The intrinsic and extrinsic effects of N-linked glycans on glycoproteostasis

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Proteins that traffic through the eukaryotic secretory pathway are commonly modified with N-linked carbohydrates. These bulky amphipathic modifications at asparagines intrinsically enhance solubility and folding energetics through carbohydrate-protein interactions. N-linked glycans can also extrinsically enhance glycoprotein folding by using the glycoprotein homeostasis or ‘glycoproteostasis’ network, which comprises numerous glycan binding and/or modification enzymes or proteins that synthesize, transfer, sculpt and use N-linked glycans to direct folding and trafficking versus degradation and trafficking of nascent N-glycoproteins through the cellular secretory pathway. If protein maturation is perturbed by misfolding, aggregation or both, stress pathways are often activated that result in transcriptional remodeling of the secretory pathway in an attempt to alleviate the insult (or insults). The inability to achieve glycoproteostasis is linked to several pathologies, including amyloidoses, cystic fibrosis and lysosomal storage diseases. Recent progress on genetic and pharmacologic adaptation of the glycoproteostasis network provides hope that drugs of this mechanistic class can be developed for these maladies in the near future.

The maintenance of the proteome is central to organismal homeostasis. As protein folding is an error-prone process, making appropriate intracellular folding-versus-degradation decisions is central to achieving protein homeostasis or proteostasis1,2. The misregulation of proteostasis is associated with a growing number of human diseases. These include loss-of-function maladies, which are the result of too much degradation and/or failure of a glycoprotein to reach its target environment as well as gain-of-toxic function disorders, which are caused by misfolding and/or aggregation that is often rooted in inappropriate folding-versus-degradation decisions. The proteostasis network, comprising thousands of proteins that make up folding, degradation and trafficking pathways, assists proteome folding and maintenance, sustaining the proteome even under conditions of stress (e.g., thermal and oxidative stress, gene multiplication or mutation, etc.). Maintenance of the proteome under stress is enabled by activating stress-responsive signaling pathways that transcriptionally remodel the proteostasis network to maintain physiologically relevant concentrations of folded and functional proteins in the face of a variety of cellular challenges3,4. Each eukaryotic sub-cellular compartment has a unique proteostasis network, made up of specialized and general components and regulated by a dedicated stress-responsive signaling pathway. The mammalian secretory pathway, which is the focus of this Review, is no exception. It has numerous specialized and common pathways and components and features a multifaceted, three-arm stress-responsive signaling pathway for regulation of secretory pathway proteostasis.

Over one-third of the mammalian proteome undergoes biogenesis and maturation through the cellular secretory pathway. The majority of these proteins have glycans covalently attached to the amide side chain of Asn harbored within an N-glycosylation ‘sequon’, or Asn-X-Ser/Thr sequence, where X is any amino acid other than Pro5,6. These so-called N-linked glycans are generally attached to largely unfolded nascent chains cotranslationally or sometimes post-translationally in the lumen of the endoplasmic reticulum (ER) by the oligosaccharyltransferase (OST)7. As the ribosome directs the secreted and secretory pathway proteome through the translocon, OST-mediated N-glycosylation has important intrinsic effects on nascent glycoproteins. N-glycosylation can intrinsically enhance the folding energetics through native state carbohydrate-protein interactions8–10. N-glycosylated proteins are also more aggregation resistant, owing to the steric bulk and hydration of the glycan. The N-glycan installed in the ER is composed of three glucose, nine mannoses and two N-acetylglucosamines (Glc3Man9GlcNAc2; Fig. 1); this core glycan is remodeled by enzymes including glycosidases and glycosyltransferases as glycoproteins move through the secretory pathway11. Thus, the N-linked glycan structure at a particular sequon within a particular protein is spatially dynamic, which provides information on the extent to which that protein has progressed through the secretory pathway as well as its folding and trafficking fitness. Core N-glycan trimming also allows N-glycoproteins to take advantage of a specialized proteostasis network within the ER, enabling folding-versus-degradation, or quality control, decisions, while also regulating the trafficking of N-glycoproteins11. In this Review, we will discuss the intrinsic and extrinsic role of N-glycans in maintaining glycoproteostasis.

