ERAD of proteins containing aberrant transmembrane domains requires ubiquitylation of cytoplasmic lysine residues

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ABSTRACT
Clearance of misfolded proteins from the endoplasmic reticulum (ER) is mediated by the ubiquitin-proteasome system in a process known as ER-associated degradation (ERAD). The mechanisms through which proteins containing aberrant transmembrane domains are degraded by ERAD are poorly understood. To address this question, we generated model ERAD substrates based on CD8 with either a non-native transmembrane domain but a folded ER luminal domain (CD8TMD*), or the native transmembrane domain but a misfolded luminal domain (CD8LUM*). Although both chimeras were degraded by ERAD, we found that the location of the folding defect determined the initial site of ubiquitylation. Ubiquitylation of cytoplasmic lysine residues was required for the extraction of CD8TMD* from the ER membrane during ERAD, whereas CD8LUM* continued to be degraded in the absence of cytoplasmic lysine residues. Cytoplasmic lysine residues were also required for degradation of an additional ERAD substrate containing an unassembled transmembrane domain and when a non-native transmembrane domain was introduced into CD8LUM*. Our results suggest that proteins with defective transmembrane domains are removed from the ER through a specific ERAD mechanism that depends upon ubiquitylation of cytoplasmic lysine residues.

KEY WORDS: ER-associated degradation, ER quality control, Membrane protein, Retrotranslocation, Transmembrane domains, Ubiquitin

INTRODUCTION
Integral membrane proteins comprise up to one-third of the human proteome (von Heijne and Gavel, 1988) and their biosynthesis involves a complex series of events including the integration of transmembrane domains (TMDs) into the lipid bilayer, folding of domains on both sides of the endoplasmic reticulum (ER) membrane, and, for multispansing or oligomeric proteins, the assembly of TMDs within the bilayer (Christis et al., 2008; Fiedler et al., 2010). Membrane proteins therefore represent a particular challenge to ER folding and quality control systems, and, perhaps unsurprisingly, many human diseases are linked to the misfolding and/or misassembly of membrane proteins (Ng et al., 2012). Misfolded proteins can disrupt ER function, and therefore it is essential that those which fail to fold or assemble correctly are quickly and efficiently removed from the ER. This is predominantly achieved through a process known as ER-associated degradation (ERAD), whereby the protein is moved back across the ER membrane for degradation by the 26S proteasome in the cytoplasm (Christianson and Ye, 2014; Vembar and Brodsky, 2008).

ERAD is initiated by recognition of the terminally misfolded protein (ERAD substrate), followed by movement across the ER membrane (retrotranslocation), ubiquitylation, extraction of the substrate from the ER membrane (dislocation) and finally targeting to the proteasome for degradation (Christianson and Ye, 2014; Ruggiano et al., 2014). These processes are mediated by a variety of ER and cytoplasmic factors that are organised around membrane-embedded E3 ubiquitin ligase complexes, which catalyse polyubiquitylation of the substrate protein and facilitate movement of the polypeptide across the ER membrane (Christianson and Ye, 2014; Ruggiano et al., 2014). Distinct combinations of ERAD factors are required for degradation of different misfolded proteins, and a key unresolved question is what dictates the requirements for degradation of the huge variety of potential ERAD substrates generated by misfolding of diverse membrane proteins. In Saccharomyces cerevisiae, the location of the folding defect is a key determinant, with distinct pathways mediating degradation of proteins with misfolded lesions in the cytoplasm (ERAD-C), membrane (ERAD-M) and lumen (ERAD-L) (Ruggiano et al., 2014). Although analogous pathways might exist in mammals, the increased number of ERAD factors, E3 ligases and potential substrates has made attempts to extrapolate these findings to mammals difficult (Christianson and Ye, 2014). Thus, defining the mechanisms through which ERAD substrates with different topologies and structural defects are recognised and retrotranslocated remains a key goal in the field.

Similar to the ERAD-L pathway described in yeast, luminal regions of membrane proteins are scrutinised by molecular chaperones such as BiP (also known as HSPA5) and lectins, including OS-9, which use exposed hydrophobic sequences and glycan-based signals, respectively, to identify misfolded conformations (Alcock and Swanton, 2009; Bernasconi et al., 2010; Burr et al., 2013; Christianson et al., 2008; Geiger et al., 2011; Otero et al., 2010) and hand them over to ERAD E3 ligase complexes that mediate retrotranslocation and polyubiquitylation. By definition, folding defects within the lipid bilayer cannot be recognised by luminal factors, and very little is known about how proteins containing non-native TMDs (potential ERAD-M substrates) are identified, ubiquitylated and removed from the ER of mammalian cells. These questions have been difficult to address in the absence of well-defined model ERAD-M substrates such as those that have allowed characterisation of the luminal quality control and ERAD machinery. The canonical mammalian ERAD-M substrate, TCRα, a type I transmembrane glycoprotein previously thought to contain a TMD-based signal for ER retention and degradation, was recently shown to translocate entirely into the ER lumen, leading to recognition by BiP and degradation through an...
defects. ERAD pathway that is mechanistically distinct from that which unassembled TMD was similarly dependent upon cytoplasmic ubiquitylation of lysine residues located in the cytoplasmic tail. Dislocation and degradation of the TMD chimera required contrast to CD8 ER quality control (ERQC) and degradation through ERAD. In luminal domain, and that the non-native TMD causes retention by non-native features such as polar residues that would normally be masked upon folding of full-length PLP (Ng et al., 2012; Swanton et al., 2003), and would therefore mimic a misassembled TMD. However, whereas wild-type CD8-HA WT* was transported to the plasma membrane of HeLa cells as expected for the correctly folded protein (Fig. 1B, left), CD8 containing the fourth TMD from PLP (CD8 LUM*) was not fully permeabilised with Triton X-100 (Fig. 1E, top). Therefore, we conclude that CD8 TMD* is integrated into the membrane, has the correct orientation (HA located in the cytoplasm and the CD8 within the lumen; Fig. 1A), and that the presence of the engineered TMD causes it to be retained intracellularly.

In order to confirm that the extracellular domain of CD8 TMD* was not misfolded, we examined whether CD8 TMD* was recognised by BiP, an Hsp70 chaperone known to bind exposed hydrophobic patches on unfolded proteins within the ER lumen (Blond-Elguindi et al., 1993; Flynn et al., 1991). To provide a control, we generated a version of CD8 (Fig. 1A, CD8 LUM*) in which folding of the extracellular domain was disrupted by mutagenising a cysteine residue known to form an intramolecular disulphide bond in the extracellular domain (Leahy et al., 1992). Co-immunoprecipitation revealed nearly twice as much BiP bound to CD8 LUM* compared to CD8TMD* (Fig. 1B,C), suggesting that CD8 TMD* exposed far fewer BiP-binding sites than CD8 LUM*. Native CD8α forms homodimers through interchain disulphide bonds (Leahy et al., 1992), and these were apparent in cells expressing CD8 WT as a 55-kDa species that was sensitive to reducing agents (Fig. S1D). CD8 TMD* also migrated as a higher molecular mass form of ~45 kDa that was lost upon reduction with dithiothreitol (Fig. S1D), consistent with formation of disulphide-linked dimers. The monomeric forms of both CD8 WT and CD8 TMD* migrated more rapidly under non-reducing conditions (Fig. S1D), suggesting that the extracellular domain of both proteins underwent oxidative folding. Thus, CD8 TMD* appears to undergo conformational maturation up to and including formation of inter- and intra-molecular disulphide bonds, indicating that the presence of the non-native TMD does not cause misfolding of the extracellular domain. Neither CD8 LUM* nor a variant possessing a second luminal mutation (CD8 LUM*W2) could be detected by an anti-CD8 monoclonal that efficiently labelled CD8 WT (Fig. S1E,F). In contrast, robust staining of CD8 TMD* was observed (Fig. S1E,F), providing further evidence that the extracellular domain of the chimera was properly folded. However, given that the epitope for this antibody is not known, a caveat to this interpretation is that the mutated cysteine residue in CD8 LUM* could form part of this epitope.

