp78 contain a number of TMDs sufficient to envision channels/passages intrinsically forming within them [113,114]. Indeed, the large 14-TMD section of Doa10 supports export/dislocation of the tail-anchored protein Ubc6 [115]. As high-resolution structures of these ER-E3s are still on the horizon, a passage-forming TMD arrangement able to dislocate substrates can only be hypothesized. Other E3s implicated in ERAD like RNF5, RNF185, or CGRRF1 however, contain far fewer TMDs and could only be envisioned to build a polypeptide conduit by forming homo- or hetero-oligomers or by co-assembling with polytopic, non-rhomboid cofactors. RNF185 contains only two TMDs but co-assembles with the polytopic proteins TMEM259 (6-8 TMDs), TMUB1 and TMUB2 (2-3 TMDs each), which are required for substrate processing but whose exact role (if any) in substrate engagement and dislocation remains unclear [86]. Recently, TMUB1 has been shown biochemically to act as an "escortase" for TMD-containing substrates [116]. Although not explicitly claimed by the authors, this activity would be consistent with a dislocation role during ERAD.

Finally, foregoing a requirement for dislocating entire membrane protein substrates intact during ERAD allow us to consider the possibility that intramembrane cleavage of TMDs occurs to lower the energetically unfavourable reaction. This cleavage could occur through engagement with the rhomboid protease RHBDL4, the intramembrane aspartyl protease signal peptide peptidase SPP (reviewed in [96]), or more recently-described signal peptidase complex (SPC, [117]). RHBDL4 is an ER-resident rhomboid protease with a ubiquitin binding domain that can cleave within the soluble parts of substrates close to the membrane surface and facilitate removal of proteolytic fragments, again likely through membrane thinning [118]. Notably, interaction of RHBDL4 with the Erlin1-Erlin2 complex also cleaves and retrotranslocates aggregation-prone

ER lumenal proteins to the cytoplasm for proteasomal degradation (**Figure 2e**) [68]. SPP removes signal peptides from nascent chains but also appears to moonlight within ERAD – associating with ER-E3s (TRC8, MARCH6) and Derlin1 and internally cleaving substrates [79,119–121]. Recently, SPC-catalysed cleavage of misfolded membrane proteins has been shown to promote their degradation from the ER by HRD1 [117]. As SPC was found coprecipitating with the HRD1 complex, this suggests that indeed, TMD cleavage occurs to lower a barrier for dislocation through this ERAD path. The paths toward ubiquitylation available to substrates during ERAD represent unique/different evolutionary solutions for transport across membranes [122].

[H2] Orienting ERAD substrates within the cytosol

Upon leaving the Hrd1-Der1 vestibule, substrates must remain soluble, with amino acid residues accepting ubiquitin modifications (i.e., lysine, serine, threonine, cysteine) remaining accessible to the ubiquitylating enzymes. A detailed analysis of these processes is still lacking, in part owing to difficulties in resolving the cytoplasmic C-terminal domain of the yeast Hrd1 complex in cryo-EM structures [93]. Recently however, deep mutational scanning found disordered portions of Hrd1's cytoplasmic domain to be involved in retrotranslocation and substrate binding [123]. Mammalian HRD1 and many of its cofactors (e.g., FAM8A1, HERPUD1, UBE2J1, see **Table 1**) similarly contain long stretches with regions predicted to be intrinsically disordered between their TMDs and rigidly folded functional domains [124]. The lack of defined secondary structure here could likewise allow adaptable, non-specific binding for emerging substrates, could help modulate retrotranslocation, or endow flexibility to arrange the HRD1 RING domain in an adaptable way to encourage ubiquitin transfer. While most ER-E3 RING domains lie at their N- or C-termini and tethered unilaterally tethered to the ER membrane, the RING domain of RNF170 lies within a cytosolic loop and is flanked by TMDs [125]. Such a position might constrain this RING domain's ability to move, perhaps limiting the range of substrates it could engage and modify.

Membrane proteins dislocated during ERAD by ER-E3s pose a different challenge. While their cytoplasmic domains/loops will remain soluble, any newly exposed hydrophobic TMDs will be aggregation prone in the aqueous cytosol. There is evidence that ubiquitin-like protein BAG6 (BCL2-associated athanogene 6; also known as TRC40) engages TMDs during ERAD to prevent aggregation [126–128]. Together with ubiquitin-like protein Ubl4A and TRC35 (also known as GET4), BAG6 forms a so-called holdase — a chaperoning complex that shields hydrophobic patches in unfolded substrates from the hydrophilic environment but doesn't facilitate the folding/unfolding of a protein. This protein assembly coordinates with gp78 [129] and perhaps other ER-E3s including Hrd1 [128] to sequester TMDs during ERAD. In fact, gp78, associated with a BAG6 complex, works together with HRD1 after retrotranslocation to process ERAD substrates [111]. Further studies will be required to delineate how the interplay of multiple ERAD routes allows spatiotemporal coordination of substrate engagement, ensures solubility, and promotes orientations that facilitates the ubiquitylation of substrates.

[H2] Access of cytoplasmic substrates to ERAD machinery

Cytoplasmic proteins have access to the majority of E3s in the cell, but some appear to be regulated by those localized to the ER and involved with ERAD. Proteins such as p53 [130], PGC1-β (peroxisome proliferator-activated receptor coactivator [131]), and METTL14 (N^6-adenosine-methyltransferase-14 [132]) are just some examples of proteins dependent (at least partially) on HRD1 for degradation. In the case of METTL14, accumulation of [137]ER lumenal protein aggregates during stress compete with it for HRD1 processing and prevent METTL14 degradation. Increasing amounts of METTL14 then induce the decay of CHOP mRNA thereby effectively blocking pro-apoptotic gene expression and allowing cellular adaptation to folding stress [132]. Yeast Doa10 engages and degrades the transcription factor Matα2 (mating type protein alpha 2), which is involved in the pheromone response pathway [133], and a mutant form of the Cbf2 (centromere binding factor 2) kinetochore protein [134]. Cytoplasmic proteins

engaging ER-E3s may do so for purposes other than degradation, as is the example for RNF26-dependent ubiquitylation of autophagy protein p62 (also known as SQSTM1), which controls perinuclear positioning of endosomes [135,136]. Broad understanding of how and why cytoplasmic substrates are targeted to these ER-E3s is not yet available. It is apparent that the ER interface is an important site for protein activity regulation, but is yet to be fully appreciated. Perhaps re-examining existing interaction mapping and functional profiling datasets of ER-E3s with a new appreciation for the cytoplasmic proteins identified might reveal the extent of this 'non-canonical' role of ERAD machinery.

[H1] Ubiquitin conjugation in ERAD

Substrate ubiquitylation for ERAD serves two main purposes: 1) to label substrate regions extruding into the cytoplasm so as to be recognized by ubiquitin-dependent unfolding machinery that then facilitates its extraction from the ER and 2) to provide the foundation for building ubiquitin chains, which represents the signal for the transport to and recognition by the proteasome. Ubiquitin chains are generated by conjugating ubiquitin moieties to an internal lysine or the N-terminal methionine residue of ubiquitin molecules already attached to substrates, giving rise to ubiquitin chains homogenous, mixed or branched linkages that direct different cellular outputs for the substrate [137]. They are highly dynamic – being built, extended, and trimmed continously - with E3 enzymes principally determining substrate specificity, E2 enzymes largely enforcing transfer to substrates, and de-ubiquitylating enzymes (DUBs) working in opposition to trim or completely remove chains.

[H2] E3 ubiquitin ligases

Ubiquitylation for ERAD requires three ER-E3s in yeast - Hrd1, Doa10, and Asi complex (built from the Asi1, Asi2 and Asi3 proteins). The Asi complex exclusively patrols proteome integrity at the inner nuclear membrane (INM) contiguous with the

ER [138,139]. In mammals, their orthologues HRD1 and MARCH6 and at least eight others among the > 25 E3 ubiquitin ligases in the metazoan ER [26,140] appear to participate (reviewed in [141,142])(**Table 1**). A metazoan orthologue for the Asi complex does not appear to be present, although recently the E3 RNF5 has been shown to perform a comparable function in the nuclear envelope - relocalising to the INM to degrade a misfolded, disease variant of the Lamin B receptor [33]. ER-E3 topologies vary widely (1 to 14 TMDs), as do the proteins with which they co-assemble and many of the substrates with which they engage (reviewed in [142]). ER-E3 diversification could reflect an evolution of bespoke regulatory strategies within metazoans to supplement general ones for fine tuning protein quality and abundance.

The ER-E3s engaged in ERAD use their encoded RING domains to promote ubiquitin transfer to substrates by activating recruited ubiquitin-bound E2s rather than by direct transfer from itself. They contain a characteristic zinc-complexing motif composed of cysteine (C) and histidine (H) residues, which stimulates ubiquitin transfer from E2s to their targets by aligning the thioester favourably towards an acceptor site and delimiting the space available to ubiquitin [24]. Similar to other RING domains, those in ER-E3s typically contain linchpin residues **[G]** that contact and allosterically activate E2~Ub thioester intermediates [143,144]. It should be noted that UBE3C, a cytoplasmic HECT type E3 that forms branched/mixed ubiquitin chains via lysines 27 and 48 (K48) in ubiquitin, has also been identified in multiple screens for ERAD substrates processed through different paths [65,86].