**Intrinsic influences of N-glycans on glycoproteostasis**

Achieving glycoproteostasis is greatly facilitated when individual N-glycosylated proteins have favorable folding energetics and low aggregation propensity1. It has been shown in some cases that the relatively rigid, largely extended and highly hydrated N-glycan intrinsically disfavors aggregation of N-glycoproteins12–15. It has also been proposed that N-glycosylation restricts the conformational entropy of the unfolded glycoprotein, increasing its free energy, which in turn would favor native folding (Fig. 2a)12,15. This effect, however, is not general15. The random introduction of N-glycans into a glycosylation-naive protein, where specific glycan–protein interactions have not evolved and are therefore relatively unlikely, usually does not substantially stabilize a protein16,17. The degree of stabilization depends on the context of

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the N-glycosylation site, arguing against a generic excluded volume effect. When N-glycosylation of a particular protein leads to a more negative (and therefore more favorable) folding free energy relative to its nonglycosylated counterpart, emerging evidence suggests that this intrinsic favorable effect on protein folding energetics is a consequence of native state stabilizing interactions between the N-glycan and the protein. Experimental evidence demonstrates that hydrogen bonds, hydrophobic contacts and CH-π interactions between the glycan and the protein contribute to a more favorable free energy of folding, as discussed in detail below.

Hydrogen bonding. Given the abundance of hydrogen bond (H bond) donors and acceptors contained in glycans and proteins, it is not surprising that H bonds are commonly invoked to explain the native state stabilizing effect of N-glycosylation. However, whether glycan-protein H bonds contribute to stabilization depends on many factors, including the nature of the H-bond donors and acceptors involved in the H bond, the gain in entropy for one type of aromatic amino acid over the others in glycan-protein interactions, except when steric considerations come into play. This hypothesis will be put to the test as more structures of carbohydrate binding proteins are lined with aromatic side chains. Furthermore, aromatic side chains as a group are highly enriched in the regions of protein surfaces that are close to glycosylation sites. Although it is tempting to view these CH-π interactions as being due to electrostatic forces between the partial positive charge on the H atom of the polarized CH bonds of carbohydrates and the partial negative charge above aromatic rings due to their π-electron systems, theoretical and experimental studies suggest that CH-π interactions are actually primarily driven by van der Waals or induced dipole–induced dipole forces. For example, the strength of a CH-π interaction (much like the one in Figure 2d) in the context of a model system for glycan-protein interactions (the WW domain) was found to depend very weakly on the electron density of the aromatic ring. This nearly complete suppression of electrostatic effects may be due to the changes in electrostatic forces having equal effects on CH-π interactions in the native state and water OH-π interactions in the denatured state. On the basis of this result, we expect there to be little preference for one type of aromatic amino acid over the others in glycan-protein interactions, except when steric considerations come into play. The net effect of N-glycosylation is stabilizing. Although the native state stabilization free energy from each source covered above can be small, their sum is often considerable (Figure 2a). This is exemplified by the family of reverse turn–based structural modules known as ‘enhanced aromatic sequons’. In enhanced aromatic sequons, an example of which is shown in Figure 2d, interactions between the N-glycan and an aromatic side chain N-terminal to the glycosylation site are enforced by the geometry of the reverse turn. Glycosylation of the Asn in this enhanced aromatic sequon stabilizes HsCD2ad by ~3 kcal mol⁻¹ (ref. 31). The hydrophobic effect and CH-π interactions between the Phe at the –2 position and the GlcNAc-1 attached to Asn contribute ~1.8 kcal mol⁻¹, whereas the Lys side chain at position –4 makes hydrophobic and possibly H-bond interactions with GlcNAc-2 that contribute the remainder of the free energy of stabilization. Glycan residues beyond the third carbohydrate ring attached to Asn (i.e., the first monosaccharide residue) do not contribute to the folding kinetics or thermodynamics of HsCD2ad. The stabilization resulting from the glycan-protein native state interactions in HsCD2ad is essential for its proper folding; in the absence of glycosylation, HsCD2ad is hydrophobic interactions tend to be less favorable than protein–protein hydrophobic interactions because it is difficult to bury the nonpolar surfaces without affecting the access of adjacent polar surfaces to water. As previously noted, a good example of a stabilizing hydrophobic glycan–protein interaction is in human chorionic gonadotropin, wherein the α-face of the first GlcNAc residue (GlcNAc-1) of the glycan is buried in a pocket formed by Pro24, Ile25 and Leu26 (Figure 2c). In addition, the hydrophobic methyl group of the N-acetyl group on GlcNAc-1 is buried from water by interactions with the Ala23, Ile25 and Val76 side chains.