Taken together, these results show that CD8 TMD* is integrated into the membrane, correctly oriented, and possesses a folded extracellular domain. Thus, we conclude that determinants located in the non-native TMD sequence are responsible for intracellular retention of CD8 TMD*. Therefore, CD8 TMD* represents a suitable model protein with which to study the quality control of TMDs in the secretory pathway.
CD8\textsuperscript{TMD\*} is localised to the ER and degraded through ERAD

At steady state, CD8\textsuperscript{TMD\*} exhibited a reticular distribution typical of the ER and showed a high degree of colocalisation with the ER marker proteins BAP31 (also known as BCAP31) and calreticulin (Fig. 2A), suggesting that the non-native TMD was recognised and retained by ERQC systems. In addition, a proportion of CD8\textsuperscript{TMD\*} colocalised with markers of the ER-Golgi intermediate compartment (ERGIC) and Golgi complex (Fig. 2B), indicating that some CD8\textsuperscript{TMD\*} might be able exit the ER and reach later stages in the secretory pathway. Following exit from the ER, CD8 undergoes O-glycosylation in the Golgi (Gill et al., 2011; Jackson et al., 1993; Pascale et al., 1992a,b), allowing the intracellular transport of CD8\textsuperscript{TMD\*} to be followed by pulse-chase labelling. CD8\textsuperscript{WT} was initially synthesised as a precursor of \(\sim 25\) kDa (Fig. 2C, lane 1) that was converted into higher molecular mass forms of \(\sim 27–28\) kDa, which were in turn replaced by a broad band at \(\sim 30\) kDa during the 90-min chase (Fig. 2C, lanes 2–7). These different forms have previously been identified as the unglycosylated precursor (u), an initially glycosylated intermediate (i) and the mature glycoform (m) of CD8, respectively (Pascale et al., 1992a,b). Consistent with this interpretation, most CD8\textsuperscript{WT} remained in the ‘u’ form when the chase was carried out at either 10°C, to
**Fig. 2.** CD8^{TMD-} is localised to the ER at steady state but partially escapes to the Golgi. (A) Cells expressing CD8^{TMD-} were fixed and labelled with anti-HA and anti-BAP31, or mouse anti-HA and anti-calreticulin antibodies. (B) Cells expressing CD8^{TMD-} were fixed and labelled with anti-HA and anti-ERGIC53, or anti-HA and anti-GM130 antibodies. Scale bars: 10 µm. (C,E) Cells expressing CD8^{WT} or CD8^{TMD-} were pulse-labelled with [35S]Met/Cys for 5 min or 90 min as indicated and chased for up to 90 min in the presence of unlabelled Met and Cys. CD8 was immunoprecipitated with anti-HA antibodies, and analysed by phosphorimaging. (D,F) Cells expressing CD8^{WT} or CD8^{TMD-} were pulse-labelled with [35S]Met/Cys for 5 or 60 min and chased for 90 min at the indicated temperature and analysed as above. (G) Lysates of cells expressing CD8^{WT} or CD8^{TMD-} were analysed by immunoblotting with anti-HA antibodies. u, i and m indicate the unglycosylated precursor, an initially glycosylated intermediate, and the mature glycoform of CD8, respectively.
inhibit ER exit (Tartakoff, 1986), or 15°C, to inhibit transport beyond the ERGIC (Fig. 2D, lanes 2 and 3), whereas the ‘i’ and ‘m’ forms predominated after chasing at 20°C, which allows transport as far as the trans-Golgi network (Matlin and Simons, 1983), or at 37°C (Fig. 2D, lanes 4 and 5). CD8TMD* was also observed as a single species immediately after the pulse (Fig. 2E, lane 2), and several additional higher molecular mass forms appeared after 15–30 min of chase, coinciding with a decrease in the intensity of the precursor (Fig. 2E, lanes 5–8). Their relative migration on SDS-PAGE and comparison with CD8WT suggested that these species represent the unmodified precursor and O-glycosylated ‘i’ and ‘m’ forms, respectively (Fig. 2E). Performing the chase at reduced temperature confirmed that the higher molecular mass forms were only produced under conditions that permit trafficking to the Golgi (Fig. 2E, lane 6). At steady state, CD8TMD* was observed primarily as the unmodified precursor with a smaller amount of the intermediate ‘i’ and very little, if any, of the ‘m’ form (Fig. 2G). Treatment of cells with brefeldin A to redistribute Golgi-resident enzymes to the ER, and very little, if any, of the CD8WT was converted all the CD8TMD* into higher molecular mass forms (Fig. S1G), providing further evidence that these represent O-glycosylated species. Taken together, these results show that CD8TMD* is not stably retained in the ER because a proportion undergoes post-translational modification in the Golgi. This might be comparable to other non-native proteins that are known to partially escape the ER and subsequently undergo retrieval from the Golgi complex (Caldwell et al., 2001; Hammond and Helenius, 1994; Pan et al., 2011; Vashist et al., 2001).

Having established that CD8TMD* was recognised and retained by quality control mechanisms in the early secretory pathway, we next examined whether the non-native TMD caused degradation of CD8 using cycloheximide chase experiments. Cells expressing CD8WT or CD8TMD* were treated with cycloheximide to block protein synthesis, chased in the presence of cycloheximide for 0–240 min, then the amount of protein remaining at each time point was determined by immunoblotting. As shown in Fig. 3A, the level of CD8WT remained relatively constant over the 4-h chase, consistent with this being a stable plasma membrane protein. In contrast, CD8TMD* was rapidly lost following addition of cycloheximide (Fig. 3A), suggesting that the chimera was degraded over time. Quantification revealed that the half-life of CD8TMD* was ~120 min compared to well over 240 min for the wild type (Fig. 3B). Aside from an initial increase in levels of the ‘i’ form, the two major forms of CD8TMD* decreased with comparable kinetics (Fig. 3A). Importantly, degradation of CD8TMD* was not due to its prolonged residence in the ER, because CD8 possessing a dilsyne ER retrieval motif (CD8KKK; Jackson et al., 1993) was stable despite being localised at the ER (Fig. S2A). Thus, in addition to causing retention in the ER, the non-native TMD present in CD8TMD* constitutes a signal for rapid degradation.