[H2] Ubiquitin conjugating enzymes (E2s)

In contrast to the variety of ERAD E3s available, the primary polyubiquitin signature on ERAD substrates is mainly generated by a functional interplay of only two ERAD-specific E2s: 1) Ubc6 (yeast) with two orthologues known in higher eukaryotes (UBE2J1 (also known as Ubc6e) and UBE2J2) and Ubc7 (yeast) and its mammalian orthologue UBE2G2. Ubc6 (UBE2J1/UBE2J2) is the only membrane embedded E2 in

514 the proteome. Ubc7 (UBE2G2) is soluble, recruited to the ER and activated by
515 conserved domains that form additional interaction surfaces, so-called “backside
516 binding” sites, within ER-E3s or associated cofactors – the G2 binding region (G2BR)
517 domains in gp78 and the UBE2G2 binding protein AUP1 in mammals [145–148] and the
518 Ubc7 binding region (U7BR) of Cue1 in yeast [149,150]. These help to align the E2 with
519 the RING domain and the substrate and thereby improve the efficient transfer of
520 ubiquitin. Yeast Ubc6 conjugates a single ubiquitin moiety to substrates, which serve
521 as primers for subsequent decoration with homotypic K48-linked polyubiquitin
522 synthesized by Ubc7 [151] (**Figure 4a**). As mammalian HRD1 complexes contain both
523 UBE2J1 and UBE2G2 and both are essential for ERAD [30,65,152], this arrangement of
524 serial ubiquitin chain building is likely conserved.
525 Employing different E2s is a common principle of RING E3s to ensure the quality of
526 the generated polyubiquitin products while maintaining a high rate of synthesis [153,154]
527 although yeast Hrd1 (and possibly also mammalian gp78) only require a dedicated
528 priming E2 enzyme for ubiquitylation of ERAD substrates lacking lysine residues [144].
529 Remarkably, Ubc6 and UBE2J1/UBE2J2 appear able to attach ubiquitin not only to
530 lysine but also to serine, threonine and possibly cysteine residues [66,151,155,156]. This
531 capability could expedite retro-/dislocation by enabling ubiquitylation of substrates
532 lacking frequent or surface-exposed lysine residues. Such activity is attractive for
533 ubiquitylation associated with HRD1, whose substrates may arrive at the site of
534 ubiquitylation in conformations or adopt positions that lack spatiotemporally available
535 lysines.
536 Why higher eukaryotes use the homologues UBE2J1 and UBE2J2 for priming
537 substrates with ubiquitin is unclear. They are differentially expressed in individual
538 tissues and show variations in their ER-membrane tethering tail anchors, which could
539 explain their distinctive preferences to collaborate with ER-E3s – UBE2J1 with HRD1
540 [30,59,73,152], RNF5 [108] and RNF26 [136] and UBE2J2 with MARCH6 [79], CGRRF1 [89,90], and
541 TMEM129 [110,157,158]. RNF26 can interact with both UBE2J1 and UBE2J2 but prefers

542 the former to ubiquitylate p62 [136], suggesting that either ubiquitin transfer is
543 incompatible with the latter or it is used for an alternative substrate/s. These differences
544 may represent adaptations to divergent demands for specific ERAD activities in
545 individual cell types.
546 Yeast Ubc1 and its mammalian homologue UBE2K have also been linked with ERAD
547 [65,86,159]. These enzymes attach ubiquitin molecules to the K48 positions of lysine 63-
548 linked polyubiquitin to generate branched chains [165]. Such ubiquitin signatures are
549 efficient proteasomal targeting signals in other cellular processes [160]. Notably,
550 depletion of UBE2K yielded monoubiquitinated $CYP51A1_{TMD}$ that suggests it acts prior
551 to processing by VCP (see next section), which requires substrates with chains of at
552 least four ubiquitins for engagement [86]. Prominent roles for other E2s in ERAD, either
553 before (or after) processing by Cdc48/VCP, are still waiting to be established.
554
555 **[H1] Extraction from the membrane and engagement of the proteasome**
556 ERAD paths converge in the cytoplasm to feed into two sequentially operational high
557 molecular weight protein complexes. Polyubiquitylated substrates are first extracted
558 from the ER by a complex containing Cdc48/VCP and are then decomposed by a multi-
559 enzyme protease termed the 26S proteasome. These events are functionally coupled
560 and because they are shared with other ubiquitin-dependent proteolytic processes,
561 they will only be briefly addressed here. Cdc48/VCP and the proteasome recognize
562 their substrates via specific ubiquitin signals and process them via unfolding by AAA-
563 ATPases. In cells, AAA-ATPases translate chemical energy into mechanical force,
564 which serves to unfold proteins and extract them from their environment [161]. They do
565 so by forming multimeric (typically hexameric) ring-shaped assemblies with a central
566 narrow pore. Each subunit exposes substrate-binding regions into that cavity.
567 Coordinated hydrolysis of ATP and the exchange of ADP with ATP in individual
568 subunits induce a piston like re-positioning in the complex that is associated with cycles
569 of substrate binding and release. The orchestrated, moving staircase-like re-

arrangements within the complex then generate the driving force that threads the substrates through the central pore and unfolds them [162,163].

Cdc48/VCP is arguably the most versatile AAA-ATPase in eukaryotes due to its large repertoire of adapter proteins and associated enzymes that integrate it into highly diverse cellular activities both in the cytoplasm and the nucleus (reviewed in [164]). Each Cdc48/VCP monomer contains a so-called N-domain for cofactor binding followed by two ATPase entities with the D1 domain regulating adapter configuration and orchestrating the activity of the complex and D2 domain providing the energy for protein unfolding [165–170]. For ERAD, ER-E3s complexes recruit the Cdc48/VCP N-domains via dedicated binding motifs present in E3s including HRD1 (VIM) and gp78 (VBM), or in associated cofactors such as Derlin1/2 (SHP), UBXD8/FAF2 (UBX) or VIMP in mammals and Dfm1 (SHP) or Ubx2 (UBX) in yeast [171–173] (**Figure 4b**). The heterodimeric polyubiquitin receptor NPL4-UFD1 (nuclear pore localisation 4 and ubiquitin fusion degradation 1; Npl4–Ufd1 in yeast).

Much of our understanding on the following steps comes from cryo-EM analysis of isolated sub-complexes from unicellular lower eukaryotes [165,170,174]. Upon binding, a ubiquitin chain is correctly positioned by Ufd1. A ubiquitin moiety within this chain unfolds within a cleft in Npl4 by a mechanism that is not yet clear, and then enters the central pore where it is engaged by the D2 domains of Cdc48 [169,170,175]. ATP hydrolysis in the D2 domains then pulls ubiquitin and the attached substrate through the narrow cavity, dissolving their folds. After passing through the Cdc48/VCP tunnel, emerging ubiquitin molecules are thought to spontaneously regain their fold. Binding of ancillary factors containing ubiquitin-like (UBL) and ubiquitin-associated (UBA) domains like mammalian BAG6 and RAD23 or yeast Rad23 and Dsk2 probably prevents refolding/aggregation of the ERAD substrate and facilitates delivery to the proteasome [176–178].

Those ubiquitin moieties residing proximal to the substrate are unfolded during processing by Cdc48/VCP, but the fate of the distal moieties is unclear. ERAD relies on the activity of the de-ubiquitylating enzymes **[G]** (DUBs) YOD1 (mammals) [179] and Otu1 (yeast) [180], which bind to the N-domain of Cdc48/VCP and cleave K48-linked ubiquitin. This implies that YOD1 and Otu1 remove ubiquitin from ERAD substrates during entry into Cdc48/VCP, which may also contribute to substrate release from the NPL4–UFD1 receptor (**Figure 4b**). Intriguingly, the ubiquitin chain on the substrate is remodelled during or after processing by Cdc48/VCP. In yeast, addition of ubiquitin moieties by Ufd2 (ubiquitin fusion degradation 2), an E3-like enzyme that generates ubiquitin chains on pre-existing ubiquitin modifications, and removing of ubiquitin by the DUB Ufd3 at the substrate exit site of Cdc48 is thought to re-shape the length and composition of the ubiquitin chain[180–182]. This probably facilitates dissociation of the substrate from the complex, prevents its re-engagement to Npl4–Ufd1, and may trigger binding to Rad23 and Dsk2 [177,178]. Whether such mechanism is conserved in higher eukaryotes is questionable, because UBE4B (ubiquitin conjugation factor E4), the human Ufd2 homologue, binds to the substrate entry site of VCP at the N-terminus and probably has no access to ubiquitin on substrates emerging at the exit site of VCP [183]. Instead, the E3 enzyme RNF126 that is associated with the BAG6 proteasomal transport factor edits the ubiquitin chain [129,184].

Substrates released from Cdc48/VCP are routed to 26S proteasomes, where they are recognized by their ubiquitin modification. Again, ubiquitin binding proteins like the RPN1, RPN10 and RPN13 (Rpn1, Rpn10 and Rpn13 in yeast) serve as receptors to position substrates for entry into an AAA-ATPase ring within the so-called regulatory particle that governs access to and feeds them into a proteolytic chamber termed the core particle (reviewed in [185]). Associated DUBs like mammalian UCHL5 and USP14 (Ubp6 in yeast) probably fulfil a proofreading function and rescue poorly ubiquitylated proteins from degradation, whereas the RPN11 DUB (Rpn11 in yeast) removes ubiquitin moieties from the substrate before proteolysis. It should be noted that

proteasomes have been observed to concentrate at localized domains of the ER [186], which could imply a direct pathway to proteolysis may exist for some ERAD substrates.