CH-π interactions. In Figure 2d, the interaction of the first GlcNAc residue of the N-glycan attached to the adhesion domain of the human protein CD2 (HsCD2ad) with the Phe side chain at the –2 position relative to the glycosylated Asn is favorable not only because of the hydrophobic burial of the aryl ring against the α-face of the GlcNAc but also because of a stabilizing CH-π interaction between the axial H atoms on the α-face of the GlcNAc and the π-electron system of the Phe side chain. CH-π interactions between carbohydrates and protein aromatic rings have long been recognized as prominent features of glycan-protein interactions. For example, the binding sites of most lectins (carbohydrate binding proteins) are lined with aromatic side chains. Furthermore, aromatic side chains as a group are highly enriched in the regions of protein surfaces that are close to glycosylation sites. Although it is tempting to view these CH-π interactions as being due to electrostatic forces between the partial positive charge on the H atom of the polarized CH bonds of carbohydrates and the partial negative charge above aromatic rings due to their π-electron systems, theoretical and experimental studies suggest that CH-π interactions are actually primarily driven by van der Waals or induced dipole–induced dipole forces. For example, the strength of a CH-π interaction (much like the one in Figure 2d) in the context of a model system for glycan-protein interactions (the WW domain) was found to depend very weakly on the electron density of the aromatic ring. This nearly complete suppression of electrostatic effects may be due to the changes in electrostatic forces having equal effects on CH-π interactions in the native state and water OH-π interactions in the denatured state. On the basis of this result, we expect there to be little preference for one type of aromatic amino acid over the others in glycan-protein interactions, except when steric considerations come into play. This hypothesis will be put to the test as more structures of glycosylated proteins are solved, enabling the enrichment of aromatic side chains near glycans to be better understood.

The net effect of N-glycosylation is stabilizing. Although the native state stabilization free energy from each source covered above can be small, their sum is often considerable (Figure 2a). This is exemplified by the family of reverse turn–based structural modules known as ‘enhanced aromatic sequons’. In enhanced aromatic sequons, an example of which is shown in Figure 2d, interactions between the N-glycan and an aromatic side chain N-terminal to the glycosylation site are enforced by the geometry of the reverse turn. Glycosylation of the Asn in this enhanced aromatic sequon stabilizes HsCD2ad by ~3 kcal mol⁻¹ (ref. 31). The hydrophobic effect and CH-π interactions between the Phe at the –2 position and the GlcNAc-1 attached to Asn contribute ~1.8 kcal mol⁻¹, whereas the Lys side chain at position –4 makes hydrophobic and possibly H-bond interactions with GlcNAc-2 that contribute the remainder of the free energy of stabilization. Glycan residues beyond the third carbohydrate ring attached to Asn (i.e., the first monosaccharide residue) do not contribute to the folding kinetics or thermodynamics of HsCD2ad. The stabilization resulting from the glycan-protein native state interactions in HsCD2ad is essential for its proper folding; in the absence of glycosylation, HsCD2ad is