In order to identify the pathways that mediate degradation of CD8TMD*, cycloheximide chase assays were carried out in the presence of a proteasome inhibitor (Z-LLF-CHO; PSI3) or a combination of leupeptin and pepstatin A to inhibit lysosomal proteolysis (Fig. 3C,D). Treatment with PSI3 substantially slowed the loss of CD8TMD* during the chase, with ~75% of the protein remaining after 120 min compared to just 45% in the absence of inhibitors (Fig. 3C). Similar results were obtained with the proteasome inhibitors bortezomib and MG132 (Fig. S2B,C), suggesting that proteasomes mediate degradation of CD8TMD*. In contrast, the lysosomal inhibitors did not obviously alter the rate at which CD8TMD* was lost during the 2-h chase period (Fig. 3C,D). Hence, CD8TMD* is degraded primarily through a proteasomal route, which, given its ER localisation, is most likely to be the ERAD pathway. Notably, proteasome inhibition stabilised the Golgi modified ‘i’ form as well as the major ‘u’ form of CD8TMD* (Fig. 3C). This indicates that CD8TMD*, which reached the Golgi, might ultimately be degraded through ERAD, supporting the view that retrieval mechanisms return some of the escaped protein to the ER. Consistent with this interpretation, CD8TMD* accumulated in the ER of proteasome inhibitor-treated cells (Fig. S2D), as would be predicted for an ERAD substrate. Under these conditions, a prominent juxtanuclear localisation of CD8TMD* was observed (Fig. S2D). This might reflect accumulation in the ER quality control compartment (ERQC), a subdomain of the ER specialised for the recognition and degradation of misfolded membrane and secretory proteins (Kamhi-Nesher et al., 2001; Leitman et al., 2014).

ERAD typically involves polyubiquitylation of substrate proteins prior to proteasomal degradation. To test whether CD8TMD* was ubiquitylated, cells expressing the chimera were treated with PSII to block proteasomal degradation of ubiquitylated proteins, and CD8TMD* was isolated by immunoprecipitation. Immunoblotting immunoprecipitated material with anti-ubiquitin antibodies revealed a broad smear of high-molecular-mass bands near the top of the gel, characteristic of polyubiquitin-conjugated proteins (Fig. 3E, lane 11). These species were only observed in immunoprecipitates from cells induced to express CD8TMD* (Fig. 3E, lane 5) and were far less abundant in the absence of proteasome inhibitor treatment (Fig. 3E, lane 8), despite equal loading of immunoprecipitated CD8TMD* (Fig. 3E, bottom panel). Thus, we conclude that CD8TMD* is polyubiquitylated en route to proteasomal degradation, consistent with it being a substrate for ERAD.

Although leupeptin and pepstatin A had no obvious effect on the stability or levels of the unmodified ‘u’ or intermediate ‘i’ forms of CD8TMD* in the short-term (Fig. 3C,F), we noticed that treatment with these inhibitors, or an alternative inhibitor chloroquine, caused a gradual accumulation of the higher-molecular-mass ‘m’ forms over time (Fig. 3F). Furthermore, CD8TMD* could be observed in lysosomes following treatment with leupeptin and pepstatin A (Fig. S2E), suggesting that a fraction of the CD8TMD* that escapes the ER is ultimately targeted to lysosomes for degradation.

Taken together, these results provide evidence that CD8TMD* is primarily degraded through the ERAD pathway, with lysosomal degradation serving as a backup pathway to eliminate CD8TMD*, which evades ER quality control (i.e. escapes retention, retrieval and ERAD). Hence, we conclude that CD8TMD* represents an authentic mammalian ERAD-M substrate given that it possesses a folded luminal domain, and determinants in its non-native TMD cause ER retention and proteasomal degradation.

Ubiquitylation of cytoplasmic lysine residues is required for dislocation and degradation of CD8TMD*

In order to characterise the requirements for degradation of CD8TMD*, we next examined the target sites for ubiquitylation. The cytoplasmic domain of CD8TMD* contains three lysine residues, and replacement of these with arginine residues (generating CD8TMD*3KR) led to a striking increase in the steady-state expression levels of the chimera (Fig. 4A). Cycloheximide chase assays revealed that CD8TMD*3KR was almost completely stable over the 2-h chase (Fig. 4B,C), suggesting that ubiquitylation of cytoplasmic lysine residues is required for ERAD of CD8TMD*. Indeed, very little polyubiquitylated CD8TMD*3KR was detected, even after treatment of cells with proteasome inhibitor, when ubiquitylated CD8TMD* was clearly observed (Fig. 4D).
Taken together, these results provide evidence that ubiquitylation of CD8\textsuperscript{TMD\textsuperscript{*}} on cytoplasmic lysine residues is a crucial step in the degradation of this ERAD-M substrate.

In addition to marking ERAD substrates for proteasomal degradation, ubiquitylation might be required for extraction of substrates from the ER membrane. We therefore examined whether cytoplasmic lysine residues were required for dislocation of CD8\textsuperscript{TMD\textsuperscript{*}} into the cytoplasm. Cells expressing CD8\textsuperscript{WT} or CD8\textsuperscript{TMD\textsuperscript{*}} were chased with cycloheximide in the continued presence of inhibitors for up to 2 h, then analysed as in A. (D) Protein levels were quantified as in B. (E) Cells expressing CD8\textsuperscript{TMD\textsuperscript{*}} were left untreated, or treated with leupeptin and pepstatin (L/P) or chloroquine for the indicated time. Cell lysates were analysed by immunoblotting with anti-HA and anti-α-tubulin antibodies. u, i and m indicate the unglycosylated precursor, an initially glycosylated intermediate, and the mature glycoform of CD8, respectively.

