Glycosylation-directed quality control of protein folding

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Abstract | Membrane-bound and soluble proteins of the secretory pathway are commonly glycosylated in the endoplasmic reticulum. These adducts have many biological functions, including, notably, their contribution to the maturation of glycoproteins. *N*-linked glycans are of oligomeric structure, forming configurations that provide blueprints to precisely instruct the folding of protein substrates and the quality control systems that scrutinize it. *O*-linked mannoses are simpler in structure and were recently found to have distinct functions in protein quality control that do not require the complex structure of *N*-linked glycans. Together, recent studies reveal the breadth and sophistication of the roles of these glycan-directed modifications in protein biogenesis.

Proteins are synthesized from cytosolic ribosomes throughout the cell and are delivered to their sites of function through encoded or post-translationally appended signals. Most secretory and membrane proteins must first cross the endoplasmic reticulum (ER) membrane using the secretory 61 (SEC61) translocon complex[1]. Unlike large nuclear pores that can accommodate folded proteins, the narrow pore-size of SEC61 requires its substrates to be unfolded. As they emerge into the ER lumen, molecular chaperones immediately engage the nascent polypeptides, assist in the translocation process and promote protein folding[2–4]. These interactions also ensure that the protein remains in the ER until its maturation is completed. As part of the maturation process, the emerging chain can be covalently modified, with some mechanisms being unique to the ER. These include signal sequence cleavage, Cys oxidation to form disulfides, ADP-ribosylation, glycosylphosphatidyl anchor addition, *O*-mannosylation and *N*-linked glycosylation.

Of all the post-translational modifications that take place in the ER, there is perhaps none more prevalent than the attachment of carbohydrates. From the complex, branched structure of *N*-linked glycans (that is, carbohydrates that are covalently bonded to the amide nitrogens of Asn side chains) to the mannose residues that are added to Ser and Thr side-chain hydroxyls in *O*-mannosylation (FIG. 1), these adducts increase protein stability and solubility. Although years of intensive research have elucidated the molecular mechanisms of glycan biosynthesis and protein glycosylation in the ER, it was only recently understood that the affixed glycans are directly involved in the folding and quality control of their recipients.

Protein-linked glycans have diverse functions, some of which are certainly yet to be discovered. Of these, perhaps the most dramatic recent advances involved their roles in protein quality control in the ER — which are the focus of this Review. In particular, we explore the remarkable mechanisms that are used to transform the conserved *N*-linked glycan structure into a molecular 'script' that directs the folding and the quality control of nascent polypeptides. First, the relatively simple budding yeast system is discussed in detail. This provides a conceptual framework for discussing the advances that have been made using higher eukaryotic systems, which remain a work in progress owing to their greater complexity. Finally, we introduce the recent findings that *O*-mannosyl groups have unexpected roles in protein quality control, which are distinct from those of *N*-glycans.

Protein glycosylation in the ER

Proteins of the secretory pathway can be glycosylated in the ER and in the Golgi. In the ER, carbohydrates are added primarily to newly synthesized, unfolded proteins. As a result, cells can use glycosylation to promote and regulate protein folding and quality control. The two currently known forms of glycosylation with these roles are *N*-linked glycosylation and *O*-mannosylation.

N-linked glycosylation. As the nascent polypeptide emerges into the ER lumen, the modifying machineries are placed in close proximity so that they can read embedded signals and act in response to them. One of the best understood of these interactions is

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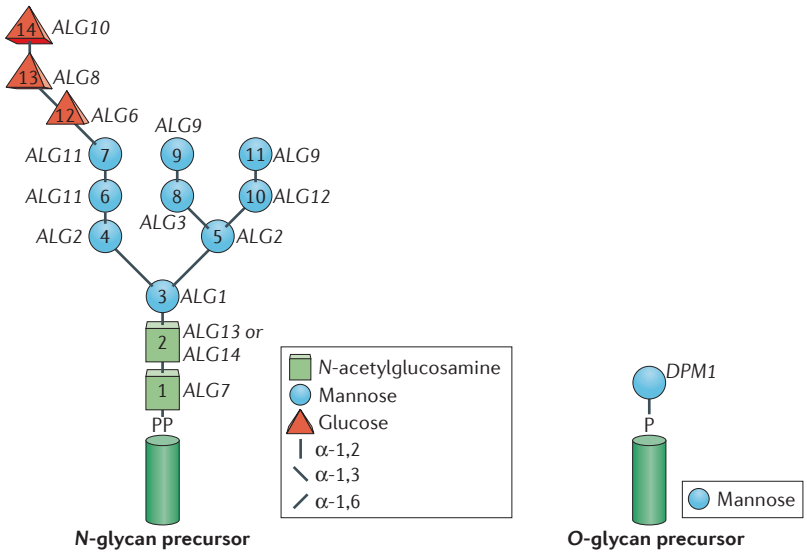

Figure 1 | **Glycan precursors in the endoplasmic reticulum.** The lipid-linked precursors of the *N*-linked core glycan (left) and the *O*-linked mannose (right) are schematically represented. The green cylinders depict the lipid, to which glycans are linked by pyrophosphate (PP) or phosphate (P) bonds. Sugar moieties are shown as coloured symbols, and specific mannosyl linkages are represented by the distinct angles with the types indicated. Genes encoding enzymes that mediate the assembly of mature precursor molecules are shown next to the respective moiety added. Numbers indicate the order and carbohydrate type added during assembly. ALG, Asn-linked glycosylation; DPM1, dolichol-phosphate mannosyltransferase subunit 1.

with the oligosaccharyl transferase (OST) complex[5]. The primary activity of the OST is to transfer the core $Glc_3Man_9GlcNAc_2$ (where Glc is glucose, Man is mannose and GlcNAc is *N*-acetylglucosamine) glycan (FIG. 1) from a lipid-linked oligosaccharide (LLO) donor to the Asn side chain when it encounters the motif Asn-X-Ser/Thr (where X is any amino acid except Pro). Owing to its proximity to the translocon, *N*-linked glycosylation occurs mostly during translocation, before the polypeptide is folded. The crystal structure of the bacterial OST shows that the peptide substrate is bound as a constrained loop[6]. This explains why Pro, which forms a kink in the peptide chain, cannot be in the second position, and why OST is restricted to unfolded substrates. In mammals, the OST complex exists in two isoforms, each containing either the STT3A or the STT3B catalytic subunit. STT3A functions co-translationally, but STT3B can glycosylate proteins post-translationally[7]. The biosynthesis of the LLO precursor dolichol (Dol) begins on the cytosolic surface of the ER membrane. There, in a series of steps, enzymes assemble Dol into the LLO intermediate dolichyl pyrophosphate (Dol-PP)-$GlcNAc_2Man_5$, which is then 'flipped' so that the oligosaccharide relocates to the ER lumen[8], where it is sequentially assembled into the final core glycan by dedicated, membrane-bound enzymes (reviewed in REFS 9,10).