[H1] Regulation of ERAD

Constitutive expression of different E3 complexes empowers baseline ERAD activity to continuously survey the ER environment, but the dynamic nature of biosynthetic/secretory flux and cellular metabolism means that the “load” placed upon different ERAD paths can fluctuate, risking disruption of homeostasis. Thus, ERAD paths must be adaptive - altering E3 complex abundance or modulating activity in response to changing conditions, to prevent overload of the ER with dysfunctional polypeptides. ERAD capacity is supplemented by 1) transcriptionally upregulating components of E3 complexes, 2) stabilizing existing complexes, 3) assembling E3 complexes from different fundamental elements (e.g. homologue substitution), or 4) modulating existing E3 complexes through PTMs. Below are highlighted several examples of how E3 complexes and their ERAD paths can be regulated.

[H2] Interplay with UPR

Accumulation of misfolded proteins in the metazoan ER is acutely sensed as proteotoxic stress through the ER-resident transduction elements - Inositol Requiring Enzyme 1 (IRE1), Activating Transcription Factor 6 alpha (ATF6) and PKR-like ER Kinase (PERK) — three arms of the UPR. Binding to BiP/Kar2 normally keeps these transducers inactive but accumulating misfolded proteins in the ER cause BiP/Kar2 dissociation inducing UPR. All UPR branches initially act in a pro-survival manner, activating transcription regulators that specifically target pro-survival genes encoding ERAD, ER chaperones, lipid synthesis, and vesicular transport factors that supplement protein quality control capacity in yeast [70,159] and mammals [71,72]. PERK and IRE1 branches can switch to activating pro-apoptotic pathways if long-term stress cannot be

652 resolved (reviewed in [187,188]). The UPR functions similarly in yeast but relies solely on
653 Ire1 [189].
654 Genes encoding HRD1 complex components contain conserved binding sites
655 recognized by both XBP1 (transcription factor activated by IRE1 via splicing of its
656 mRNA) and ATF6 [190,191]. Transcriptional activation leads to concomitant upregulation
657 of most HRD1 complex components (e.g. SEL1L, UBE2J1, OS-9, etc.). Coordinated
658 upregulation during the UPR is key as SEL1L is an important determinant of HRD1
659 stability and hence functionality [192]. Of note, both HERPUD1 and Derlin3 are two of
660 most highly upregulated genes by the UPR during ER stress [69]. They compete with
661 basally expressed homologues HERPUD2 and Derlin2 respectively, for assembly into
662 HRD1 complexes [103]. HERPUD1 and HERPUD2 play an important role in the
663 assembly, localization, and activity of Hrd1 complexes for ERAD [103,124,193–195]. Whether
664 these new “ER stress-induced” HRD1 assemblies provide superior or different ERAD
665 selectivity/activity, has not yet been firmly established but remains an appealing
666 possibility. Intriguingly, both IRE1 and ATF6 become targets for ERAD via HRD1,
667 providing an effective autoregulatory negative-feedback loop [196,197]. Moreover, the
668 translation product of unspliced XBP1 RNA ($XBP1_U$), which itself serves as a negative
669 regulator of the UPR, is degraded by TRC8, together with SPP and Derlin1, as another
670 feedback mechanism within ERAD to tune the UPR [119]. Other ER-E3s (e.g. BFAR,
671 RNF183, RNF13) are regulated by ER stress [198] and some play a role in stress
672 resolution, by modulating stability of ER-resident factors participating in apoptotic
673 signalling [199–201].
674
675 *[H2] Role of PTMs*
676 Although systematic analysis is just at its beginnings, several examples demonstrate
677 regulation of ERAD complexes by PTMs. There is compelling evidence from yeast
678 Hrd1 reconstituted in lipid bilayers that opening/closing of its passage is gated by
679 cycles of auto-ubiquitylation and deubiquitylation on lysines within its RING domain

and modulated by both Hrd3 and the DUB Ubp1 [202–204]. While ubiquitylation of Hrd1 has been reported [148], conservation of such a mechanism in higher eukaryotes has not yet been confirmed. Negative regulation of an ER-E3 by ubiquitylation is exemplified by UBE2J2, which initiates degradation of TRC8 and thereby counteracts MHC Class I downregulation via the viral protein US2 (Box 1) [205]. Another example of ERAD component regulated by PTMs is UBE2J1. It is first phosphorylated by MAP kinase, which leads to its auto-ubiquitylation and degradation, facilitating recovery of cells from ER stress[206–209].

[H2] Interplay with lipid metabolism

ERAD serves as key post-translational regulatory feedback mechanism during metabolic and environmental fluctuations. To ensure this adaptability, executioners of ERAD need to sense and be regulated by metabolic cues. We discuss here one prominent example of such concerted action: interplay of ERAD with lipid metabolism. Sterols are essential components of membranes that regulate their biophysical properties, such as fluidity, rigidity, and permeability and their abundance and localization is tightly regulated. While normally kept low, accumulation of free sterols is toxic to both cells and whole organisms [210,211]. Consequently, the ER-E3s regulate abundance of ER-resident, rate-limiting enzymes within the sterol biosynthetic pathway — most prominently HMGCR and SM — through degradation (reviewed in [12]). Briefly, high levels of ergosterol in yeast cause structural re-arrangements in HMGCR, facilitating ERAD by Hrd1 [8]. In mammals, cholesterol induces binding of ER-resident INSIG1/2 to SCAP (Sterol regulatory element-binding protein cleavage-activating protein), which prevents its ubiquitylation by gp78 [212]. Increased amounts of INSIG1/2 then recruit RNF145 and gp78 to HMGCR to attenuate cholesterol biosynthesis [8,32,76]. Similarly, the downstream-lying SM is an ERAD target for Doa10 (yeast) and MARCH6 (mammals) when sterol levels are high, thus offering a secondary regulatory point within the pathways [85,213]. Cellular cholesterol levels also

influence intrinsic stability of ERAD E3s directly. RNF145 accumulates upon sterol depletion [32] while MARCH6 is stabilized by cholesterol [214]. RNF145 can also be transcriptionally controlled by the sterol-responsive Liver X receptor (LXR)-dependent pathway [215].

Lipid composition in the ER membrane also regulates ERAD paths with elevated ceramide **[G]** levels compromising Hrd1-mediated ERAD by reducing substrate extraction in yeast [99]. Saturating membranes with fatty acids in mammals leads to RNF145 auto-ubiquitylation and degradation, stabilizing its substrate ADIPOR2 and restoring lipid homeostasis [16]. Clearly the extent of the relationship between ERAD and lipid regulation is just beginning to be appreciated, and will be an area of intense future work. Undoubtedly there are other sensing mechanisms that will regulate ERAD paths and perhaps with an increased appreciation of their different substrates, a better understanding of their control can be reached. One example here is degradation of activated IP_3Rs by RNF170 in response to Ca^{2+} stimulation [87,125]. In a similar fashion in yeast, SPP-like protease Ypf1 (yeast presenilin-like family 1) uses regulated ERAD by Doa10 and Dfm1 to control levels of plasma membrane transporters when intracellular Zn^{2+} levels are high [216].

[H1] Conclusions and perspectives

More than 25 years have passed since the first genetic screens in yeast identified Hrd1/Der3 as a model ubiquitin ligase in the ER for ERAD. Since then, advances in proteomics and mammalian genome manipulation have filled in details conventional biochemistry and yeast genetics could not. Now, cryo-EM structures and *in vitro* reconstitution are giving us unprecedented insight into the mechanistic details of substrate retrotranslocation during ERAD while *in vivo* mouse models inform on the broad roles of ERAD in physiology (reviewed in [14]). The emerging snapshots have revealed unexpected complexities within the Hrd1 architecture, most notably thinning

of the local membrane and formation of substrate-accepting vestibules by the ER-E3 and Der1. Yet, our understanding of other ER-E3s underpinning the ERAD disposal routes operating in parallel still trails behind, but perhaps not for long. As we begin to better appreciate the different substrate recognition and dislocation strategies that have evolved within other ER-E3s to accomplish ERAD, the full picture of how this specialized UPS network functions will come into full focus.

Evolution has greatly expanded the repertoire of ERAD functional modalities in metazoans, and only by extending our investigations to the compositions and structures of other ER-E3 complexes will we begin to understand the diversity of ERAD solutions. Currently we are only availed with advanced prediction algorithms (e.g. AlphaFold, Meta AI) to envision the structures of these ER-E3s complexes [113]. With the technological advances and breakthroughs of recent years in cryo-EM, proteomics, an increasingly holistic view of ERAD paths and the mechanisms underlying substrate recognition, dislocation, and ubiquitin processing is now within reach. Next, the functional reconstitution of each ERAD path will help to better capture the dynamic nature of these processing steps and reveal the individual contribution of the components involved. Moreover, further studies should enlighten us of the spectrum of retrotranslocation/dislocation strategies at work in the ER. In the future, ERAD might be better viewed as a common degradation strategy executed by a collective of mechanistically distinct routes toward ubiquitylation, rather than an array of ubiquitylation pathways varying only in their substrate specificity.