Fig. 3. CD8\textsuperscript{TMD\textsuperscript{*}} is an ERAD substrate. (A) Measurement of protein degradation by cycloheximide chase assays. Cells expressing CD8\textsuperscript{WT} or CD8\textsuperscript{TMD\textsuperscript{*}} were chased by incubation with cycloheximide for up to 4 h. Cell lysates were analysed by immunoblotting (IB) with anti-HA and anti-actin antibodies, followed by secondary antibodies labelled with infrared fluorophores. (B) The anti-HA antibody signal normalised relative to the anti-actin signal is expressed as a percentage of that present at the start of the chase. Graphs represent the means±s.e.m. of three independent experiments. (C) Cells expressing CD8\textsuperscript{TMD\textsuperscript{*}} were left untreated or treated with leupeptin and pepstatin A (L/P) or PSII for 2 h, then chased with cycloheximide in the continued presence of inhibitors for up to 2 h, then analysed as in A. (D) Protein levels were quantified as in B. (E) Cells expressing CD8\textsuperscript{TMD\textsuperscript{*}} were left untreated or treated with leupeptin and pepstatin A (L/P) or PSII for 2 h, then chased with cycloheximide in the continued presence of inhibitors for up to 2 h, then analysed as in A. (D) Protein levels were quantified as in B. (E) Cells expressing CD8\textsuperscript{TMD\textsuperscript{*}} were left untreated or treated with leupeptin and pepstatin A (L/P) or PSII for 2 h, then chased with cycloheximide in the continued presence of inhibitors for up to 2 h, then analysed as in A. (D) Protein levels were quantified as in B. (E) Cells expressing CD8\textsuperscript{TMD\textsuperscript{*}} were left untreated or treated with leupeptin and pepstatin A (L/P) or PSII for 2 h, then chased with cycloheximide in the continued presence of inhibitors for up to 2 h, then analysed as in A. (D) Protein levels were quantified as in B. (E) Cells expressing CD8\textsuperscript{TMD\textsuperscript{*}} were left untreated or treated with leupeptin and pepstatin A (L/P) or PSII for 2 h, then chased with cycloheximide in the continued presence of inhibitors for up to 2 h, then analysed as in A. (D) Protein levels were quantified as in B. (E) Cells expressing CD8\textsuperscript{TMD\textsuperscript{*}} were left untreated or treated with leupeptin and pepstatin A (L/P) or PSII for 2 h, then chased with cycloheximide in the continued presence of inhibitors for up to 2 h, then analysed as in A. (D) Protein levels were quantified as in B. (E) Cells expressing CD8\textsuperscript{TMD\textsuperscript{*}} were left untreated or treated with leupeptin and pepstatin A (L/P) or PSII for 2 h, then chased with cycloheximide in the continued presence of inhibitors for up to 2 h, then analysed as in A. (D) Protein levels were quantified as in B. (E) Cells expressing CD8\textsuperscript{TMD\textsuperscript{*}} were left untreated or treated with leupeptin and pepstatin A (L/P) or PSII for 2 h, then chased with cycloheximide in the continued presence of inhibitors for up to 2 h, then analysed as in A. (D) Protein levels were quantified as in B. (E) Cells expressing CD8\textsuperscript{TMD\textsuperscript{*}} were left untreated or treated with leupeptin and pepstatin A (L/P) or PSII for 2 h, then chased with cycloheximide in the continued presence of inhibitors for up to 2 h, then analysed as in A. (D) Protein levels were quantified as in B. (E) Cells expressing CD8\textsuperscript{TMD\textsuperscript{*}} were left untreated or treated with leupeptin and pepstatin A (L/P) or PSII for 2 h, then chased with cycloheximide in the continued presence of inhibitors for up to 2 h, then analysed as in A. (D) Protein levels were quantified as in B.
Fig. 4. Ubiquitylation of cytoplasmic lysine residues is required for dislocation and degradation of CD8
TMD*. (A) Lysates of cells expressing CD8
TMD* or CD8
TMD*3KR were analysed by immunoblotting (IB) with anti-HA and anti-actin antibodies. (B,C) Degradation of CD8
TMD* and CD8
TMD*3KR was measured by cycloheximide chase assays as in Fig. 3A,B. (D) Cells expressing CD8
TMD* or CD8
TMD*3KR were treated with or without PSII for 8 h, lysed and the CD8 immunoprecipitated (IP) with anti-HA antibodies. Samples were analysed by immunoblotting with anti-ubiquitin and anti-HA antibodies. *HC, IgG heavy chain; Ubn, polyubiquitylated proteins; T, 5% of the total input; IP, immunoprecipitated sample. (E) The anti-ubiquitin and anti-HA antibody signals from the immunoprecipitated samples were quantified and expressed as a ratio of Ubn:HA. (F) Cells expressing CD8
TMD* or CD8
TMD*3KR were left untreated or treated with PSII for 2 h prior to carbonate extraction as in Fig. 1C. Equivalent proportions of the initial lysis supernatant and the membrane fraction for each condition were analysed by immunoblotting with antibodies against HA. Loading controls and subcellular fractionation markers are shown in Fig. S3. u, the unglycosylated precursor of CD8. (G) Cells were induced to express CD8
TMD* or CD8
TMD*3KR for 48 h prior to fixation. Cells were labelled with anti-HA and anti-BAP31 antibodies. Scale bars: 10 µm. (H) Cells expressing CD8
TMD* or CD8
TMD*3KR were plated at the same time, induced at 24 h intervals up to a maximum total induction time of 96 h or left uninduced, and viable cells determined by MTT assays. The amount of viable cells remaining after induction was expressed relative to cultures that remained uninduced throughout the 96 h. Graphs represent the mean±s.e.m. of three independent experiments.
of 96 h, the number of viable cells was determined using MTT substrates containing defective TMDs (CD8*; Tanaka et al., 2002; Valetti et al., 1991). Cells containing CD8* lane 1 and 5). Treatment with PSII led to the appearance of CD8*2014; Valetti et al., 1991). Cells containing CD8* structures were positive for the ER marker BAP31, consistent with strikingly different localisation after 48 h of continued expression, confirming that the majority of the KR mutant had failed to undergo dislocation. Hence, we conclude that ubiquitylation of cytoplasmic lysine residues is an early event in CD8* degradation and is required for dislocation into the cytoplasm.

The observation that mutation of the cytoplasmic lysine residues effectively inhibited CD8* degradation allowed us to examine the consequences of failing to remove protein containing aberrant TMDs from the ER. Like CD8*, CD8*LUM* was distributed throughout the ER after 24 h of expression, as shown by colocalisation with BAP31 (Fig. S3E). The subcellular distribution of CD8*TMD* did not change dramatically over time following induction with tetracycline, and remained dispersed through the ER (Fig. 4G, top). In contrast, CD8*TMD*KR had a strikingly different localisation after 48 h of continued expression, appearing in large intracellular inclusions (Fig. 4G, bottom). These structures were positive for the ER marker BAP31, consistent with CD8*TMD*KR failing to undergo dislocation into the cytoplasm and thus remaining in the ER membrane. Although the precise nature of these inclusions is not known, they might represent a subcompartment of the ER containing aggregates of CD8*(LUM*). Further evidence for dislocation of CD8* was provided by performing radioactive pulse-chase assays (Fig. 6). Although the consequences of the misfolded luminal domain in CD8*LUM* were studied in detail, the role of ubiquitylation of cytoplasmic lysine residues for degradation is not well understood. This is interesting because it indicates that, in the context of CD8*, the presence of a non-native TMD constitutes a dominant signal that prevents ubiquitylation and dislocation of CD8*.

To examine whether the presence of the misfolded luminal domain in CD8*LUM* could overcome the requirement for ubiquitylation of the cytoplasmic region during ERAD. However, as seen for CD8*TMD*, replacing the cytoplasmic lysine residues with arginine residues inhibited ubiquitylation (Fig. 5A), confirming that CD8*LUM* is an ERAD substrate. We then tested whether the presence of the misfolded luminal domain in CD8*LUM* could overcome the requirement for ubiquitylation of the cytoplasmic lysine residues for degradation. This is interesting because it indicates that, in the context of CD8, the presence of a non-native TMD constitutes a dominant signal that commits the protein to a specific degradation pathway distinct from that which mediates degradation of substrates with solely luminal folding defects.