***O*-mannosylation.** Depending on the organism, Ser and Thr residues of proteins in the ER can be modified by various carbohydrates, including fucose, glucose, mannose and GlcNAc[11]. These can be extended further in the Golgi apparatus, where previously unmodified residues can also be modified by various glycans. Glycosylation by mannose in the ER, termed protein *O*-mannosylation, is emerging as a key player in protein quality control in budding yeast. Unlike the hetero-oligomeric structure of *N*-linked glycans, the *O*-mannosylation process involves a single mannose residue linked to Ser and Thr side-chain hydroxyls in the α-configuration (FIG. 1). This moiety can be extended further if the recipient protein is trafficked to the Golgi. Despite the simplicity of these moieties, some proteins are modified so heavily that the *O*-mannosyl groups comprise most of their total mass. Discovery of this modification originated from the analysis of yeast cell wall preparations, in which *O*-mannosyl glycans were sometimes found covalently attached to Ser and Thr side-chain hydroxyls[12]. The peak of protein mannosyltransferase activity was accompanied by ER markers, suggesting that *O*-mannosylation occurs in the ER[13]. Using vesicle-transport mutants, it was determined that a single mannosyl residue is attached to acceptor hydroxyls in the α-1 configuration in the ER. The *O*-linked mannose can be lengthened by adding mannose in the Golgi through α-1,2 and α-1,3 linkages[14].

The mannosyl donor is Dol phosphate β-D-mannose (Dol-P-Man). Dol-P-Man is synthesized by the GDP-α-D-mannose Dol-P β-D-mannosyltransferase, which catalyses the transfer of mannose from GDP-α-D-mannose to Dol-P[15]. Dol-P-Man is made on the cytosolic side of the ER membrane and is later flipped into the luminal face. Dol-P-Man is also the mannose donor that is used in the biosynthesis of *N*-glycans and glycosylphosphatidylinositol (GPI) anchors. In *Saccharomyces cerevisiae*, the *O*-mannosyltransferase (PMT) protein family transfers mannose from Dol-P-Man to peptides[16,17]. Altogether, there are six established members of this family (Pmt1 to Pmt6) in budding yeast that form functional hetero- and homodimeric complexes[18,19]. There is also a seventh putative member (Pmt7), which remains uncharacterized. Three subfamilies were distinguished on the basis of sequence identity: PMT1 (consisting of Pmt1p and Pmt5p); PMT2 (Pmt2p, Pmt3p and Pmt6p); and PMT4 (Pmt4p)[20–22]. Higher eukaryotes have members of the PMT2 and PMT4 subfamilies, whereas the PMT1 subfamily is only found in fungi[22]. Pmt1p forms a heterodimer with Pmt2p in budding yeast, and Pmt3p interacts with Pmt5p[20,23]. Minor concentrations of Pmt1–Pmt3 and Pmt2–Pmt5 complexes are also detectable, but their specific roles, if any, are unclear[20]. Unlike the others, Pmt4p primarily forms homodimers[20]. By contrast, mammalian PMTs do not follow this pattern. POMT1, which is a member of the PMT4 family in mammals, forms a complex with POMT2; formation of this heterodimer is essential for function *in vivo*[24]. Interestingly, the Pmt1–Pmt2 dimer specifically modifies misfolded proteins, even those that are not usually *O*-mannosylated[25–31].

Quality control for protein folding in the ER

Although estimates of its efficiency vary, protein folding is a high-fidelity process[32]. Nonetheless, a small proportion of proteins always misfold, owing to genetic mutations, errors in transcription and translation, cell stress

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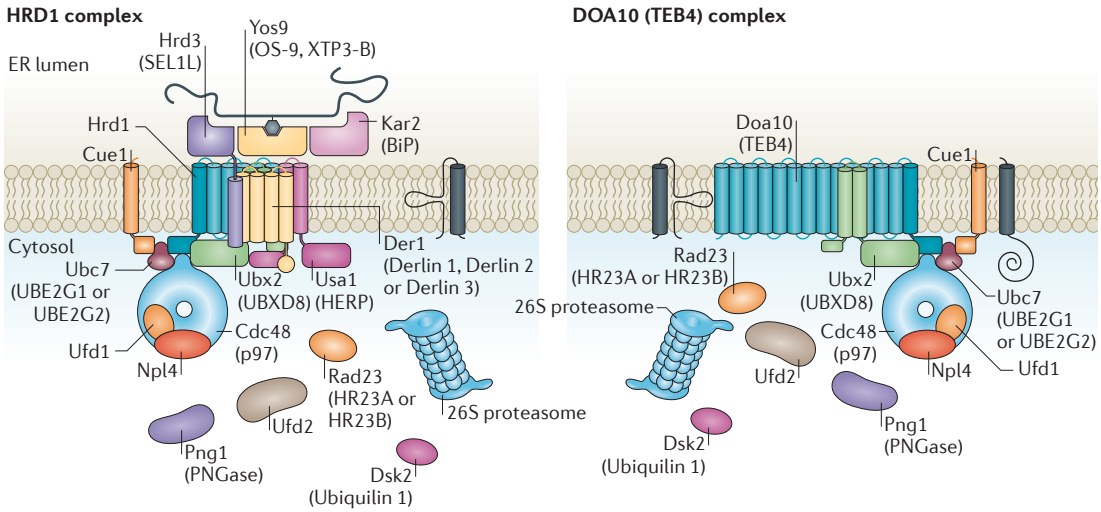

Figure 2 | **The Doa10 and HMG-CoA reductase degradation 1 (Hrd1) endoplasmic reticulum (ER)-associated degradation (ERAD) complexes and their downstream effectors.** The membrane-based Hrd1 (left) and Doa10 (right) E3 ubiquitin ligase complexes are shown. Cytosolic effectors of each pathway are depicted below the membrane complexes. ERAD substrates are shown in grey, with misfolded domains as lines and the glycan signal ligand as a hexagon. Individual factor names are indicated (yeast, with corresponding mammalian homologue names in parentheses where these are different). Cdc48, cell division cycle 48; Cue1, coupling of ubiquitin conjugation to ER degradation 1; Der1, degradation in the ER 1; Kar2, karyogamy 2; Npl4, nuclear protein localization 4; Png1, peptide *N*-glycanase; Rad23, radiation-sensitive 23; Ubc7, ubiquitin-conjugating enzyme 7; Ubx2, UBX domain-containing 2; Ufd, ubiquitin fusion degradation; Usa1, U1-Snp1-associating 1; Yos9, yeast osteosarcoma 9.

and the stochastic effects that arise in a complex process. Without countermeasures, the accumulation of aberrant proteins can be toxic. Accordingly, sophisticated quality control mechanisms are in place to recognize, segregate and degrade misfolded proteins. Although protein synthesis occurs at multiple cellular locations, the quality control system of the ER is perhaps the most complex. The localization of chaperones, folding catalysts and protein-modifying enzymes in the ER requires a sorting mechanism that allows only mature proteins to exit the ER. Proteins that fail to fold must be retained and recognized by the ER-associated degradation (ERAD) mechanisms for turnover[4,33–37].