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**Figure 1** The initial composition of an N-linked glycan. The primary structure of the GlcNAc₉Man₉Glc, glycan transferred to Asn residues within glycosylation sequons (Asn-Xxx-Ser/Thr sequences, where Xxx is any amino acid other than Pro). Symbols and colors for monosaccharides are those recommended by the Consortium for Functional Glycomics (http://www.functionalglycomics.org/). The modes of linkage for the residues of the glycan are indicated next to the lines joining the symbols (e.g., β4 indicates a β-1-4 linkage, etc.).
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Figure 2 | Intrinsic effects of N-glycosylation on protein folding. (a) A free energy diagram illustrating the change in energy of the unfolded (U) and native (N) states upon N-glycosylation. The energy of the unfolded state of the nonglycosylated protein (G_{un}) tends to increase upon N-glycosylation (G_{nst}), whereas the energy of the native state of the nonglycosylated protein (G_{nst}) tends to decrease (G_{nst}). The effect of N-glycosylation on the free energy of folding (\Delta G_{folding} versus \Delta G_{folding}) is the sum of these effects and can be on the order of several kcal mol^{-1}. (b) Glycan-protein H bonds in the mature, complex-type N-glycan in the Fc fragment of human IgG1 (Protein Data Bank (PDB) code 1FC1). Note that the N-acetyl group of GlcNAc-1 is shown in the energetically unfavorable cis conformation; this may be a misassignment because the electron densities of the acetyl methyl and carbonyl O groups are likely to be similar at this resolution. Fuc, fucose. (c) Glycan-protein hydrophobic burial in human choriconic gonadotropin (PDB code IHCN). The hydrophobic α-face of GlcNAc-1 is buried in a pocket formed by Pro24, Ile25 and Leu26, whereas the N-acetyl methyl group is buried in an adjacent pocket formed by Ala23, Ile25 and Val76. (d) A glycan-protein CH-π interaction in the adhesion domain of the human protein CD2 (HsCD2ad; PDB code 1GYA). The hydrogen atom on C5 of GlcNAc-1 interacts with the aromatic side chain of Phe63. The structural module shown is known as an enhanced aromatic sequon. Only the first GlcNAc of the glycan is shown for clarity.

Impossible to fold in vitro under normal, native conditions\textsuperscript{31,34}. The effect of glycosylation on the proteostasis of HsCD2 in vivo is correspondingly profound. Loss of glycosylation causes the expression levels of HsCD2 to decrease to ~50% of that of the wild-type\textsuperscript{34}. The intrinsic effects of N-glycosylation are similarly important to the proteostasis of other N-glycoproteins, including the cystic fibrosis transmembrane conductance regulator (CFTR)\textsuperscript{35} and rhodopsin\textsuperscript{36} (although it should be noted that in each of these examples extrinsic effects of N-glycosylation also contribute to the proteostasis of these glycoproteins).

Extrinsic influence of N-glycans on glycoproteostasis

Another important function of N-glycans on secreted proteins is to allow the N-glycoproteins to use a proteostasis network reserved for them. Enzymatically trimmed core glycans allow N-glycoproteins to recruit an array of macromolecular folding assistants and quality control carbohydrate-binding proteins comprising the specialized glycoproteostasis network of the ER. The glycoproteostasis network assists N-glycoprotein folding, quality control and degradation. Removing the two terminal A-branch glucose residues (Fig. 1) allows N-glycoprotein interactions with the lectin chaperones, which facilitate N-glycoprotein folding. In contrast, removal of mannose residues from the core glycan targets N-glycoproteins for anterograde or retrograde exit from the ER\textsuperscript{37}.