Although the conditions used for the cycloheximide chase assays did not induce ER stress or apoptosis (data not shown), treatment with cycloheximide might deplete short-lived ERAD factors. In order to rule out the possibility that the increased stability of CD8*TMD*KR and CD8*LUM*KR was due to depletion of factors required for degradation of these ERAD substrates (but not the other substrates examined), we examined the turnover of each of the substrates by radioactive pulse-chase assays (Fig. 6). Although the absolute rates of degradation measured using this approach were different to those obtained from cycloheximide chase assays, the requirement for lysine residues in the cytoplasmic tail was strikingly consistent. Hence, whereas mutation of lysine residues in the cytoplasmic tail markedly stabilised CD8*TMD* and CD8*LUM* (Fig. 6A,B), the mutant possessing only a luminal defect, CD8*LUM*, continued to be rapidly degraded in the absence of cytoplasmic lysine residues (Fig. 6C).

In order to test whether these findings can be extended to other proteins containing non-native TMDs, we utilised OP91, a truncated form of the GPCR rhodopsin composed of the first and part of the second TMD (Fig. 7A). A proportion of OP91 is integrated into the ER membrane and undergoes N-glycosylation...
and is subsequently degraded through a proteasomal pathway, suggesting it is a substrate for ERAD (Fig. 7C,D). As was seen for CD8αTMD*, replacing the two cytoplasmic lysine residues in OP91 with arginine residues caused a marked stabilisation of the resulting protein OP912KR (Fig. 7C,D). The lysine to arginine mutation specifically stabilised the N-glycosylated (and thus ER-integrated) forms of OP91 (Fig. 7C, OP91-1CHO and OP91-2CHO; Fig. 7D) but not the non-glycosylated form of the protein (Fig. 7C, OP91-0CHO; Fig. 7D). This is an important observation as it shows that the two cytoplasmic lysine residues are specifically required for ERAD of membrane-integrated OP91, but are not essential for proteasomal degradation of this polypeptide per se. These results provide further support for our hypothesis that ERAD substrates containing TMD defects are degraded through a distinct pathway that depends upon ubiquitylation of cytoplasmic lysine residues.

**DISCUSSION**

The mechanisms by which proteins that contain defective TMDs are recognised and removed from the ER are poorly understood. Here, we generated a model protein to study TMD quality control by replacing the endogenous TMD of the type I membrane protein CD8α with an exogenous sequence derived from a polytopic membrane protein. The non-native TMD caused recognition by ERQC systems, leading to rapid degradation through the ERAD pathway. Degradation of CD8αTMD*, as well as a second transmembrane ERAD substrate containing an unassembled TMD, was dependent upon the ubiquitylation of lysine residues within the cytoplasmic domain. In contrast, a version of CD8 containing the native TMD but a misfolded luminal domain (CD8LUM*) was efficiently degraded in the absence of cytoplasmic lysine residues. Our findings suggest that proteins with defective TMDs are removed from the ER of mammalian cells through a
distinct ERAD pathway in which ubiquitylation of cytoplasmic residues is crucial for extraction from the ER membrane (Fig. 8).

In order for CD8\textsuperscript{TMD*} to represent a suitable model for studying the quality control of TMDs, it is important that the extracellular and luminal domains are folded, and several lines of evidence support this. Previous studies have shown that the luminal domain of CD8 folds independently of the rest of the molecule, and that replacing the TMD with exogenous sequences does not necessarily prevent transport to the cell surface (Li et al., 2010; Munro, 1995). In addition, the lack of recognition by BiP binding, recognition of the extracellular domain by anti-CD8 antibodies and the formation of interchain disulphide bonds all suggest that the luminal domain of CD8\textsuperscript{TMD*} was folded. Thus, we conclude that the major structural defect in this protein lies in the non-native TMD sequence.

The non-native TMD caused CD8\textsuperscript{TMD*} to be localised to the ER and targeted for degradation through the ubiquitin-proteasome system. This sequence, derived from the fourth TMD of PLP, contains five weakly polar residues and one highly polar residue that could potentially act as signals for ER localisation and ERAD (Houck and Cyr, 2011; Ng et al., 2012). In addition, residues located between the transmembrane and cytosolic or luminal domains might influence the behaviour of integral membrane proteins, and thus defects at the TMD junctions could also contribute to the recognition of CD8\textsuperscript{TMD*} by the ERQC machinery. Charged and polar residues within TMDs have long been thought to cause ER retention and degradation of membrane proteins, including unassembled T-cell receptor (TCR) subunits, the IgE receptor, membrane-bound IgM and several engineered proteins (Bonifacino et al., 1991, 1990; Cauvi et al., 2006; Fayadat and Kopito, 2003; Li et al., 2010; Williams et al., 1990). However, recent work suggests that in at least some cases, ER retention is due to translocation of the TMD into the ER lumen, leading to recognition by BiP and targeting for ERAD (Fayadat and Kopito, 2003; Feige and Hendershot, 2013; Shin et al., 1993). Our findings with CD8\textsuperscript{TMD*}, which we show is stably integrated into the ER membrane, demonstrate that determinants embedded within the lipid bilayer can also lead to ER retention and ERAD of proteins containing non-native TMDs. Potential candidates for mediating TMD-based retention of CD8\textsuperscript{TMD*} include Rer1, calnexin and the E3 ligase Hrd1 (also known as SYVN1), which have been implicated in the ER retrieval, ER retention and ubiquitylation, respectively, of proteins containing non-native or misassembled TMDs (Cannon and Cresswell, 2001; Kaether et al., 2007; Li et al., 2010; Sato et al., 2009, 2003; Swanton et al., 2003). Future studies aimed at defining the role of these and other factors in ER retention and ERAD targeting of CD8\textsuperscript{TMD*} will provide new insight into the molecular basis for quality control of TMDs within the lipid bilayer.

In S. cerevisiae, proteins with misfolded membrane segments are degraded through a distinct ERAD-M pathway, which requires the E3 ligase Hrd1p, but not luminal factors, such as Yos9p, that target proteins with misfolded domains in the ER lumen for ERAD-L (Carvalho et al., 2006; Sato et al., 2009). It is not clear whether a similar distinction between ERAD pathways for proteins with transmembrane or luminal defects can be made in mammalian cells. Analysis of CD8\textsuperscript{TMD*} and CD8\textsuperscript{LUM*} allowed us to compare degradation of a single integral membrane protein containing defects in different regions of the polypeptide. We found that degradation of CD8\textsuperscript{TMD*} but not CD8\textsuperscript{LUM*} was dependent upon the presence of lysine residues in the cytoplasmic tail, suggesting that the location of a folding defect can influence the ERAD mechanism used. Replacement of cytoplasmic lysine residues with arginine residues inhibited ubiquitylation and extraction of CD8\textsuperscript{TMD*} from the ER membrane, leading to recognition by BiP and targeting for ERAD (Fayadat and Kopito, 2003; Feige and Hendershot, 2013; Shin et al., 1993). Our findings with CD8\textsuperscript{TMD*}, which we show is stably integrated into the ER membrane, demonstrate that determinants embedded within the lipid bilayer can also lead to ER retention and ERAD of proteins containing non-native TMDs. Potential candidates for mediating TMD-based retention of CD8\textsuperscript{TMD*} include Rer1, calnexin and the E3 ligase Hrd1 (also known as SYVN1), which have been implicated in the ER retrieval, ER retention and ubiquitylation, respectively, of proteins containing non-native or misassembled TMDs (Cannon and Cresswell, 2001; Kaether et al., 2007; Li et al., 2010; Sato et al., 2009, 2003; Swanton et al., 2003). Future studies aimed at defining the role of these and other factors in ER retention and ERAD targeting of CD8\textsuperscript{TMD*} will provide new insight into the molecular basis for quality control of TMDs within the lipid bilayer.