The simplest ERAD system was first discovered in *S. cerevisiae*[38–40] and has since been shown to be largely conserved in higher eukaryotes, although with a notable expansion in the number of factors, which is presumably to handle the greater diversity of proteins traversing the metazoan ER. Perhaps the most important early revelation was that the degradation of ER-localized proteins was performed by the cytosolic ubiquitin–proteasome system[40], which raised intriguing questions such as how ER-localized proteins are recognized, retained and re-translocated back to the cytosol.

The substrate range requires that ER quality control systems recognize conformational determinants in the lumen, in the lipid bilayer and on the cytosolic side of the membrane. Integral membrane proteins can have ordered domains in all three zones simultaneously, whereas soluble proteins in the ER have elements only in the lumen. In yeast, three pathways emerged, which are termed ERAD-L (luminal ERAD), ERAD-M (membrane ERAD) and ERAD-C (cytosolic ERAD)[41,42]. The ERAD-L pathway primarily pivots around the membrane E3 ubiquitin ligase HMG-CoA reductase degradation 1 (Hrd1). ERAD-C depends on the Doa10 E3 ligase (also known as SSM4; MARCH6 in mammals), which forms a complex that is altogether distinct from the HRD1 complex, even though they have several components in common[41–43] (FIG. 2). Although it was originally thought that ERAD-M was carried out by Hrd1, a recent study demonstrated that unassembled Sec61β homologue 2 (Sbh2), which is a component of the SEC61 translocon complex, relies on Doa10 for its degradation[41,43,44]. Thus, both Hrd1 and Doa10 have the capacity to recognize aberrant or unassembled transmembrane peptides, and together these mechanisms provide the coverage that is needed to monitor all types of nascent proteins in the ER.

Roles of* N*-glycans in ER protein folding and quality control. A requirement for *N*-glycans in protein folding was first demonstrated for the vesicular stomatitis virus G (VSVG) and influenza HA glycoproteins, which are classic models of ER protein folding[45,46]. The subsequent discovery of two ER lectin-like proteins that specifically bind to unfolded glycoproteins suggested the existence of *trans*-acting folding factors working through target glycans. Indeed, this interaction is part of an orchestrated binding-and-release cycle that culminates in a folded glycoprotein (reviewed in REFS 47–49).

The mechanism of glycan-directed protein folding begins soon after translation and glycosylation, with glucosidase I initiating a glycan-trimming cascade by removing the terminal glucose residue from the A-branch of the glycan (FIGS. 3,4). This is followed by

HRD1 complex
Multi-subunit membrane protein complex in the endoplasmic reticulum (ER) that is organized around the E3 ubiquitin ligase HMG-CoA reductase degradation 1 (Hrd1). The HRD1 complex receives, retrotranslocates and ubiquitylates substrates for degradation by ER-associated degradation (ERAD).

Lectin
Member of a class of proteins that bind to carbohydrates. Lectins usually have high specificity for sugar type and/or glycan-linkage configuration.

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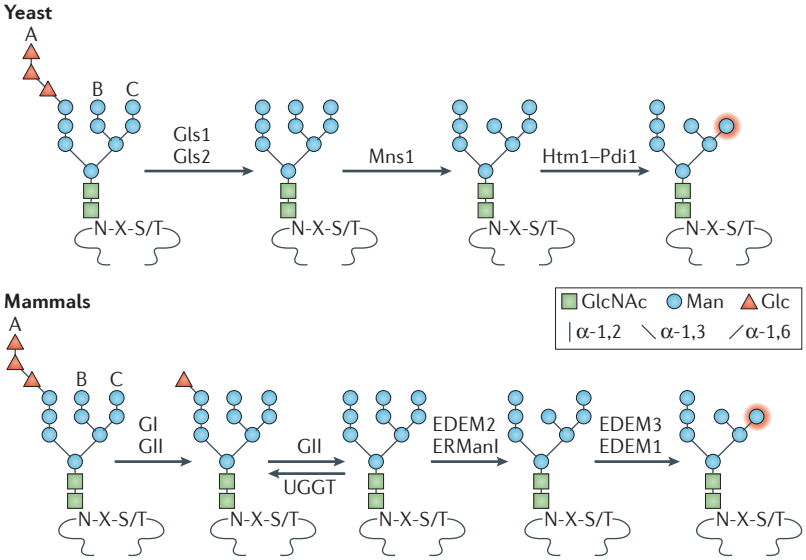

Figure 3 | ***N*-linked glycan trimming in the endoplasmic reticulum (ER).** The upper panel depicts the yeast pathway of glycan trimming, and the lower panel depicts the mammalian pathway. Coloured symbols represent specific sugar moieties and the bond angles indicate the specific type of mannose linkage. See the main text for a description of the enzymes that mediate each step. The blue sphere outlined in red ($Man_7GlcNAc_2$ glycan; right) indicates the terminal α-1,6-linked mannose ligand of the yeast osteosarcoma 9 (Yos9; OS-9 and XTP3-B in mammals) ER-associated degradation (ERAD) receptor. EDEM, ER degradation-enhancing α-mannosidase-like protein; ERManI, ER mannosidase I; GI, glucosidase I; GII, glucosidase II; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Gls, glucan synthase of cerevisiae protein; Man, mannose; Mns1, mannosidase I; Pdi1, protein disulfide isomerase; UGGT, UDP-glucose:glycoprotein glucosyltransferase.