Our appreciation for the protein forms whose quality and quantity are ensured by ER-E3s and ERAD is growing. While exogenously expressed mutants have been valuable model substrates for charting the basic ERAD mechanisms, it is the endogenous membrane proteins with important physiological roles that should now be garnering greater attention. Fundamental understanding of specialized ERAD processes will also create the motivation necessary to identify and develop small molecules that target

764 these pathways and offer novel strategies for therapeutic intervention, something that
765 is already beginning [217,218]. Compounds able to interfere with regulated ERAD of
766 metabolic factors, redirect undesired secretory proteins for ERAD, help disease-
767 causing mutant evade premature ERAD, or prevent the hijacking of ERAD by microbial
768 pathogens, could be valuable alternative strategies for treatment of a plethora of
769 diseases.
770

Table 1. ER-resident E3s and their reported interactors

ER-E3s		COFACTORS				Cdc48/VCP recruitment (via)	Reference
mammalian	**TMDs**	**Recognition**	**Organization**	**Dislocation & Retrotranslocation**	**E2**		
HRD1	8	OS9, XTP3-B, SEL1L, EDEM1-3, ERDJ5, BiP	FAM8A1, HERPUD1, HERPUD2, FAF2/UBXD8, AUP1, VIMP	DERL2, DERL3, DERL1(?)	UBE2J1, UBE2G2	DERL1, DERL2, HRD1, FAF2/UBXD8	30,55,103,124,152,219,220
gp78/AMFR	8	INSIG1, INSIG2, LMBRL	UBAC2	DERL1	UBE2G2	gp78	30,111,221–223
MARCH6/TEB4	14	-	-	-	UBE2J2 UBE2G2	-	79
RNF5/RMA1	2	-	-	DERL1	UBE2D3, UBE2J1	DERL1	33,108
CGRRF1	1	ERLIN1	FAF2/UBXD8, UBXN4, UBXN6,	DERL3, TMUB2	UBE2J2, UBE2N, UBE2K	UBXN4	89,90
TRC8/RNF139	12	US2*, DERL1, SPP	AUP1	DERL1(?)	UBE2J2, UBE2G2	-	79,119,205
RNF145	14	INSIG1, INSIG2	FAF2/UBXD8	-	UBE2G2	-	32,76
RNF170	3	ERLIN1, ERLIN2	-	-	-	-	88,125
RNF185	2	TMEM259	-	TMUB1, TMUB2	UBE2K, UBE2D3	-	86
TMEM129	3	US11*	-	DERL1, DERL2, VIMP	UBE2J2 UEB2K	-	110,157
RNF26	4	-	TMEM33, TMEM43, ENDOD1, TMED1	-	UBE2J1	-	26,136
BFAR	3-4	-	-	-	-	-	198,200
TRIM13	1	-	-	-	-	-	224
ZNRF4	1	-	-	-	-	-	140
RNF19B	2	-	-	-	UBE2L3, UBE2L6	-	198,225
RNF133	1	-	-	-	UBE2J1	-	226
RNFT1	6	-	-	-	-	-	198
yeast	**TMDs**	**Recognition**	**Organisation**	**Dislocation & Retrotranslocation**	**E2**	**Cdc48/VCP recruitment**	**Reference**
Hrd1	8	Hrd3, Yos9, Kar2, Htm1	Usa1, Cue1	Der1, Dfm1*	Ubc6, Ubc7	Ubx2, Dfm1	61,105,171,172,227–232
Doa10	14	-	Cue1	Dfm1*	Ubc6, Ubc7	Ubx2, Dfm1*	31,133,134,151,231
Asi1-Asi3	5-5	Asi2	-	-	Ubc6, Ubc7	-	138,139,233

Table legend:

ERAD routes defined by ER-E3s and their associated proteins in mammals and yeast. TMDs gives the number of (predicted) transmembrane domains in the E3 enzymes. Co-factors with documented function in substrate recognition (Recognition), assembly of the E3 complex (Organisation), mobilisation of substrates from the ER (Dislocation and Retrotranslocation), substrate ubiquitylation (E2) and the recruitment of the Cdc48/VCP AAA-ATPase are given for each E3 complex. Asterisks refer to proteins with weak or transient binding to the E3 complexes (Dfm1) or to factors that are encoded by pathogens and are not expressed in uninfected cells (US2, US11).

Mammalian proteins:
AUP1: ancient ubiquitous protein 1
BFAR: bifunctional apoptosis regulator
BiP: immunoglobulin heavy chain-binding protein homolog
CGRRF1: cell growth regulator with RING finger domain protein 1
DERL1, DERL2, DERL3: Derlin 1, 2, 3, Der1-like proteins 1, 2 and 3
EDEM1, EDEM2 and EDEM3: ER degradation enhancing alpha-mannosidase-like proteins 1, 2, and 3
ENDOD1: endonuclease domain-containing 1

ERDJ5: ER DNA J domain-containing protein 5
ERLIN1, ERLIN2: ER lipid raft-associated proteins 1 and 2
FAF2/UBXD8: Fas associated factor family member 2/UBX domain-containing protein 8
FAM8A1: family with sequence similarity 8, member A1
gp78/AMFR: glycoprotein 78/autocrine motility factor receptor
HERPUD1, HERPUD2: homocysteine inducible ER proteins with ubiquitin like domain 1 and 2
HRD1: HMG-CoA reductase degradation 1
OS9: amplified in osteosarcoma 9
INSIG1, INSIG2: insulin induced gene 1 and 2 proteins
LMBRL: Limb region 1 protein homolog-like
MARCH6/TEB4: membrane-associated RING finger protein 6/
RNF133: RING-finger protein 133
RNF145: RING-finger protein 145
RNF170: RING-finger protein 170
RNF185: RING-finger protein 185
RNF19B: RING-finger protein 19B
RNF26: RING-finger protein 26
RNF5/RMA1: RING-finger protein 5/ RING membrane-anchor 1
RNFT1: RING finger and transmembrane domain-containing protein 1
SEL1L: suppressor of lin-12-like protein 1
SPP: signal peptide peptidase
TMED1: transmembrane emp24 domain-containing protein 1
TMEM33: transmembrane protein 33
TMEM43: transmembrane protein 43
TMEM259: transmembrane protein 259
TMUB2: transmembrane and ubiquitin-like domain-containing protein 2
TRC8/RNF139: translocation in renal carcinoma on chromosome 8/RING-finger protein 139
TRIM13: tripartite motif-containing protein 13
UBAC2: ubiquitin-associated domain-containing protein 2
UBE2J1, UBE2JJ2, UBE2JG2, UBE2JD3, UBE2JK, UBE2L3, UBE2L6, UBE2JN: ubiquitin E2 enzymes J1, J2, G2, D3, K, L3, L6, and N
UBXN4, UBXN6: UBX domain containing proteins 4 and 6
US2, US11: Herpes simplex proteins US2 and 11
VIMP: VCP interacting membrane protein
XTP3B: XTP3-transactivated protein B
ZNRF4: Zinc/RING finger protein 4

yeast proteins:
Asi1, Asi2, Asi3: amino acid sensor-independent proteins 1, 2, and 3
Cue1: coupling of ubiquitin conjugation to ER degradation
Der1: degradation in the ER 1
Dfm1: Der1-like family member protein 1
Doa10: degradation of alpha protein 10
Hrd1: HMG-CoA reductase degradation 1
Hrd3: HMG-CoA reductase degradation 3
Htm1: homologous to mannosidase 1
Kar2: karyogamy-deficient 2
Ubc6, Ubc7: ubiquitin conjugating enzymes 6 and 7
Ubx2: UBX domain containing protein 2
Usa1: U1-Snp1 Associating 1
Yos9: yeast OS9 homolog

Glossary

26S proteasome: ubiquitin specific protease complex that specifically recognizes ubiquitylated proteins and cleaves them into peptide fragments.

AAA-ATPase: protein containing a conserved motif that is organized in multimeric (typically homo-hexameric) assemblies with a central pore; structural re-arrangements within these complexes upon coordinated ATP hydrolysis thread protein substrates through the pore and unfold them thereby extracting them from cellular structures like protein complexes, DNA, or membranes.

Ceramide: lipid molecules built up of a N-acetylspingosine and a fatty acid that serve as second messengers and play a role in the structural organisation of cellular membranes.

De-ubiquitylating enzymes (DUBs): ubiquitin-specific proteases that cleave ubiquitin from ubiquitylated proteins.

Degron: an amino acid sequence or structural motif within a protein that targets it to an E3 enzyme for ubiquitylation and degradation; degrons are inherent elements that, when fused to a non-related protein in the same cellular compartment, confer UPS-mediated degradation

Dislocation: eviction of transmembrane domain/s from lipid bilayer during ERAD

E1 enzyme: activates ubiquitin for the transfer onto substrates by forming a thioester linkage with the C-terminal glycine residue in ubiquitin and a cysteine residue in its active site centre upon hydrolysis of ATP

E2 enzyme: also termed ubiquitin conjugating enzyme; conserved group of enzymes that is charged with a ubiquitin thioester on their active site cysteine residues by E1 enzymes and either directly conjugate ubiquitin onto substrates or transfer the ubiquitin thioester onto a dedicated E3 enzyme.