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the ER membrane, consistent with the view that membrane-spanning ERAD substrates are initially ubiquitylated on domains located in the cytoplasm, leading to recruitment of p97 (also known as VCP), which pulls other regions of the protein across the ER membrane to the cytoplasm for degradation (Christianson and Ye, 2014; Ye et al., 2001). In contrast, degradation of CD8LUM*, which contains the native TMD but a misfolded luminal domain, was not dependent on cytoplasmic lysine residues, indicating that the site of substrate ubiquitylation might be determined by the position of the non-native domain, at least in the context of CD8 (see model in Fig. 8). CD8LUM* might be ubiquitylated initially on lysine residues in the extracellular or luminal domain as recently shown for the unassembled MHC I heavy chain (Burr et al., 2013), or could potentially undergo non-canonical ubiquitylation of serine or cysteine residues in its cytoplasmic tail (Shimizu et al., 2010).

To our knowledge, the target sites for ubiquitylation have only been identified for three transmembrane proteins that are degraded by the cellular ERAD machinery. Nonetheless, these examples are consistent with a model whereby the location of the folding defect determines the initial site of ubiquitylation during ERAD. As observed for CD8LUM*, degradation of unassembled MHC I heavy chain, another type I membrane protein, does not require ubiquitylation of lysine residues in its cytoplasmic tail (Burr et al., 2013). Instead residues in its luminal domain are preferentially ubiquitylated during ERAD. The determinants for ERAD targeting were shown to lie solely within the luminal domain of the MHC I heavy chain, and it has been suggested that recognition by luminal adaptors initiates an early retrotranslocation event that exposes the luminal domain to the cytoplasm for ubiquitylation (Burr et al., 2013). In contrast, the type I membrane protein TCRα, which is targeted for ERAD owing to the presence of charged residues within the TMD, is ubiquitylated on residues in its cytoplasmic domain (Ishikura et al., 2010). If the TMD is translocated fully into the ER lumen, as recently suggested (Feige and Hendershot, 2013; Shin et al., 1993), both the structural defect and site for ubiquitylation would be located within the lumen. Sterol-induced ERAD of the polytopic membrane protein HMG-CoA reductase is dependent upon ubiquitylation of two lysine residues located in its cytoplasmic domain (Sever et al., 2003). ERAD targeting is dependent upon a series of sterol-regulated interactions between the TMDs of the regulatory protein Insig-1, HMG-CoA reductase and the ERAD E3 ligase gp78 (also known as AMFR), suggesting that the determinants for ERAD lie at least partly within the lipid bilayer (Lee et al., 2007; Sever et al., 2003).
As observed for CD8\(^{TMD*}\), mutation of these specific lysine residues effectively blocks dislocation and degradation of HMG-CoA reductase (Sever et al., 2003). Finally, as we show here, degradation of OP91, an ERAD substrate containing an unassembled TMD, is also dependent upon cytoplasmically located lysine residues. These ERAD substrates therefore appear to fall into two distinct classes, those that possess TMD-based ERAD signals (CD8\(^{TMD*}\), OP91, HMG-CoA reductase) and require ubiquitylation of cytoplasmic residues, and those that contain luminal defects and do not (CD8\(^{LUM*}\) and MHC I heavy chain). The variable requirement for ubiquitylation of cytoplasmic lysine residues indicates that these ERAD substrates are degraded by distinct ERAD mechanisms, either through distinct E3 ligases or alternatively by the same E3 ligase associated with different ERAD factors (Christianson et al., 2012).

On the basis of these observations, we propose a model whereby integral membrane proteins containing non-native determinants within their TMD(s) are recruited to ERAD complexes that mediate ubiquitylation on cytoplasmic regions, providing a handle for p97-mediated extraction and proteasomal degradation (Fig. 8A). In contrast, membrane proteins with folding defects in their luminal domain(s) might be targeted to ERAD complexes that mediate initial retrotranslocation of a luminal region of the polypeptide prior to its ubiquitylation and recruitment of p97 (Fig. 8B). The latter mechanism is conceptually similar to that which operates for soluble ERAD substrates (Christianson and Ye, 2014), and, as shown for unassembled MHC I heavy chain (Burr et al., 2013), is likely to involve recognition of the misfolded luminal domain by luminal adaptors such as OS-9 and XTP3-B. How proteins with TMD defects are targeted for ubiquitylation is not known, but this process could potentially involve direct recognition of signals within the bilayer by membrane-spanning E3 ligase complexes as has been shown for the Hrd1p in \textit{S. cerevisiae} (Sato et al., 2009).

Interestingly, we found that ERAD of CD8-containing defects in the TMD and in the luminal domain (CD8\(^{LUM*}\)) required ubiquitylation of cytoplasmic lysine residues. Hence, the TMD-located ERAD signal appears to be dominant in the context of this type I membrane protein. During membrane protein biosynthesis, the folding of domains within the cytoplasm, membrane and ER lumen might be interdependent (Skach, 2009), and therefore it is likely that some ERAD substrates will have defects located in more than one region of the polypeptide. Future work will be aimed at identifying whether the proposed model can be extended to explain ERAD of other misfolded membrane proteins in mammalian cells, and defining the molecular mechanisms and components that mediate degradation of proteins with defective TMDs.

**MATERIALS AND METHODS**

**Reagents and antibodies**

Antibodies against CD8 and rabbit HA were from Sigma, antibodies against BAP31, LAMP1, actin, Hsp70 and \(\beta\)-tubulin were from AbCam, anti-ERGIC53 antibody was from Alexis, mouse anti-HA antibody was from Santa Cruz Biotechnology, anti-BiP antibody was from Cell Signaling, anti-CNX and -CRT antibodies for immunoblotting were from Stressgen, anti-CRT antibody for immunofluorescence was from Thermo Scientific and anti-GM130 antibody was from BD Biosciences. Antibodies against opsin and STT3B antibodies were provided by Stephen High (University of Manchester, Manchester, UK). IRDye 800 CW and IRDye 680 RD were from LI-COR, and secondary antibodies for microscopy were from Jackson Laboratories (Stratech Scientific). The inhibitors leupeptin (Enzo Life Sciences), pepstatin A (Sigma), Z-LLF-CHO (PSII, Calbiochem), chloroquine (Sigma) and cycloheximide (CHX, Sigma) were used at 0.5 mM, 1 \(\mu\)g/ml, 10 \(\mu\)M, 5 mM and 100 \(\mu\)g/ml, respectively.
DNA constructs

CD8\textsuperscript{TMD} was generated by PCR overlap extension using human CD8\alpha and human PLP as templates to insert the TMD sequence LFIAAUTFGAAAYLSLTFMIAATYNPAL, and was cloned into pcDNA5/FRT/TO (Invitrogen). OP91, an N-terminal fragment (residues 1–91) of bovine rhodopsin (Wunderley et al., 2014), was provided by Stephen High. Other constructs were generated by site-directed mutagenesis, and were verified by DNA sequencing.