the removal of the second residue by glucosidase II. The lectin-like ER proteins calnexin and calreticulin bind to the remaining glucose residue in the $Glc_1Man_9GlcNAc_2$ structure[50–52]. Calnexin is a type I integral membrane protein with a large luminal domain and a short carboxy-terminal cytoplasmic tail. Its lectin domain binds to the glycan and to an extended 'arm' (the A-branch) of the *N*-glycan (FIG. 3) that interacts with chaperones and with other parts of the substrate molecule. Calreticulin is the soluble homologue of calnexin, and is retained in the ER through its C-terminal KDEL retention signal. It is this configuration, together with associated factors such as the oxidoreductase ERp57 (also known as PDIA3) that promotes the folding and oxidation of bound substrates[53,54]. This is a transient interaction, which allows the cleavage of the last glucose by glucosidase II, thereby preventing re-engagement of calnexin. Subsequently, UDP-glucose:glycoprotein glucosyltransferase (UGGT), which is a large ER protein, 'inspects' the released proteins; those still bearing unfolded domains are re-glucosylated by UGGT at the A-branch, restoring the calnexin-binding site for another round of binding and release[55–58] (FIG. 4). This process continues until the protein is fully folded or until quality control mechanisms break the cycle. Although the calnexin cycle is conserved among diverse organisms, a role for calnexin in protein folding in budding yeast remains to be determined[39]. Taken together, the available data reveal a remarkable level of regulation orchestrating the folding process, using just the three terminal glucose residues of the A-branch. The decision to transition from protein folding to degradation of terminally misfolded molecules, which is the defining step of quality control, is fittingly directed by the remaining $Man_9GlcNAc_2$ glycan. Glycoprotein quality control has been extensively studied both in budding yeast and in mammalian tissue culture systems, and the data that have been gathered so far have indicated that the basic machinery is largely conserved. However, the proposed quality control mechanisms differed, which was perplexing given the extent of conservation. Thus, it could be instructive to re-examine the advances that have been made in understanding the yeast and mammalian systems, to assess whether the differences reflect evolutionary divergence or simply differences in experimental conditions.

Glycan-directed ERAD in budding yeast. Although the process of glycan trimming in the ER was mostly solved, the functional importance of each step was understood only recently. The first steps, removal of glucosyl residues, are a prerequisite for entry into glycan-dependent ERAD[59] (FIGS 3,4). This is effectively an early ERAD checkpoint that functions until the cessation of folding[52,59]. Following removal of the glucose residues, mannosidase I (Mns1) removes the α-1,2-linked terminal mannose residue from the B-branch to yield $Man_8GlcNAc_2$ (REF. 60). This glycan structure is shared between folded proteins exiting the ER and ER-retained misfolded proteins, indicating that Mns1 does not discriminate between substrates on the basis of protein structure[61,62]. Accordingly, the $Man_8GlcNAc_2$ glycan alone does not function as a transport signal. Instead, that role is attributed to conformational protein determinants (sometimes in conjunction with glycans) that are recognized by ER-to-Golgi cargo receptors[2–4,63]. This is an important mechanism of ER quality control that makes use of positive export signals that are formed on protein surfaces only upon folding. Conversely, unfolded proteins expose determinants that tend to be hydrophobic. These are recognized by ER chaperones that retain them for further folding attempts or for degradation. For glycoproteins, however, the requirement for Mns1 in ERAD and the predominant $Man_8GlcNAc_2$ glycoform of carboxypeptidase Y (also known as CPY*) led to the proposal of a glycan degradation signal[61,64]. The proposed mechanism was consistent with the data that were available at the time, but it failed to explain how folded proteins bearing the same glycans escape ERAD. In contrast to budding yeast, the fission yeast *Schizosaccharomyces pombe* exhibits little α-mannosidase activity against unfolded glycoproteins[65]. Because a knockout of the corresponding α-mannosidase-like gene disrupted ERAD, it was proposed that it functions as a lectin in ERAD. Whether these two yeasts evolved different roles from their ancestral *MNS1* gene remains to be determined.

To validate the hypothesis that ERAD was activated by a glycan signal, an ERAD glycan receptor needed to be identified. Yeast osteosarcoma 9 (Yos9), which is an ER resident[66] that is required for glycoprotein ERAD, emerged as a strong candidate[67–70]. The *YOS9* gene was previously identified as a homologue of the human *OS9*

Oxidoreductase
Member of a class of enzymes that mediate the transfer of electrons from one molecule to another. In the endoplasmic reticulum, most oxidoreductases form and break disulfide bonds.

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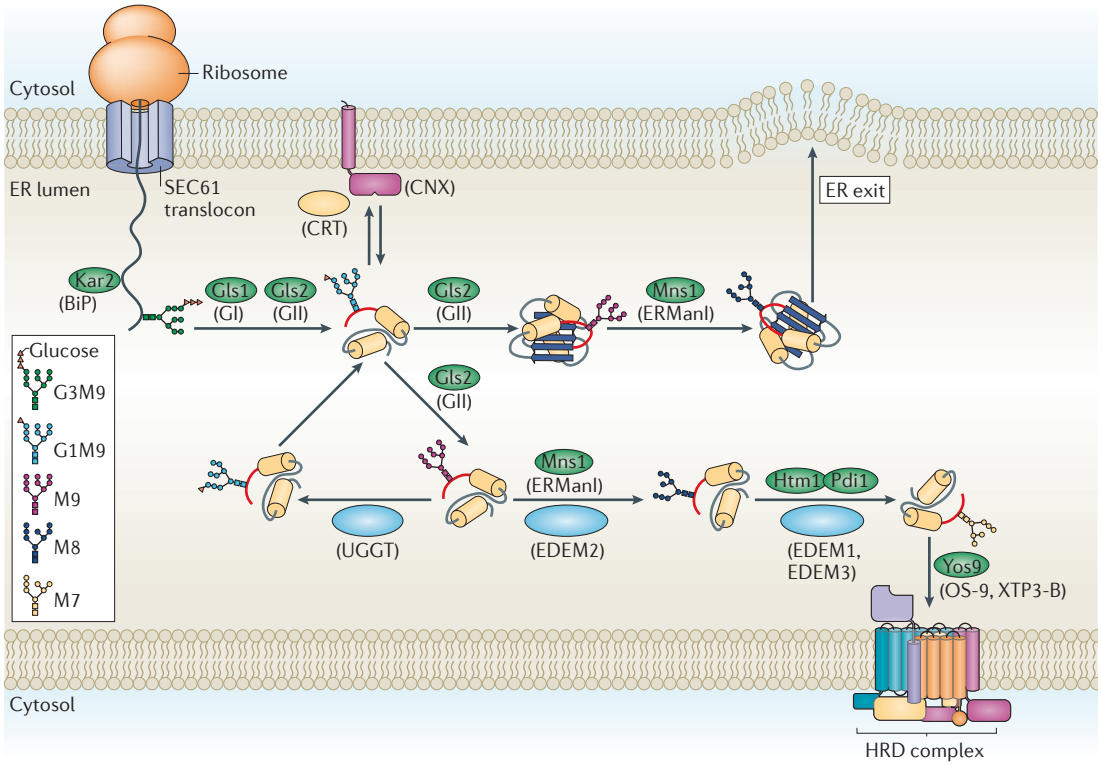