E3 enzyme: also termed ubiquitin ligases; single proteins or protein complexes that recognize substrates and either activate E2 enzymes for attaching ubiquitin onto a

878 substrate (RING-type E3) or themselves form a thioester with ubiquitin and then
879 transfer ubiquitin (HECT- and RBR-type E3s)
880 **ER-lysosomal degradation (ERLAD) pathway**: Collection of mechanistically diverse
881 ER-phagy pathways that captured misfoled ER proteins in double membrane
882 autophagosomes and deliver them to degradative organelles for proteolysis.
883 **Lectin:** glycan binding protein that recognize specific carbohydrate molecule but do
884 not harbour enzymatic activity.
885 **Linchpin residue**s: A particular amino acid position adjacent to the ultimate cysteine
886 residue in most RING-finger domains that interacts with both the E2 enzyme and the
887 ubiquitin thioester thereby delimitating the space available for the ubiquitin thioester
888 and coordinatesfacilitating its conjugation to a substrate.
889 **Mannose-6-phosphate receptor homology (MRH) domains**: regions in a protein
890 that binds a specific structure within a high-mannose N-glycan.
891 **Microautophagy:** Highly conserved cellular process causing the engulfment of
892 cytoplasmic material with lysosomal/vacuolar membranes and its degradation
893 **Molecular glues**: a typically small molecule that assists in establishing a physical
894 association of two proteins that do not normally interact.
895 **Poly-ubiquitylation**: decoration of substrates with complex poly-ubiquitin molecules
896 by linking the C-terminal glycine residues of ubiquitin molecules to one of seven
897 internal lysine or the N-terminal methionine residue of already conjugated ubiquitin
898 moieties.
899 **Really Interesting New Gene (RING) finger domain:** a structural protein motif of
900 about 30 to 60 amino acids typically containing seven cysteine and one histidine
901 residues for complexing two zinc cations that is characteristically found in a class of
902 E3 ubiquitylating enzymes.
903 **Retrotranslocation**: transport of lumenal entire proteins or domains of misfolded
904 proteins across the ER membrane into the cytoplasm during ERAD

Rhomboid intramembrane proteases Structurally conserved family of integral membrane serine proteases that mostly target membrane proteins and cleave them at sites within or close to the lipid bilayer.

Rhomboid pseudoprotease: catalytically inactive variant of inner-membrane rhomboid proteases sharing with their catalytic-active relatives the overall membrane topology and fold.

Sec61 translocon complex: An ER-resident protein complex composed of the Sec61alpha, Sec61beta, and Sec61gamma subunits that forms a polypeptide conducting channel through the ER membrane for the import of secretory proteins into the ER.

Ubiquitin- proteasome system (UPS): sum of all proteins involved in the generation, decoding and processing of ubiquitin and ubiquitylated proteins.

Figure legends

Figure 1: Overview of the principal steps in ERAD.

Step 1: Proteins destined for the secretory pathway enter the ER lumen (a) or are integrated into the ER-membrane (b) in an unfolded state, fold to attain their native structure, and eventually assemble into complexes. Protein maturation can be compromised by mutations in the encoding genes, environmental stress, or unbalanced synthesis of partner proteins. Moreover, conditionally unstable proteins to be targeted for degradation in response to cell signals may adopt characteristics that simulate a defective fold. Polypeptides that misfold, are targeted for degradation by leveraging features such as prolonged association with folding enzymes, exposed hydrophobic patches, or a specific N-linked oligosaccharide structure to yield degrons. Soluble proteins in the ER lumen are primarily routed to the HRD1 E3 complex, whereas integral membrane proteins are processed via multiple ERAD paths. **Steps 2 and 3**: ER membrane bound E3s (ERAD complexes) promote transport of these polypeptides to the cytoplasm (Step 2) via retrotranslocation (a) or dislocation (b) and decorate them with polyubiquitin adducts (Step 3). Integral membrane proteins may be directly ubiquitylated by E3 enzymes at cytoplasmically-exposed regions without preceding dislocation or in their lumenal domains once exposed. **Step 3** is tightly connected to **steps 4 and 5**: the extraction of the polyubiquitylated substrates by the ubiquitin-dependent AAA-ATPase Cdc48/VCP (step 4) and rearrangements of the ubiquitin signal on the substrate (remodelling) by de-ubiquitylating and ubiquitin chain elongating enzymes (step 5). Aggregation of the emerging substrate is prevented by ubiquitin binding proteins, which also assist in the subsequent transfer to the proteasome. Finally, at **step 6** cytoplasmic proteins direct the newly polyubiquitylated substrate to 26S proteasomes to be degraded. Different factors may be employed for the recognition and recruitment of an individual ERAD substrate (step 1). Distinct ERAD paths then utilize a discrete set of components and mechanistic strategies for step 2 whereas they all employ a shared set of E2 enzymes in step 3. Processing of

all membrane-bound ERAD substrates merges at the extraction of substrates from the ER by Cdc48/VCP (step 4). This and the subsequent steps 5 and 6 are also shared with other ubiquitin/proteasome-dependent processes. c) Selected ERAD E3s are also involved in the ubiquitylation of cytosolic and nuclear proteins that often expose specific degradation signals. Typically, these substrates are directly routed to proteasomes without unfolding by Cdc48/VCP.

Figure 2: ER-E3 complexes follow different strategies for substrate recruitment.

a. After insertion into the ER, secretory proteins destined to be modified with an asparagine- (N-) linked glycan are first decorated with a $GlcNAc_2Man_9Glc_3$ polysaccharide structure. This becomes rapidly de-glucosylated into a $GlcNAc_2Man_9$ saccharide by Glucosidases. ER-Mannosidase I (ERManI) then slowly removes a proximal Mannose residue generating a $GlcNAc_2Man_8$ glycan during folding and maturation of the protein. Proteins leaving the ER are equipped with this glycan species. Futile folding attempts marked by multiple cycles of binding/unbinding events of ER chaperone BiP and the protein oxidoreductase PDI eventually recruit BiP-associated disulfide reductases such ERdj5, TXNDC11, and ERp46 to the protein, which themselves interact with the EDEM mannosidases. The EDEMs remove further mannose moieties from the glycan thereby generating a polysaccharide exposing a terminal alpha1,6 mannose residue ($GlcNAc_2Man_{5-7}$). This is the glycan structure recognized by OS-9 and XTP3-B ERAD substrate receptors, which assemble into the HRD1 E3 complex by binding to SEL1L. After binding to OS-9-XTP3-B, the substrate aligns with a proteinaceous conduit in the ER membrane possibly formed by the TMDs of the rhomboid pseudoprotease Derlin2, HRD1, and HERP as deduced from topological similarities to the yeast complex.

b. Non-glycosylated soluble proteins are recruited to the HRD1 E3 complex by SEL1L, which either directly binds unfolded proteins or recognizes them indirectly via

interaction with BiP. Dislocation into the cytoplasm also involves the Derlin2-HRD1 channel. Recruitment of membrane proteins by HRD1 follows either the same rule as for soluble glycosylated and non-glycosylated proteins but may also involve direct binding of TMDs in the substrate to TMD sections of the HRD1 E3 complex (not shown).

c. High levels of sterols like cholesterol prevent the degradation of INSIG1 and INSIG2 – regulators of lipid metabolism. INSIG1 then recruits 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) to the E3 gp78 for ubiquitylation and ERAD to reduce cholesterol biosynthesis. Both INSIG1 and INSIG2 also serve as adaptors for HMGCR ubiquitylation by RNF145 (not shown).

D. The yeast Doa10 ER-E3 targets integral membrane proteins and soluble proteins from the cytoplasm and nucleoplasm. Most of these proteins expose amphipathic helices, which are recognized by cytoplasmic Hsp70- and Hsp40-type chaperones. Binding of the chaperones is also required for degradation. A C-terminal extension in Doa10 (CTE) may serve as a recruitment platform for the chaperone-bound substrates. The topologically-related ER-E3 MARCH6 in mammalian cells contains a strongly related CTE, which may be involved in a similar activity [82]. In yeast, recognition of tail-anchored membrane proteins by Doa10 involves specific attributes in or near their membrane-embedded domain and does not require cytoplasmic chaperones or the CTE region. The mechanisms underlying direct detection of TMD sections by Doa10 are not known as indicated by a question mark.

E. Aggregation-prone soluble proteins in the ER lumen are bound by a complex containing ERLIN1 and 2 and the rhomboid protease RHBDL4. Cleavage of the substrate precedes its transport into the cytoplasm, where it is degraded by proteasomes. It is unclear, whether this process involves the activity of an E3 enzyme.

F. Upon binding to inositol-tri-phosphate (IP_3) the tetrameric IP_3R receptor undergoes conformational changes in the ER-luminal domains. The ERLIN1 and 2 proteins recruit

these aberrant conformers to the RNF170 E3 for ubiquitylation. The Involvement of the RNF170 partner protein Derlin1 in IP_3R degradation remains to be shown.

Figure 3: Topological organization of the Hrd1 ER-E3 complex.