Cell culture, transfection and stable cell line generation

To generate stable cell lines, HeLa TrEx Flp-In host cells (provided by Stephen Taylor, University of Manchester, Manchester, UK) were transfected with CD8 constructs. Stably transfected cells were selected using hygromycin B (ForMedium) and blasticidin (InvivoGen). Cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% non-essential amino acids at 37°C and under 8% CO\textsubscript{2}. Experiments were performed after inducing expression with 1 µg/ml tetracycline for 10–20 h unless otherwise stated. For transient transfections, HeLa cells were transfected using Lipofectamine LTX (Invitrogen) and analysed after 16–20 h.

Cycloheximide chase analysis of protein stability

Cells were treated with 100 µg/ml cycloheximide (CHX) to inhibit protein synthesis, and harvested immediately or at 60-min intervals following addition of CHX, by lysing directly in SDS-PAGE sample buffer (30 mM Tris-HCl pH 7.6, 2% SDS, 5% glycerol, 0.01% Bromophenol Blue and 100 mM DTT). Where indicated, inhibitors were added at 2 h prior to CHX and included throughout the chase. Samples were analysed by immunoblotting with anti-HA and anti-actin or -\alpha-tubulin antibodies followed by IRDye-conjugated secondary antibodies and visualised using an Odyssey\textsuperscript{TM} Sa Infrared Imaging System (LI-COR). Anti-HA antibody signal intensity was quantified and normalised relative to the loading control then expressed as a percentage of that present at the start of the chase. For EndoH treatment, cells were lysed in sample buffer and incubated with EndoH (1000 U/ml) (New England Biolabs) at 37°C overnight.

Radiolabelling and pulse-chase analysis

Cells were grown in DMEM lacking Met and Cys (GIBCO) for 30 min, and then pulse-labelled in DMEM containing 22 µCi/ml \textsuperscript{35}S Met/Cys EasyTag\textsuperscript{TM} EXPRESS35S protein labelling mix (PerkinElmer) at 37°C for 5–10 min for protein maturation or 60 min for protein degradation assays, then chased in complete DMEM supplemented with 10 mM unlabelled Met and Cys for up to 90 min at the indicated temperature for protein maturation or up to 4 h at 37°C for protein degradation. At each time point, cells were lysed in IP-Tx buffer [10 mM Tris-HCl pH 7.6, 140 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100 and 1 mM PMSF]. Lysates were clarified by centrifugation at 5000 g for 10 min at 4°C, and immunoprecipitated with anti-HA antibodies and protein-A-Sepharose (Genscript). Immunoprecipitated material was analysed by SDS-PAGE and phosphorimaging.

Detection of substrate ubiquitylation

Cells were incubated with tetracycline for 16 h, and treated with or without PSII (10 µM) for 8 h. Cells were incubated in PBS containing 20 mM NEM for 5 min then lysed in Ub-IP buffer [25 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% (v/v) Na-deoxycholate, 1% (v/v) Triton X-100 (2% for CD8\textsuperscript{TMD}), 0.1% (v/v) SDS, 5 mM NEM and 1 mM PMSF], and lysates were incubated on ice for 1 h with intermittent vortexing. Lysates were clarified by centrifugation at 5000 g for 10 min at 4°C, then immunoprecipitated with anti-HA antibody followed by protein A or G sepharose beads. Immunoprecipitated material was analysed by SDS-PAGE (10% polyacrylamide gels) and immunoblotting with anti-ubiquitin antibody.

Carbonate extraction

Cells were trypsinised and resuspended in HIM buffer (10 mM HEPES pH 7.5, 200 mM mannitol, 70 mM sucrose, 1 mM EGTA and 1 mM PMSF), then homogenised by being passed 20 times through a 25G needle. Extracts were centrifuged at 1500 g for 15 min, and the supernatant spun at 100,000 g for 30 min. Membrane pellets were subjected to two rounds of carbonate extraction, consisting of 1 h incubation on ice in 200 µl of 100 mM NaCO\textsubscript{3} followed by spinning at 100,000 g for 1 h. The final membrane pellet and each supernatant were analysed by SDS-PAGE.

Immunofluorescence microscopy

Cells were fixed for 15 min in 3% formaldehyde (Sigma), quenched with glycine and permeabilised for 4 min in 0.1% (v/v) Triton X-100 in PBS. Cells were labelled with primary antibodies for 30–60 min followed by Alexa-Fluor-594- or Alexa-Fluor-488-conjugated secondary antibodies for 30 min. Coverslips were mounted in ProLong Gold with DAPI (Molecular Probes) and viewed with an Olympus BX60 upright microscope using a 60×1.40 N.A. PlanApo objective. Images were taken with a CoolSNAP Ez camera (Photometrics) using MetaMorph software (MDS Analytical Technologies). All image processing was performed using ImageJ (http://rsweb.nih.gov/ij/).

MTT assay of viable cells

Cells were seeded at 8000 cells/well in a 24-well dish, and induced with tetracycline at 24-h intervals for a maximum of 96 h. Cells were incubated for 2 h in serum-free DMEM containing 0.5 mg/ml MTT, and the resulting formazan crystals dissolved in 500 µl DMSO. Triplicate samples were transferred into a 96-well plate and the absorbance at 570 nm measured using a Synergy H1 Hybrid reader (BioTek).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

K.B. and E.S. contributed to experimental design, data analysis and manuscript preparation. K.B. performed the majority of the experiments. Y.-H.K. and Y.O. carried out selected experiments.

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Supplementary information

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References

Alcock, F. and Swanton, E. (2009). Mammalian OS-9 is upregulated in response to endoplasmic reticulum stress and facilitates ubiquitination of misfolded glycoproteins. J. Mol. Biol. 385, 1032-1042.

Bernasconi, R., Galli, C., Calanca, V., Nakajima, T. and Molinari, M., (2010). Stringent requirement for HRD1, SEL1L, and OS-9/XTP3-B for disposal of ER-LS substrates. J. Cell Biol. 188, 223-235.

Blond-Elguedi, S., Cwirka, S. E., Dower, W. J., Lipshutz, R. J., Sprang, S. R., Sambrook, J. F. and Gething, M.-J.H. (1993). Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. Cell 75, 717-728.

Bonifacio, J. S., Suzuki, C. K. and Klausner, R. D. (1990). A peptide sequence confers retention and rapid degradation in the endoplasmic reticulum. Science 247, 79-82.

Bonifacio, J. S., Coisson, P., Shah, N. and Klausner, R. D. (1991). Role of potentially charged transmembrane residues in targeting proteins for retention and degradation within the endoplasmic reticulum. EMBO J. 10, 2783-2793.