Figure 4 | ***N*-glycan directed protein folding and quality control.** The productive glycoprotein-folding pathway (top) and the pathway of a protein-folding failure (bottom) are depicted. Symbols representing glycan structures G3M9 ($Glc_3Man_8GlcNAc_2$), G1M9 ($Glc_1Man_8GlcNAc_2$), M9 ($Man_9GlcNAc_2$), M8 ($Man_8GlcNAc_2$) and M7 ($Man_7GlcNAc_2$) are shown on the left. Yeast proteins are represented by green ovals (with the names of their mammalian homologues below them in parentheses). Folded protein structures are depicted with cylinders and sheets; unfolded structures are represented as cylinders and lines. CNX, calnexin; CRT, calreticulin; ER, endoplasmic reticulum; ERManI, ER mannosidase I; Gls, glucan synthase of cerevisiae protein; HRD, HMG-CoA reductase degradation; Kar2, karyogamy 2; Mns1, mannosidase I; Pdi1, protein disulfide isomerase; Sec61, secretory 61; Yos9, yeast osteosarcoma 9.

gene that is amplified in some cancers[71]. Notably, Yos9 contains the mannose-6-phosphate receptor homology (MRH) domain[66], which is configured for glycan binding. Yos9 binds to glycosylated CPY* *in vivo*, but not to a glycan-free variant, suggesting that glycans specify at least part of its substrate recognition. In support of this notion, mutation of key MRH-domain residues abolishes the ability of Yos9 to bind to substrate and to mediate degradation. Its role as a lectin-like receptor was fully validated through biochemical analyses using purified recombinant Yos9 and synthetic branched glycans. Yos9 had the greatest affinity for glycans containing a terminal α-1,6-linked mannose residue at any position[72] (FIG. 3; red circle). Taken together, the data suggested two possibilities. The first is that the substrates could acquire terminal α-1,6-linked mannose residues following transport and retrieval from the Golgi apparatus, where mannosyltransferases that extend *N*-glycans are located. A second possibility is the modification of substrate glycans in the ER through a novel mechanism of addition or removal of mannose.

The question of how the α-1,6-linked mannose-containing ligand of Yos9 is generated shifted the focus to Htm1 (also known as Mnl1) because of its α-mannosidase homology domain. Overexpression of Htm1 in cells caused a shift from $Man_8GlcNAc_2$ to $Man_7GlcNAc_2$ in the population of protein-linked oligosaccharides, owing to removal of the C-branch α-1,2-linked mannose, which exposed a terminal α-1,6-linked mannose[73]. Knocking out the oligosaccharide biosynthetic gene Asn-linked glycosylation 3 (*ALG3*) modifies all *N*-glycans to be of the $Man_5GlcNAc_2$ form (FIG. 1), which contains a terminal α-1,6-linked mannose. Although the Yos9 ligand is at a different position, this glycoform bypasses the requirement for Htm1 in ERAD. *In vitro* reconstitution experiments demonstrated that the α-1,2-mannosidase activity of Htm1 generates the $Man_7GlcNAc_2$ structure with a C-branch-terminal α-1,6-linked mannose[61,62] (FIGS 3,4). Htm1 forms a complex with the ER chaperone protein disulfide isomerase (Pdi1), which is an oxidoreductase[73], and this interaction is essential for Htm1 function *in vivo* and *in vitro*[61,62]. There are two requirements for the generation of the $Man_7GlcNAc_2$ ligand by Htm1. The first is the $Man_8GlcNAc_2$ structure, which is processed by glucan synthase of cerevisiae protein 1 (Gls1; also known as Cwh41), Gls2 and Mns1 (REFS 74,59,64,75) (FIGS 3,4). The second is that the $Man_8GlcNAc_2$ substrate is attached to an unfolded peptide segment[62,76]. Although it remains unclear how the complex recognizes unfolded proteins, Pdi1 probably provides this function, as it binds

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independently to unfolded proteins, whereas Htm1 alone cannot process substrates[62,73,77]. Other ER luminal chaperones, including karyogamy 2 (Kar2; known as GRP78 or BiP in mammals) and its modulators, and the luminal Hsp70 Lhs1 (known as GRP170 or HYOU1 in mammals), are required by different substrates for ERAD[78–80]. Their roles are less well defined, but they are certainly required to maintain substrates in the soluble state that is necessary for ERAD. These chaperones may also have the role of retaining unfolded proteins in the ER for modification by the Htm1–Pdi1 complex. Together, the sequential reactions that are mediated by Gls1, Gls2 and Mns1 set a time window for protein folding (FIGS 3,4). If unfolded proteins are detected outside this window, nascent glycoproteins become vulnerable to further glycan modification and ERAD. Thus, the system does not differentiate whether the substrate is irreversibly misfolded or just folding at a slower rate. The mechanism seems wasteful, but it facilitates the discrimination of irreversibly misfolded proteins with high fidelity. Given the toxicity of misfolded proteins, the cost of degrading a small number of slow but potentially properly folding proteins seems acceptable.

Interestingly, *cis*-Golgi mannosyltransferases, which modify both folded and unfolded proteins, decorate glycoproteins with α-1,6-linked mannose residues, providing an alternative mechanism for marking unfolded molecules that exit the ER and thus can be later retrieved[81–83]. Indeed, analysis of cytosolic glycans that were released from ERAD substrates identified a substantial proportion that contained Golgi modifications, indicating that this route might be physiologically relevant *in vivo*[83]. This was supported by mammalian experiments suggesting that the retrieval of ERAD substrates from the Golgi is more common than was previously thought[84].

Yos9 is a member of the ERAD-L HRD1 complex[41,85–87] (FIG. 2). The yeast HRD1 membrane complex is composed of the Hrd1 E3 ligase, directly associated with Hrd3. Hrd3 contains a large luminal domain that anchors Yos9 to the complex and can recognize non-glycosylated substrates independently of Yos9 (REFS 88,87,89). However, recent reports indicate that Yos9 itself can interact directly with peptides, and its absence marginally decreases the degradation of non-glycosylated substrates[90,91]. U1 Snp1-associating 1 (Usa1) links Hrd1 to Der1 (degradation in the endoplasmic reticulum 1; derlins in mammals)[41,92]. Der1 is required for luminal substrates (glycosylated and non-glycosylated) and facilitates their translocation to the Hrd1 ligase for ubiquitylation[42,93,94]. Crosslinking experiments indicated that Der1 might directly bind to and guide the substrate across the membrane into the cytosol, similar to the mechanism that was originally proposed for its mammalian homologue Derlin 1 (REFS 94,95). The identity of the actual channel proteins, however, remains unknown.