A. Cartoon representing the structural build of assorted constituents (TMDs of Hrd1, Der1, Usa1 and the soluble portions of Hrd3 and Yos9) of the yeast Hrd1 complex reconstituted from purified components as determined by single particle cryo-EM. Distortion of lipids around the transmembrane domains of Hrd1 and Der1 locally reduces the thickness of the ER membrane. At the ER luminal side, Der1 forms a shallow hydrophilic pit in the membrane into which a substrate polypeptide docking at the Hrd3 and Yos9 receptor proteins immerses as a loop. A proteinaceous channel lined by TMDs 2 and 5 of Der1 and TMDs 3 and 8 of Hrd1 then facilitates transport of the substrate into the cytoplasm. TMD 8 and 3 of Hrd1 and TMD 2 and 5 of Der1 (and probably also parts of Usa1) also form a cytoplasmic cavity that thins the membrane and probably decreases the energy barrier for dislocation. Location of selected Hrd1 and Der1 TMDs is indicated. The position of an arrested substrate was derived from *in vivo* crosslinking data and is indicated as an orange line. The crosslinking experiments cover only selected areas of the investigated proteins and the position of the substrate can only be inaccurately given for most regions (indicated by a dashed line). A glycan structure on the substrate and its association with the oligosaccharide binding MRH domain of Yos9 is given. It is important to note that cryo-EM structures were determined from reconstituted particles lacking most of the cytoplasmically-exposed parts in the Hrd1 complex, and hence the holo-complex may adopt a different conformation. Figure adapted from [93].

B. Putative configurations of the yeast Hrd1 channel for the dislocation of TMD-containing proteins. Der1 is dispensable for the degradation of TMD proteins in yeast and the rhomboid pseudoprotease Dfm1 may be utilized instead. It is unclear, whether

Dfm1 directly teams up with Hrd1 for channel formation (left;[231]), or independently forms a separate pore by oligomerization (middle; [234]). Dfm1 function is bypassed by elevated levels of Hrd1 [235]. Since Hrd1 is organized in multimeric assemblies [230], it may also form a channel on its own for the removal of TMD substrates (right; [100]). Whether this is conserved in mammals is unclear.

Figure 4: Polyubiquitylation drives substrate extraction from the ER through the Cdc48/VCP AAA-ATPase complex.

a. Most ER-E3 complexes employ distinct E2 enzymes in subsequent ubiquitylation reactions to generate a Lys48 (K48)-linked polyubiquitin signal on their targets. The yeast E2 enzyme Ubc6 (UBE2J1 and UBE2J2 in mammals) first attaches a single ubiquitin moiety to the substrate. Remarkably, these enzymes conjugate ubiquitin not only to lysines, but also to other amino acids like serine and threonine residues. At least Ubc6 presents ubiquitin in the highly reactive, closed state and does not need further stimulation by a RING domain for the transfer to the substrate [144]. In yeast, the E2 enzyme Ubc7 (mammalian UBE2G2) is recruited to the ERAD E3 enzymes by binding to its co-factor Cue1 (AUP1 in mammals or a dedicated binding site in gp78). Binding to Cue1 via the U7BR region (G2BR in AUP1 or gp78) also induces structural changes in the E2 enzyme thereby increasing its ubiquitylating activity but not forcing the ubiquitin thioester into a closed conformation and thereby leaving it in a rather inactive state for the transfer onto a substrate. Dedicated E3 enzymes harbouring a RING domain with a particular conformation in the linchpin position force ubiquitin on Ubc7 (and possibly also UBE2G2) into the closed state and stimulate direct conjugation on lysine residues of substrates. Ubc7 then synthesizes a K48-linked polyubiquitin chain on priming ubiquitin moieties. At the early steps of chain building, the RING domain of the E3 enzyme largely stimulates the activity of Ubc7. Binding of the CUE domain of Cue1 (and AUP1 or gp78) to the chain positions Ubc7 for ubiquitin transfer and facilitates elongation independently of the RING domain [236].

b. The homo-hexameric AAA-APTase Cdc48 (VCP in mammals) complex is recruited to the ER-E3s via multiple binding sites in the E3s or in associated co-factors like yeast Ubx2 and Dfm1 (UBXD8, Derlin1, Derlin2 and VIMP in mammals). The Cdc48 adaptor proteins Npl4 and Ufd1 dock to the substrate entry site of the Cdc48 complex and bind K48-linked polyubiquitin on the substrate (**Step 1**). A ubiquitin moiety within the polyubiquitin chain then unfolds in a cleft in Npl4 and enters a narrow channel built by the AAA assembly, where it interacts with channel-exposed regions in the D2 domains (**Step 2**). Orchestrated ATP hydrolysis in the D2 domains drive structural rearrangements in the hexameric complex and cycles of substrate binding and release generate a pulling force (**Step 3**). This threads ubiquitin moieties distal to the substrate and the attached substrate through the narrow pore and unfolds them. At the exit site of the Cdc48 conduit, ubiquitin is thought to regain its structure, whereas binding of ancillary factors like ubiquitin-binding proteins Rad23 and Dsk2 (in mammals BAG6 and RAD23) prevents folding/aggregation of the substrate (**Step 4**). Ubiquitin that did not enter the Cdc48 pore is probably cleaved off by the Otu1 de-ubiquitylating enzyme (YOD1 in mammals), which may also contribute to detach the substrate from the Npl4 receptor. In yeast, the functional interplay of the Ufd2 E3-like enzyme and the Ufd3 DUB at the substrate exit site of Cdc48 most likely generates the ubiquitin signature on the substrate required for proteasomal targeting (**Step 5**). In mammals, the RNF126 E3 enzyme that is associated with BAG6 serves this purpose. Delivery to the proteasome is facilitated by the ubiquitin binding proteins Rad23 and Dsk2 in yeast and RAD23 and BAG6 in mammals, which bind ubiquitylated proteins and the proteasome (**Step 6**).

Box 1*: Exploitation of ERAD by viruses and other pathogens*

Many pathogens have evolved strategies to leverage ERAD within their host cell to promote their survival (reviewed in [237]). One well-studied example is human

cytomegalovirus (HCMV), which encodes two proteins US2 and US11, acting as recruitment factors that cause premature degradation of MHC Class I (MHC-I)[238]. US2 and US11 hijack distinct ERAD paths to downregulate MHC-I. US2 brings MHC-I to TRC8 and UBE2G2 for ubiquitylation [205,239]. How ubiquitylated MHC-I is then dislocated from the ER is unclear. Signal peptide peptidase associates with US2 and TRC8 [121,239], but it is dispensable for US2-mediated MHC-I degradation [240]. Instead, VCP is required, albeit not its adapter proteins NPL4 and UFD1, nor the pore-forming rhomboid pseudoprotease Derlin1 [121,241]. US11 recruits TMEM129, an ER-E3 containing a non-canonical, cysteine-only RING domain, along with UBE2J2, Derlin1 and Derlin2 to promote MHC-I ubiquitylation and dislocation [110,157]. TMEM129 also associates with VCP but its functional contribution to US11-mediated MHC-I downregulation is unclear[157]. Degradation of unassembled MHC-I molecules utilizes the HRD1 ERAD complex [73], indicating that MHC-I removal induced by US2 and US11 is a gain of function. One might envision these viral proteins as prototypes for the behaviour of "molecular glues **[G]**".

Murine gamma herpesvirus 68 expresses a RING-type E3 enzyme mK3 that integrates into the ER membrane and engages the antigen peptide transport/loading complex for MHC-I molecules called TAP[242]. mK3 then facilitates ubiquitylation of MHC-I, the TAP complex, and the adapter protein tapasin which routes them to proteasomal degradation. Degradation of MHC-I via mK3 shares mechanistic similarities with processing of ERAD substrates because mK3 appears to hijack and associate with Derlin1 and VCP [243].

HIV-1 encodes Vpu (viral protein U), which integrates into the ER membrane and binds the CD4 receptor protein precursor. Vpu also recruits a cytoplasmic E3 ligase complex built up of the SCF complex (Skp, Cullin, F-box containing complex) and the F-box protein β_{TRCP} (beta-transducin repeat containing E3 ubiquitin protein ligase) to

the ER, which promotes ubiquitylation and proteasomal degradation of CD4 [244]. VCP facilitates the extraction of CD4 from the ER [245]. Removing CD4 from the host T cell is thought to prevent over-infection by the virus and promote release of new virions. Vpu-mediated CD4 downregulation demonstrates that cytoplasmic E3s can initiate degradation of ER membrane proteins.

Transport of uncoated polyoma viruses from the ER into the cytoplasm involves selected components of the ERAD machinery [246]. These viruses enter the host cell by docking to receptor proteins at the cell surface and are internalized by endocytosis. After vesicle fusion with the ER, uncoated virus particles are released into the ER lumen. Transport of these across the ER membrane into the cytoplasm has been best studied for Simian virus 40 (SV40) and while not identical to other polyoma viruses, they follow common principles in the events in the ER lumen. The SV40 capsid is engaged by ER-resident redox proteins like PDI, Erp57 and ERdJ5, which change the structure of the virus particle by reducing and isomerizing disulfide bonds [247,248]. Consequently, the hydrophobic virus proteins VP2 and VP3 are exposed at the surface and are recognized by BiP. Binding by BiP prevents aggregation of virus particles while also facilitating recruitment to the ER membrane by binding to the SEL1L ERAD receptor. The nucleotide exchange factor Grp170 triggers release of the virus from BiP and allows insertion of the hydrophobic virus particles into the ER membrane. The particles are not further processed by the ERAD machinery but instead penetrate the ER membrane by an unknown mechanism, involving the recruitment of the membrane proteins BAP31, EMC1 (a subunit of the EMC complex that confers integration of newly-synthesized TMD-containing proteins into the ER membrane), and the activity of cytoplasmic chaperones [249].