Quality control in the ER. ER glucosidases initially act cotranslationally on the core glycan that is transferred to N-glycoprotein nascent chains as they are inserted into the ER lumen\textsuperscript{1}. This yields monoglucosylated A-branch glycoproteins that are substrates for the membrane-integrated lectin chaperone calnexin and its soluble paralog calreticulin (Fig. 3). Lectin chaperone binding and conformational cycling to promote folding is antagonized by removal of the final A-branch glucose by glucosidase II. In contrast, glucose readdition to the A-branch by UDP-Glc-glycoprotein glucosyltransferase 1 (UGT1; Figs. 1 and 3) redirects improperly folded N-glycoproteins back into the calnexin-calreticulin folding pathway by a mechanism requiring recognition that the N-glycoprotein client is not properly folded\textsuperscript{38}. Thus, UGT1 acts as a folding sensor and modifies nonnatively folded N-glycoproteins that lack an A-branch glucose by re-adding an A-branch glucose, enabling reengagement by the lectin chaperones.

The conserved globular β-sandwich domains of calnexin and calreticulin contain a single carbohydrate-binding site that interacts with the GlcMan3 tetrasaccharide with a micromolar dissociation constant\textsuperscript{39,40}. A proline-rich arm (P-domain) extends away from the globular domain of calnexin and calreticulin. This arm comprises a cofactor interaction site at the tip that supports binding to the foldases ERp57 and cyclophilin B (CyPB)\textsuperscript{41,42}. ERp57 is an oxidoeductase that facilitates disulfide formation and/or rearrangement, and CyPB is a peptidyl proline isomerase that catalyzes cis-trans proline isomerization.

The lectin chaperone–foldase complexes promote the folding and assembly of functional N-glycoproteins through several mechanisms\textsuperscript{43,44}. The complexes are localized to the ER and thus retain non-native N-glycoproteins in the optimal folding environment of the ER, providing additional opportunities for client N-glycoproteins to reach their native state. For example, UGT1 acts on N-glycoprotein clients that have most likely been through a failed calnexin-calreticulin folding cycle. Chaperone binding also prevents the aggregation of N-glycoproteins for which the intrinsic aggregation-inhibiting effect of N-glycans is insufficient to maintain them in the soluble state and slows the folding reaction\textsuperscript{45-47}. Monoglucosylated N-glycoproteins that persistently bind calnexin-calreticulin, as directed by UGT1, are proposed to fold more slowly than unglycosylated or unglycosylated proteins. Trapping folding substrates in the monoglucosylated state results in continual chaperone binding and arrests distal folding events, as probed by oxidation\textsuperscript{48}. Calnexin and calreticulin...
ER exit to the Golgi. ER mannosidase I (MAN1B1) removes mannose residues from properly folded proteins to mark them for anterograde trafficking (Fig. 3). Depending on the N-glycoprotein cargo, ER exit can either simply result from the bulk flow of substrates that are no longer retained in the ER or from the selective recognition by sorting receptors. ERGIC-53, VIPL and VIP36 are examples of N-glycoprotein cargo receptors that recognize natively folded mannoside-trimmed substrates and package them into COPII vesicles. ERGIC-53 cycles between the ER and ER-Golgi-intermediate compartment (ERGIC), supporting the trafficking of glycosylated substrates such as α-1-antitrypsin, coagulation factor V and VIII and cathepsin Z. The pH and calcium sensitivity of ERGIC-53 are proposed to support substrate binding in the neutral-pH and calcium-rich environment of the ER and release clients in the slightly more acidic ERGIC, assisting in the anterograde trafficking of properly folded N-glycoproteins.

Degradation. Proteins that fail to reach their native state exit the ER to the cytoplasm by a retrograde route, where they are ubiquitinated and then degraded by the proteasome through a process known as ER-associated protein degradation (ERAD). Extensive demannosylation targets N-glycoproteins for degradation, as evidenced by mannoside inhibitors delaying the degradation of glycosylated ERAD substrates. ERAD-directed glycopeptides have Man5GlcNAc2 or Man6GlcNAc2 glycoforms. Demannosylation by glycosylhydrolase 47 family members including MAN1B1, and possibly EDEM1–3, aids the ERAD process by reducing the size of the substrate to facilitate dislocation and removal of the client N-glycoproteins from the lectin chaperone binding and folding cycle. Removing mannose residues also creates glycoforms that are recognized by downstream ERAD carbohydrate-binding receptors. Of special note is the controversy over the function and localization of MAN1B1, as recent studies have localized it to the Golgi and questioned the involvement of its carbohydrate-binding receptors. Of special note is the controversy over the function and localization of MAN1B1, as recent studies have localized it to the Golgi and questioned the involvement of its carbohydrate-binding receptors.