In addition to Der1 and derlins, other candidates are proposed to retrotranslocate substrates from the lumen across the ER membrane. The import translocon composed of Sec61 proteins is required for the efficient degradation of some substrates, including non-glycosylated pro-α factor and Deg1–Sec62^{ProtA} (REFS 96–100). An intriguing candidate is the Hrd1 ligase itself. Upon Hrd1 overexpression, substrate degradation can take place even in the absence of Usa1, Der1, Hrd3 and Yos9 (REF. 101). However, other components of the Hrd1 complex, including ubiquitin-conjugating enzyme 7 (Ubc7)[102] and the cell division cycle 48 (Cdc48) complex (an ATPase of the AAA (ATPases associated with diverse cellular activities) family that is required for ERAD substrate extraction)[103–106] are required, as is the ligase activity of Hrd1, ruling out an alternative pathway for substrate degradation. Hrd1 contains multiple transmembrane segments, so it was proposed to form all or part of a protein-conducting channel for ERAD[101]. Recently, the Hrd1 minimal system was reconstituted from purified components, consisting of Hrd1, the Ubc7–Cue1 (coupling of ubiquitin conjugation to ER degradation 1) dimer and the Cdc48 complex (consisting of Cdc48, UBX domain-containing 2 (Ubx2) and ubiquitin fusion degradation 1 (Ufd1)–nuclear protein localization 4 (Npl4)) (FIG. 2). In detergent-solubilized complexes and reconstituted proteoliposomes, this complex binds specifically to unfolded substrate, ubiquitylates it and extracts it in a Cdc48-dependent and energy-dependent fashion[107]. Together, these data show that the role of Hrd1 in ERAD-L goes beyond its known function as an E3 ligase. Hrd1 also receives and regulates or mediates the retrotranslocation and extraction of substrates at the ER membrane. Although the *in vitro* system described above lacks many crucial factors of ERAD-L, it provides an estimate of the type of experimental strategy that is needed to uncover mechanistic details that are not accessible using intact cells.

Once extracted into the cytosol, glycoprotein substrates are deglycosylated by cytosolic peptide *N*-glycanase (PNGase; Png1 in yeast)[108,109]. PNGase displays a preference for unfolded glycoproteins, and deglycosylation enhances proteasomal substrate turnover[110]. Finally, cytosolic chaperones, including heat shock proteins Hsp70, Hsp90, Hsp26 and Hsp42 and the ubiquitin-related factors Dsk2 and radiation-sensitive 23 (Rad23), prevent protein aggregation and facilitate delivery of substrates to the proteasome for degradation[43,111–116] (FIG. 2).

Glycan-directed ERAD in mammals. Glycan-directed quality control components are generally conserved from yeast to mammals, with some notable differences. One clear difference is the greater number of factors that are devoted to ERAD in mammals. This is not surprising, given the greater range of mammalian substrates and cell types. The reasons for other differences are less clear, for example, there is disagreement concerning the role of the mammalian Mns1 homologue ER mannosidase I (ERManI; also known as MAN1B1). Whereas *in vitro* experiments using the luminal domain of human ERManI showed conversion of the $Man_9GlcNAc_2$ substrate exclusively to $Man_8GlcNAc_2$ at branch B[117], *in vivo* experiments demonstrated a mannose-trimming activity resulting in glycans ranging from $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ (REF. 118). The localization of ERManI is

Proteoliposomes
Particles composed of proteins and lipids, which are usually reconstituted from purified components.

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also controversial. Originally it was found to be localized at the ER in various organisms[117–119]; however, recent studies have suggested alternative localizations, such as the Golgi membranes in HeLa cells, where it contributes to retrieving unfolded proteins to the ER for ERAD[120,121]. In a second study, ERManI was found to be localized to novel compartments in NIH 3T3 and HEK293 cells that the authors termed quality control vesicles (QCVs)[122]. Using a combination of ERManI antibodies and fluorescent fusion proteins, the investigators localized ERManI to various positions that are distinct from ER and Golgi markers. The same ERManI antibodies were used in both studies[120,122], so it was surprising that distinct localizations were obtained. However, as different cell lines were used, it is possible that the location varies among cell types.

Various factors specialize in performing the steps following ERManI in mammalian ERAD. For Htm1, there are three homologues, ER degradation-enhancing α-mannosidase-like protein 1 (EDEM1), EDEM2 and EDEM3. EDEM1 (or just EDEM in earlier reports) has homology to class I α-1,2-mannosidases (which constitute the glycosyl hydrolase family 47). EDEM1 can give rise to soluble and membrane forms because of its inefficiently cleaved ER signal sequence[123,124]. As with ERManI, the role of EDEM1 varies depending on the substrate examined. For some substrates, EDEM1 enhances degradation of the glycosylated species but has no effect on the degradation of their non-glycosylated variants[125,126]. Other studies reported a requirement of EDEM1 for degradation of some non-glycosylated proteins as well[127,128].

Like Htm1, EDEM1 functions with an oxidoreductase partner, the DnaJ protein ERdj5 (also known as DNAJC10)[129]. ERdj5 plays a key part in ERAD by reducing disulfide bonds of misfolded proteins in preparation for retrotranslocation to the cytosol. This is a crucial activity, because substrates bearing intra- and intermolecular disulfide bonds would require a conduit so large that maintaining the ionic barrier between the ER lumen and the cytosol would be difficult. ERdj5 also interacts with the ER chaperone BiP through its J domain. Although it was first thought that EDEM1 lacks mannosidase activity, overexpression studies demonstrated an increase of $Glc_1Man_8GlcNAc_2$ glycans on the NHK substrate. This activity was not observed when a mannosidase catalytic mutant was expressed. Furthermore, overexpression led to an overall increase of protein-linked $Man_7GlcNAc_2$ and $Man_6GlcNAc_2$ glycans, in which the branch C α-1,2-linked mannose was removed[130]. Although this activity is similar to that of yeast Htm1–Pdi1, whether the Pdi1 subunit plays a similar role in reducing disulfide bonds in yeast is currently not known.

EDEM2 and EDEM3 are soluble proteins, and despite their shared homology with EDEM1 in their glycosyl hydrolase domain, each member is distinct. EDEM2 can be found associated with substrates, and EDEM2 overexpression will specifically accelerate glycosylated substrate degradation[131,132]. However, how EDEM2 could enhance degradation is unclear, as no α-1,2-mannosidase activity could be detected[132]. EDEM3 overexpression caused significant glycan trimming of the NHK substrate to $Man_7GlcNAc_2$ and $Man_6GlcNAc_2$, whereas few $Man_8GlcNAc_2$ forms were observed[133]. EDEM3 overexpression also accelerated the degradation of NHK and of the unassembled α-subunit of the T cell receptor, but not of non-glycosylated NHK. An EDEM3 catalytic mutant trimmed glycans poorly and failed to accelerate ERAD, suggesting that it possesses mannosidase activity.