Burr, M. L., van den Boomen, D. J. H., Bye, H., Antrubos, R., Wiertz, E. J. and Lehner, P. J. (2013). MHC class I molecules are preferentially ubiquitinated on endoplasmic reticulum luminal residues during HRD1 ubiquitin E3 ligase-mediated dislocation. Proc. Natl. Acad. Sci. USA 110, 14290-14295.

Calderwood, S. R., Hill, K. J. and Cooper, A. A. (2001). Degradation of endoplasmic reticulum (ER) quality control substrates requires transport between the ER and Golgi. J. Biol. Chem. 276, 23296-23303.

Cannon, K. S. and Cresswell, P. (2001). Quality control of transmembrane domain assembly in the tetraspanin CD82. EMBO J. 20, 2443-2453.
Carvalho, P., Goder, V. and Rapoport, T. A. (2006). Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell 126*, 361-373.

Cauil, D. M., Tian, X., von Loehnseysen, K. and Robertson, M. W. (2006). Transport of the fast-rector alpha-chain is controlled by a multicomponent intracellular retention signal. *J. Biol. Chem. 281*, 10448-10460.

Christianson, J. C. and Ye, Y. (2014). Cleaning up in the endoplasmic reticulum: ubiquitin in charge. *Nat. Struct. Mol. Biol. 21*, 325-335.

Christianson, J. C., Shaler, T. A., Tyler, R. E. and Kopito, R. R. (2008). OS-9 and GRP94 define a functional alpha-lipochainopathy to the Hrd1p-SEL1L ubiquitin ligase complex for ERAD. *Nat. Cell Biol. 10*, 272-282.

Christianson, J. C., Olzmann, J. A., Sowa, M. E., Bennett, E. J., Richter, C. M., Tyler, R. E., Greenblatt, E. J., Wade Harper, J. and Kopito, R. R. (2012). Defining human ERAD networks through an integrative mapping strategy. *Nat. Cell Biol. 14*, 93-105.

Christis, C., Lubesn, N. H. and Braakman, I. (2008). Protein folding includes oligomerization - examples from the endoplasmic reticulum and cytosol. *FEBS J. 275*, 4700-4727.

Fayadat, L. and Kopito, R. R. (2003). Recognition of a single transmembrane domain by sequential quality control checkpoints. *Mol. Biol. Cell 14*, 1268-1278.

Feige, M. J. and Hendershot, L. M. (2013). Quality control of integral membrane proteins by assembly-dependent membrane integration. *Mol. Cell 51*, 297-309.

Fiedler, S., Broecker, J. and Keller, S. (2010). Protein folding in membranes. *Cell Mol. Life Sci. 67*, 1777-1798.

Flynn, G. C., Pohl, J., Flocco, M. T. and Rothman, J. E. (1991). Peptide-binding specificity of the molecular chaperone BiP. *Nature 353*, 726-730.

Fu, L. and Sztul, E. (2003). Traffic-independent function of the Sar1p/COPII machinery in prorateosomal sorting of the cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem. 278*, 16007-16015.

Geiger, R., Andritschke, D., Friese, S., Herzog, F., Lusioni, S., Heger, T. and Helenius, A. (2011). BAP31 and BiP are essential for dislocation of SV40 from the endoplasmic reticulum to the cytosol. *Nat. Cell Biol. 13*, 1305-1314.

Gill, D. J., Clausen, H. and Bard, F. (2011). Location, location, location: new insights into O-GalNAc protein glycosylation. *Trends Cell Biol. 21*, 149-158.

Hammond, C. and Helenius, A. (1994). Quality control in the secretory pathway: retention of a misfolded viral membrane glycoprotein involves cycling between the ER, intermediate compartment, and Golgi apparatus. *J. Cell Biol. 126*, 41-52.

Houck, S. A. and Cyr, D. M. (2011). Mechanisms for quality control of misfolded transmembrane proteins. *Biochim. Biophys. Acta 1818*, 1108-1114.

Ishikura, S., Weissman, A. M. and Bonifacino, J. S. (2003). Reduced temperature prevents transfer of a retention signal from the Golgi to the endoplasmic reticulum. *Nat. Struct. Mol. Biol. 10*, 222-227.

Ishikura, S., Weissman, A. M. and Bonifacino, J. S. (2003). Recognition of a single transmembrane alpha-lipochainopathy to the Hrd1p-SEL1L ubiquitin ligase complex for ERAD. *Nat. Cell Biol. 10*, 272-282.

Joukovski, M., Hendershot, L. M. and Lederkremer, G. Z. (2012). Golgi localization of ERManI defines spatial separation of the mammalian glycoprotein quality control system. *Mol. Biol. Cell 22*, 2810-2822.

Kamboh-Nesher, S., Shenkman, M., Tolchinsky, S., Fromm, S. V., Ehrlich, R. and Kaether, C., Scheuermann, J., Fassler, M., Zilow, S., Shirotani, K., Valkova, C., Lee, P. C. W., Nguyen, A. D. and Debose-Boyd, R. A. (2000). Membrane protein misassembly in disease. *Biochim. Biophys. Acta 1818*, 1115-1122.

Kamisawa, T., Shaler, T. A., Tyler, R. E. and Kopito, R. R. (2006). Ubiquitination of a BiP substrate. *Sem. Cell Dev. Biol. 21*, 472-478.

Kapustka, M. C., Erra, M. C., Malagolini, N., Serafini-Cessi, F., Leone, A. and Bonatti, S. (1992a). Post-translational processing of an O-glycosylated protein, the human CD8 glycoprotein, during the intracellular transport to the plasma membrane. *J. Biol. Chem. 267*, 25196-25201.

Kemp, C. M., Malagolini, N., Serafini-Cessi, F., Migliaccioc, G., Leone, A. and Bonatti, S. (1992b). Biosynthesis and oligosaccharide structure of human CD8 glycoprotein expressed in a rat epithelial cell line. *J. Biol. Chem. 267*, 9940-9947.

Kemp, C. M., Malagolini, N., Serafini-Cessi, F., Migliaccioc, G., Leone, A. and Bonatti, S. (1992b). Biosynthesis and oligosaccharide structure of human CD8 glycoprotein expressed in a rat epithelial cell line. *J. Biol. Chem. 267*, 9940-9947.

Kemp, C. M., Malagolini, N., Serafini-Cessi, F., Migliaccioc, G., Leone, A. and Bonatti, S. (1992b). Biosynthesis and oligosaccharide structure of human CD8 glycoprotein expressed in a rat epithelial cell line. *J. Biol. Chem. 267*, 9940-9947.

Kemp, C. M., Malagolini, N., Serafini-Cessi, F., Migliaccioc, G., Leone, A. and Bonatti, S. (1992b). Biosynthesis and oligosaccharide structure of human CD8 glycoprotein expressed in a rat epithelial cell line. *J. Biol. Chem. 267*, 9940-9947.

Kemp, C. M., Malagolini, N., Serafini-Cessi, F., Migliaccioc, G., Leone, A. and Bonatti, S. (1992b). Biosynthesis and oligosaccharide structure of human CD8 glycoprotein expressed in a rat epithelial cell line. *J. Biol. Chem. 267*, 9940-9947.