AB-type toxins including bacterial cholera (CT) and shiga toxins (ST) and plant ricin, exploit the ERAD machinery to enter the cytosol by mimicking ERAD substrates

(reviewed in [250]). These protein toxins are secreted as a complex composed of an inactive form of the toxic A subunit and multiple B factors. The B subunits initiate entry into the host cell by docking to receptors at the plasma membrane. The A subunit dissociates from B and is internalized into vesicles, which fuse with the ER and release it into the ER lumen. Proteolytic cleavage gives rise to two fragments of the A subunit, the toxic component A1 and the A2 part, which remains associated with A1 by a disulfide bond and keeps the toxin inactive. Upon entry into the ER, the A1-A2 dimer is recognized as being unfolded and engaged by peptidyl disulfide isomerases such as PDI and ERp57. Reduction of the disulfide bond releases the A1 chain. Binding to BiP maintains A1 in an inactive form and routes it to SEL1L. How A1 then passes through the ER membrane into the cytoplasm is unknown but this process seemingly involves Derlin1 but does not rely on VCP nor the ubiquitylation of A1 [251–253].

In addition to the examples above, ERAD paths play important roles in propagation of different flaviviruses (via HRD1) [218,254,255] and more recently SARS-CoV2 (via RNF5 and RNF185) [256,257]. A growing appreciation of their roles has prompted investigations into the feasibility of ERAD paths as novel targets for antivirals [218,256].

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Ethics Declaration

Competing interests – The authors declare no competing interests.

Fig 1

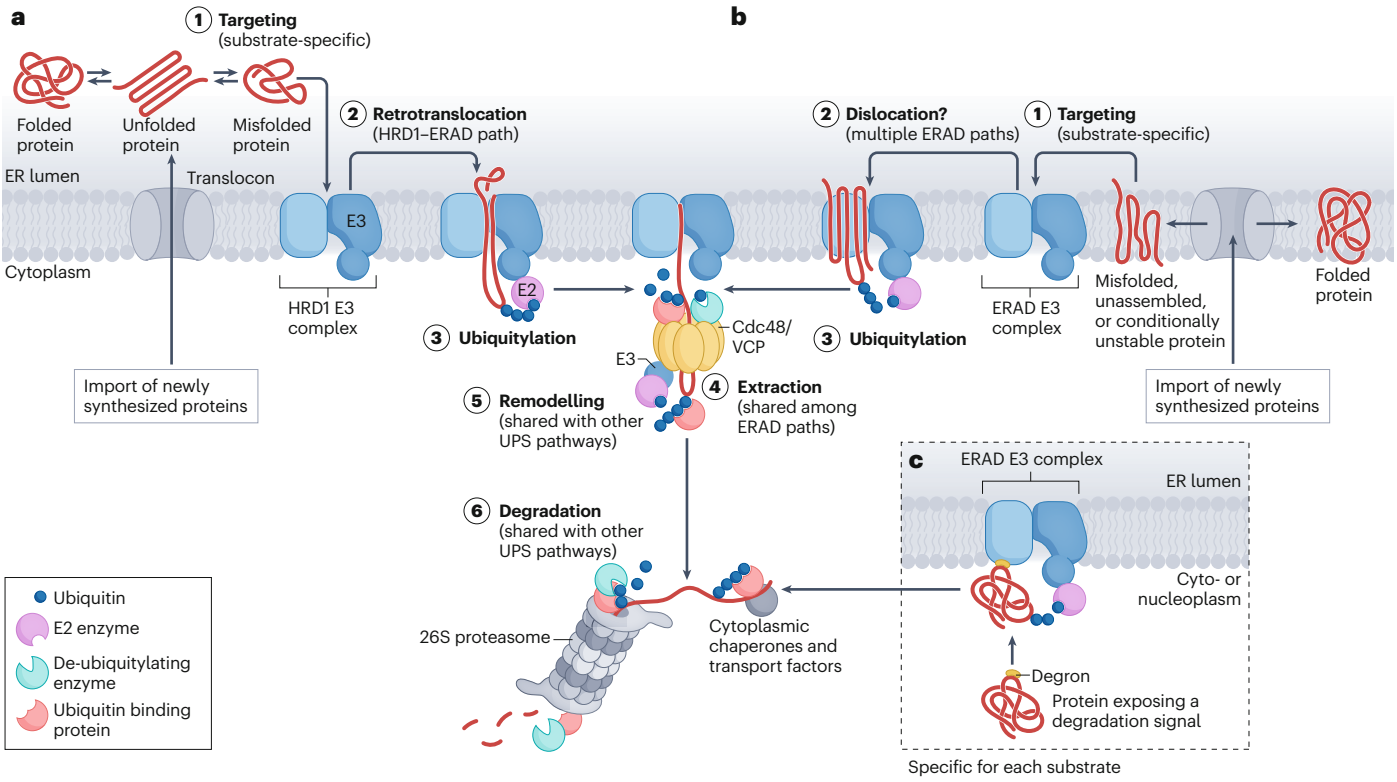

a
1 Targeting
(substrate-specific)
Folded protein
Unfolded protein
Misfolded protein
ER lumen
Translocon
Cytoplasm
2 Retrotranslocation
(HRD1–ERAD path)
E3
HRD1 E3 complex
E2
3 Ubiquitylation
Import of newly synthesized proteins
b
2 Dislocation?
(multiple ERAD paths)
1 Targeting
(substrate-specific)
ERAD E3 complex
Misfolded, unassembled, or conditionally unstable protein
Folded protein
3 Ubiquitylation
Import of newly synthesized proteins
Cdc48/ VCP
E3
4 Extraction
(shared among ERAD paths)
5 Remodelling
(shared with other UPS pathways)
6 Degradation
(shared with other UPS pathways)
26S proteasome
Cytoplasmic chaperones and transport factors
c
ERAD E3 complex
ER lumen
Cyto- or nucleoplasm
Degron
Protein exposing a degradation signal
Specific for each substrate
Ubiquitin
E2 enzyme
De-ubiquitylating enzyme
Ubiquitin binding protein