The reasons for the diversity of reported functions for ERManI and EDEM family members are unclear. For this reason, a recent study took a systematic approach to examine the functions of ERManI, EDEM1, EDEM2 and EDEM3 (REF. 134). Using distinct avian and human cell lines, deletions of each gene were generated and analysed. Unexpectedly, in cells lacking ERManI, only limited effects on the levels of $Man_9GlcNAc_2$ were observed, suggesting that there is functional redundancy or that the contribution of ERManI to trimming the branch B mannose is limited. The recent reports showing ERManI to be a non-ER enzyme support the latter option, because glycans of folded proteins leaving the ER are already trimmed to $Man_8GlcNAc_2$ (REFS 120,122). Conversely, loss of EDEM2 did cause a substantial accumulation of $Man_9GlcNAc_2$, supporting the idea that EDEM2 catalyses the first mannose-trimming step, which in yeast is performed by Mns1 (FIGS 3,4). In cells lacking EDEM1 or EDEM3, $Man_8GlcNAc_2$ glycans accumulated, which indicates that they have a role in the second step of mannose trimming. Interestingly, although the mannosidase activity of EDEM1 is weaker than that of EDEM3, the strong effects on ERAD observed in the knockout lines supports previous reports that EDEM1 has a role in ERAD in addition to the glycan-trimming activity. The authors propose a model in which the EDEM family functions downstream of glucosidase I and glucosidase II to convert $Man_9GlcNAc_2$ glycans of unfolded proteins to $Man_7GlcNAc_2$, in a two-step process. The relatively slow activities of EDEM proteins allow nascent proteins to escape ERAD if they fold before the final hydrolysis by EDEM1 or EDEM3 (FIG. 4). Taken together, these studies show that the general strategy of using *N*-linked glycan structures to regulate the progress of protein folding is evolutionarily conserved. Not only do *N*-glycans provide an 'instruction manual' for folding attached to each protein, they also function as a destruction signal should the process fail.

OS-9 and XTP3-B (also known as ERLEC1), which are the mammalian homologues of Yos9, also function as lectin-like receptors in ERAD[135,86,136,137]. Human *OS9* is expressed as three splice variants, each containing a single amino-proximal MRH domain. XTP3-B is a shorter protein that contains two MRH domains[86,136]. OS-9 variants and XTP3-B interact independently with the luminal domain of SEL1L, which is the mammalian homologue of yeast Hrd3 (REFS 86,138) (FIG. 2). In co-immunoprecipitation experiments, many proteins are detected in association with OS-9 and XTP3-B[86]. Whereas some, such as SEL1L, are functional partners, others could be substrates. For example, CD147 was found to be an endogenous substrate of OS-9, even though its normal

DnaJ protein
A protein containing a 'J domain', which interacts with Hsp70 chaperones and stimulates their ATPase activity. Many DnaJ proteins are chaperones and directly bind to substrates.

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function as an assembly factor might have suggested a role as a partner[139]. Similarly, the ER chaperone 94 kDa glucose-regulated protein (GRP94; also known as endoplasmin) was once proposed to be a functional partner of OS-9 (REF. 86). Recent studies demonstrated that an abnormally hyper-glycosylated GRP94 subspecies accounts for the interaction, making it an OS-9 substrate instead of a partner[140]. OS-9 is required for degrading a range of glycosylated substrates, showing specificity over XTP3-B for some substrates[86,136,138,141,142]. Biochemical analyses of OS-9 proteins and domains revealed a binding preference for terminal α-1,6-linked mannose moieties in oligosaccharides[135,143]. This was confirmed by a high-resolution crystal structure showing the binding site within the MRH domain[144]. Hydrophobic effects and hydrogen bonds form to stabilize the interaction between this domain and the terminal α-1,6-linked and penultimate mannose residues on the C-branch of the $Man_7GlcNAc_2$ glycan. Together, these findings support a model in which Htm1 and EDEM1 and EDEM3 expose the ERAD signal by removing the α-1,2-linked mannose 'cap' (FIG. 3). The structure was pivotal in demonstrating how the α-1,2-linked mannose prevents binding to the lectin receptor to avoid ERAD.

The role of the OS-9 homologue XTP3-B is less clear. For some substrates, XTP3-B is functionally redundant to OS-9 in ERAD[141]. For others, XTP3-B is dispensable for degradation, even though it can bind to the substrates[86,139]. This might be explained by the C-terminal MRH domain of XTP3-B, which binds to $Man_9GlcNAc_2$ glycans *in vitro* and *in vivo*[138], thereby protecting $Man_9GlcNAc_2$-containing glycoproteins undergoing folding from degradation.

Unfolded protein *O*-mannosylation (UPOM)

The ease by which the folding capacity of polypeptides can be eliminated by mutations has given rise to a wide range of model ERAD substrates. These have been instrumental in uncovering commonalities as well as varying substrate-specific requirements for their degradation. Interestingly, a common post-translational modification of ERAD substrates in yeast is ER *O*-mannosylation, even if their native, folded counterparts are not *O*-mannosylated[25–31].

Quality control by protein* O-*mannosylation. UPOM is a seemingly ubiquitous event in the yeast ER[25–31]. Although the ER *O*-mannosylation machinery is conserved to mammals, there are no reports of an equivalent UPOM activity in higher eukaryotes[18]. In yeast, UPOM is carried out exclusively by the Pmt1–Pmt2 protein complex. Because *PMT1* and *PMT2* are non-essential genes that are regulated by the unfolded protein response (UPR), the role of UPOM in quality control can be analysed in *PMT*$^-$ knockout strains[145]. Using this approach, the effect of UPOM on ERAD was found to vary depending on the substrate. For KHN (which is the soluble form of parainfluenza 5 haemagglutinin-neuraminidase glycoprotein) and ΔG(QQQ)pαF (non-glycosylated pro α-factor), UPOM promotes efficient ERAD because it maintains substrate solubility[27,28]. Gas1* (misfolded GPI-anchored surface glycoprotein) requires UPOM for ER retention and ERAD[30]. For Δgpαf (similar to ΔG(QQQ)pαF), UPOM can be inhibitory for ERAD[26]. When translocated into wild-type ER microsomes, Δgpαf is *O*-mannosylated in a Pmt2-dependent manner. In cells lacking Pmt2, however, Δgpαf degradation is not inhibited, but is slightly enhanced. The A-chain of Shiga-like toxin (SLTxA1(N$^-$)) is *O*-mannosylated and degraded by ERAD when expressed in budding yeast[29]. In this case, although UPOM only slightly improves ERAD of SLTxA1(N$^-$), *O*-mannosylation inhibits the toxic form of the protein after export to the cytosol. Similarly, Deg1–Sec62, which is a membrane protein ERAD substrate, is *O*-mannosylated but does not require *O*-mannosylation for efficient degradation[25]. Importantly, the Pmt1–Pmt2 complex is associated with factors involved in ER protein processing, including the p24 membrane protein family, the oxidoreductases Ero1 and Pdi1 and the GPI-remodelling factor Ted1. In addition to these interactions, the activity of the ER chaperone Kar2 is required for UPOM[28,146]. Taken together, these studies show that UPOM activity is functionally and physically associated with the ER quality control process in yeast.

UPOM terminates futile protein folding cycles. Recently, a new activity was uncovered for UPOM. Expression of GFP in the mammalian and yeast ER (ER-GFP) results in poorly folded protein[147,148]. Biochemical analysis showed that ER-GFP in yeast cells is *O*-mannosylated, whereas the fast-folding variant ER-GFP^{fast} folds efficiently and is not *O*-mannosylated[149]. These data raised the possibility that *O*-mannosylation could be a mechanism that terminates the folding of 'kinetic laggards'. Indeed, elimination of *O*-mannosylation *in vivo* allowed ER-GFP to fold to completion. *In vitro* folding assays demonstrated that unmodified ER-GFP folds efficiently, whereas *O*-mannosylated ER-GFP is incapable of folding. These findings show that the modification disrupts the intrinsic folding capacity of substrates. Furthermore, *O*-mannosylation reduces substrate interactions with Kar2, which suggests that *O*-mannosylation has a role in removing substrates from futile protein folding cycles (FIG. 5). Although this has been shown in yeast cells, whether the folding defect of ER-GFP in mammalian cells is caused in part by *O*-mannosylation, is currently unknown. In summary, these studies indicate that UPOM plays a part in the early stages of ER quality control and also facilitates entry into ERAD for some substrates.

Conclusion and perspectives

Recent advances in the understanding of glycoprotein synthesis have shown that the complex structures of core *N*-linked glycans function as a script to guide protein folding and quality control. Each glycoform, from $Glc_3Man_9GlcNAc_2$ to $Man_7GlcNAc_2$ (and sometimes beyond) informs the biosynthetic machinery of the status of the nascent polypeptide so that the appropriate action is taken. In addition, the simpler UPOM modification adds to the intricacy of ER quality control.

Unfolded protein response (UPR). A signalling response that is triggered by the accumulation of misfolded or unfolded proteins in the endoplasmic reticulum.

Microsomes
Vesicles that are formed from endoplasmic reticulum membranes after mechanical cell disruption.

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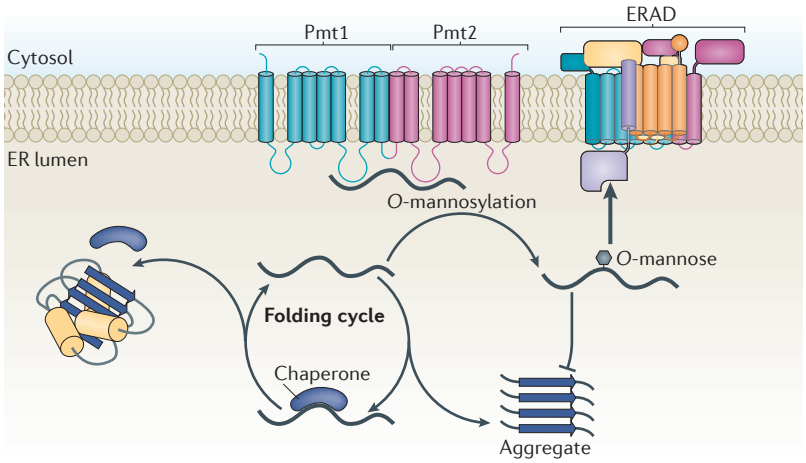

Figure 5 | **The unfolded protein *O*-mannosylation pathway.** A newly synthesized unfolded protein (wavy black line) engages a chaperone to begin its folding. It can either fold (left) or re-engage the chaperone for another round of folding. Failure to fold within a defined time window makes the unfolded protein a substrate for *O*-mannosylation by the protein *O*-mannosyltransferase 1 (Pmt1)–Pmt2 complex. *O*-mannosylation takes the substrate out of the folding cycle, inhibits its aggregation and facilitates its entry into endoplasmic reticulum (ER)-associated degradation (ERAD).

O-mannosylation can function early in the quality control process to terminate futile protein folding cycles, and also in the terminal phase by facilitating protein retention in the ER and ERAD. Although these pathways do not seem to be functionally interdependent, they are certainly complementary.

Important questions remain regarding the role of glycans in protein folding and quality control. How proteins transition from actively folding molecules to ERAD substrates remains unclear. Although the glycan-trimming cascade provides important insights into the process, some details do not resolve neatly. For example, calnexin and calreticulin alternate binding between $Glc_1Man_9GlcNAc_2$ and $Man_9GlcNAc_2$ protein-linked glycans to mediate substrate folding. ERManI or EDEM2 interrupts these cycles at the appropriate time (intervening too early destroys potentially functional proteins and risks ER stress, whereas intervening too late might result in protein aggregation) to trigger ERAD, but how they do so is unknown. Perhaps the most immediate need is to determine the exact activities of ERManI, EDEM1, EDEM2 and EDEM3, and of OS-9 and XTP3-B. Currently, it is unclear whether the reported differences are cell type-dependent and/or species-dependent, or whether they originate in varying substrate requirements.

Free oligosaccharides released from substrates in yeast and mammalian cells show that some ERAD substrates are first retrieved from the Golgi apparatus[83,84]. These studies raise the question of whether retrieval is obligatory for some substrates or simply reflects the existence of a proportion of substrates escaping ER retention mechanisms. With the report of potentially relevant glycan-modifying enzymes in post-ER compartments, the circumstantial evidence is compelling for the former[81,82,118,120–122].

Perhaps the broadest remaining question is how quality control facilitators recognize unfolded proteins. Folded proteins with terminal α-1,6-linked mannose-containing glycans are ignored by ERAD, emphasizing the importance of the unfolded state[76]. In two independent steps, trimming from $Man_8GlcNAc_2$ to $Man_7GlcNAc_2$ and substrate recognition by the HRD1 complex require unfolded protein recognition. In the first step, the oxidoreductases Pdi1 and ERdj5 are likely to play a part through their interactions with their lectins[62,73,129]. However, substrates also bind to the chaperone Kar2, which is also required for ERAD[78–80,89]. At the HRD1 complex, both Hrd3 and Hrd1 have been shown to recognize unfolded proteins, and one or both probably collaborates with Yos9 (OS-9 in mammals) in substrate recognition[87,89,101,150]. For UPOM, substrate recognition remains mysterious. However, as in the case of *N*-glycans, it is likely to involve factors associated with the Pmt1–Pmt2 complex[146]. These roles await experimental confirmation.

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Competing interests statement

The authors declare no competing interests.

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