Fig 2

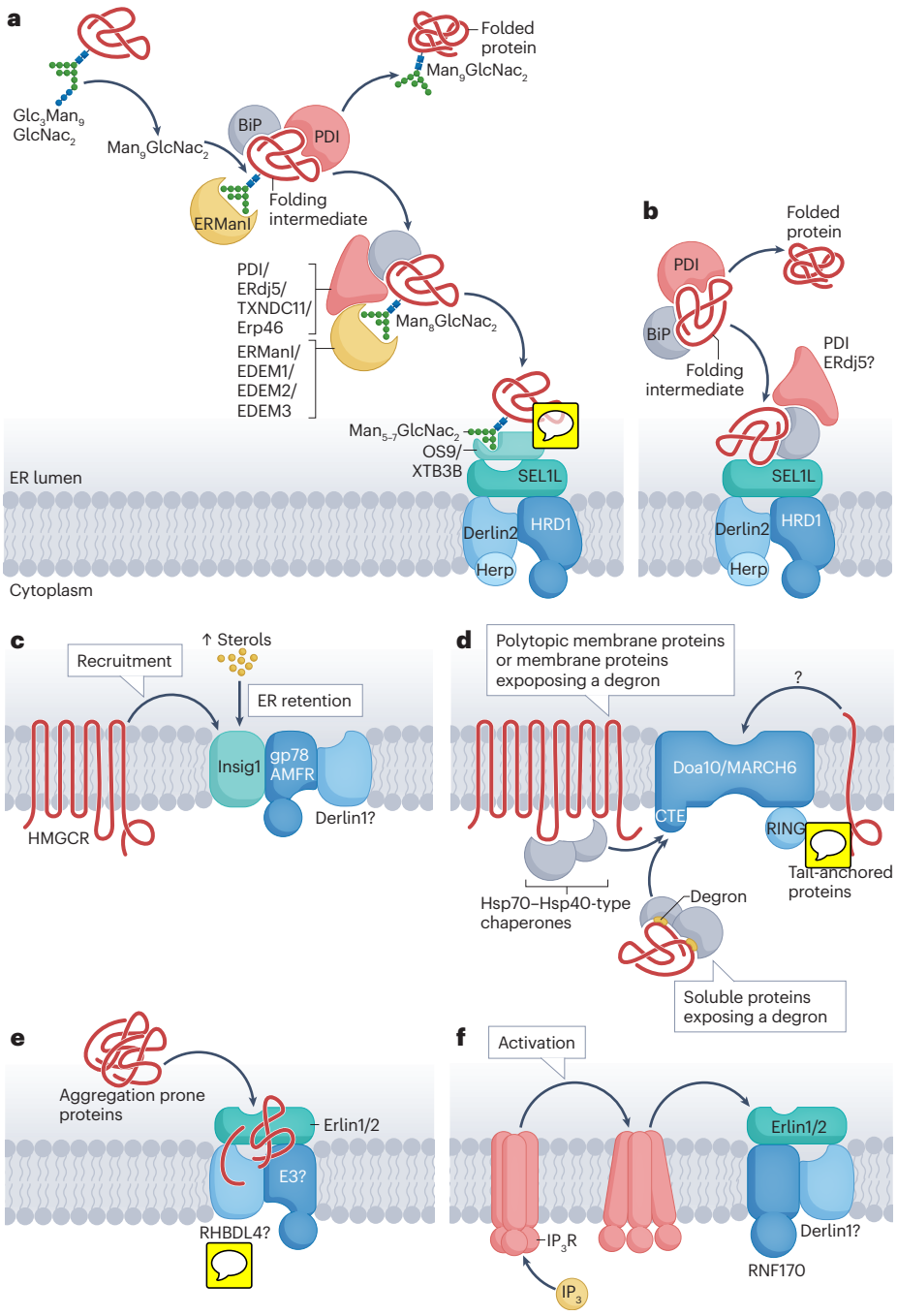

a
Glc3Man9GlcNac2
Man9GlcNac2
BiP
PDI
Folded protein
Man9GlcNac2
ERManI
Folding intermediate
PDI/ERdj5/TXNDC11/Erp46
ERManI/EDEM1/EDEM2/EDEM3
Man8GlcNac2
Man5–7GlcNac2
OS9/XTB3B
SEL1L
Derlin2
HRD1
Herp
ER lumen
Cytoplasm
b
Folded protein
PDI
BiP
Folding intermediate
PDI ERdj5?
SEL1L
Derlin2
HRD1
Herp
c
Recruitment
↑ Sterols
ER retention
Insig1
gp78
AMFR
Derlin1?
HMGCR
d
Polytopic membrane proteins or membrane proteins expoposing a degron
?
Doa10/MARCH6
CTE
RING
Tail-anchored proteins
Hsp70–Hsp40-type chaperones
Degron
Soluble proteins exposing a degron
e
Aggregation prone proteins
Erlin1/2
E3?
RHBDL4?
f
Activation
Erlin1/2
IP3R
Derlin1?
RNF170
IP3

Fig 3

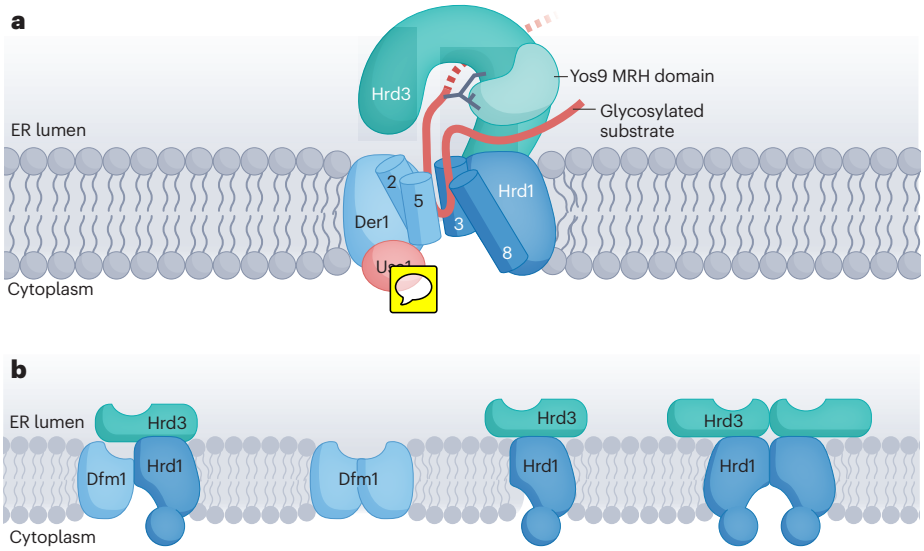
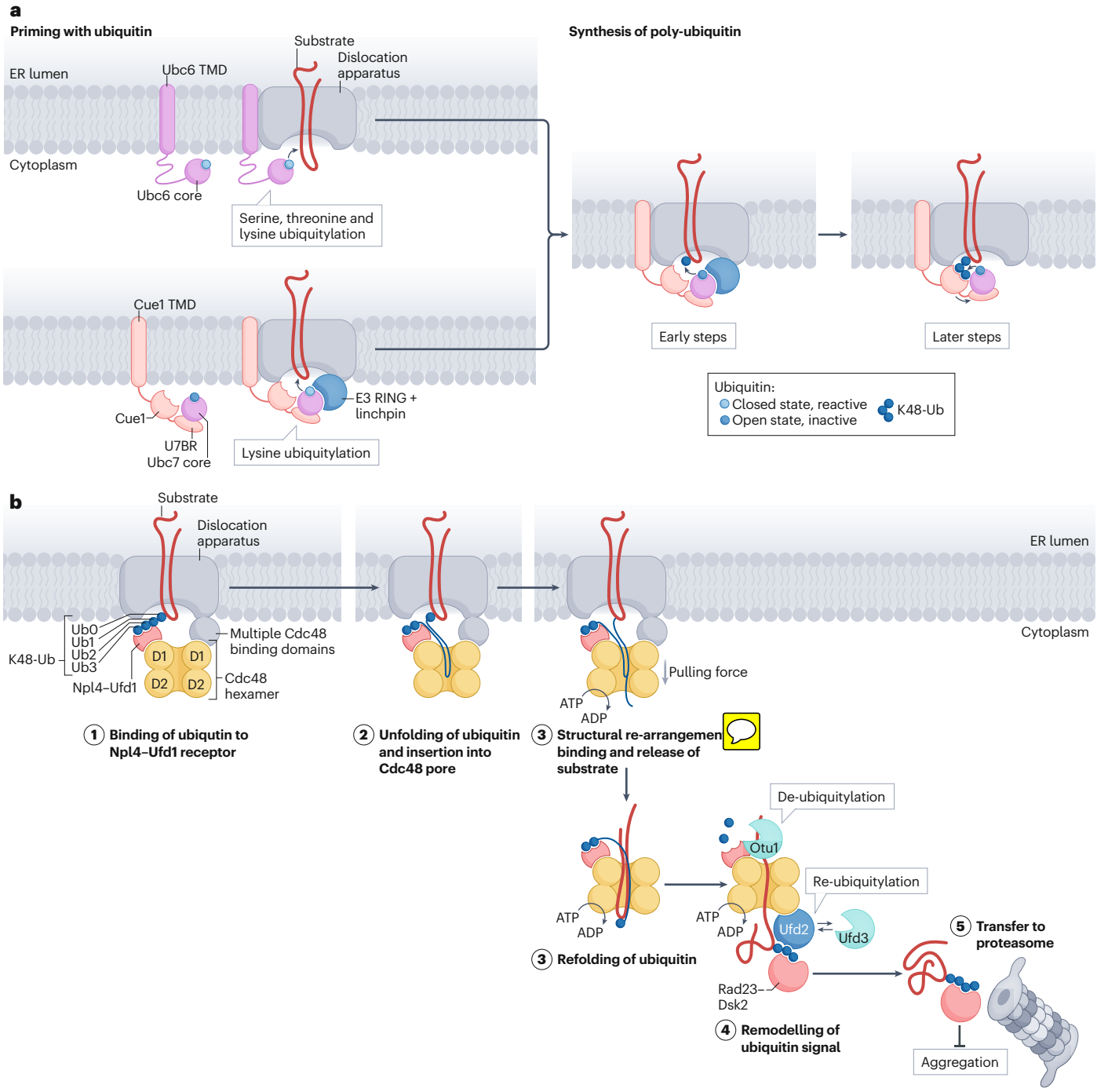

a
Hrd3
Yos9 MRH domain
Glycosylated substrate
ER lumen
2
5
Der1
3
Hrd1
8
Cytoplasm
b
ER lumen
Hrd3
Dfm1
Hrd1
Dfm1
Hrd3
Hrd1
Hrd3
Hrd1
Cytoplasm

Fig 4


a
Priming with ubiquitin
Synthesis of poly-ubiquitin
ER lumen
Cytoplasm
Ubc6 TMD
Substrate
Dislocation apparatus
Ubc6 core
Serine, threonine and lysine ubiquitylation
Cue1 TMD
Cue1
U7BR
Ubc7 core
E3 RING + linchpin
Lysine ubiquitylation
Early steps
Later steps
Ubiquitin:
Closed state, reactive
Open state, inactive
K48-Ub
b
Substrate
Dislocation apparatus
Ub0
Ub1
Ub2
Ub3
K48-Ub
Npl4–Ufd1
D1
D2
Multiple Cdc48 binding domains
Cdc48 hexamer
ER lumen
Cytoplasm
Pulling force
ATP
ADP
1 Binding of ubiqutin to Npl4–Ufd1 receptor
2 Unfolding of ubiquitin and insertion into Cdc48 pore
3 Structural re-arrangement binding and release of substrate
3 Refolding of ubiquitin
De-ubiquitylation
Otu1
Re-ubiquitylation
Ufd2
Ufd3
Rad23–Dsk2
4 Remodelling of ubiquitin signal
5 Transfer to proteasome
Aggregation