For this model, the associated lectin would directly bind the ERAD receptor and interact with SEL1L in a glycan-dependent manner. ERAD also appears to serve this role as they both bind ERAD substrates and interact with SEL1L to work in concert to recruit the OS-9– or XTP3-B–substrate complex to the ERAD complex and to pass the substrate along to the degradation machinery for dislocation and ubiquitination. The removal of a C-branch terminal α(1,2)-linked mannose exposes α(1,6)-linked mannose residues, generating substrates for the lectin ERAD receptors OS-9 and XTP3-B (Fig. 3). The carbohydrate-binding ERAD receptors OS-9 and XTP3-B contain one and two mannose-6-phosphate receptor homology (MRH) domains, respectively. The MRH domain from OS-9 has high affinity for glycoforms with exposed C-branch α(1,6)-linked mannoses, whereas the carbohydrate binding specificity for XTP3-B is controversial. OS-9 and XTP3-B deliver misfolded proteins to an ER membrane ERAD complex that contains the machinery required for dislocation and ubiquitination. This supramolecular ERAD complex is nucleated by the membrane protein SEL1L. The luminal N-terminal domain of SEL1L contains 11 tritericopeptide repeats and 5 possible N-glycosylation sites that seem to work in concert to recruit the OS-9– or XTP3-B–substrate complex to the ERAD complex and to pass the substrate along to the dislocation and ubiquitination machinery. EDEM1 and EDEM3 also appear to serve this role as they both bind ERAD substrates and interact with SEL1L in a glycan-dependent manner. ERAD substrates are prepared for dislocation by associated factors, such as ERdj5 and BiP for EDEM1 and possibly GRP94 for OS-9. Alternatively, these factors may recognize the aberrant structures using the traditional chaperone queries of exposed hydrophobic residues or free thiols for oxidoreductase searches.

The regulation of glycoproteostasis networks

The accumulation of misfolded proteins in the ER disrupts the efficient maturation of additional entering nascent chains,
leading to a breakdown in protein biogenesis within the secretory pathway. To circumvent such disturbances and maintain cellular homeostasis, the unfolded protein response (UPR) signaling pathway is activated, which regulates ER proteostasis\textsuperscript{4,4}. Enhancing ER proteostasis capacity by UPR activation helps to alleviate stress by upregulating the clearance of defective substrates, by translational attenuation and by increasing the capacity of the ER proteostasis network by transcriptional remodeling. If the stress persists, apoptosis or cell death is induced to preserve organismal homeostasis.

Activation of one or more of the three distinct ER-membrane integrated stress sensors induces signaling in the corresponding arm of the UPR. These stress sensors include the double-stranded RNA–activated protein kinase-like eukaryotic initiation factor 2α kinase (PERK), the inositol-requiring transmembrane kinase/endoRNase 1 (IRE1) and the activating transcription factor-6 (ATF6)\textsuperscript{4,4}. The induction of each branch or arm of the UPR leads to the generation of a transcription factor that upregulates UPR genes involved in protein folding, quality control, degradation and lipid production. For detailed descriptions of the signaling pathways comprising the individual branches of UPR, readers are directed to reviews that concentrate on these topics\textsuperscript{4,4}. Here we will focus on how UPR activation influences the glycoproteostasis network.

The burden of accumulating misfolded or aggregated proteins within the ER can be reduced by translation attenuation, wherein the activity of the translation machinery is reduced. Transcription levels are also reduced by an enzymatic process\textsuperscript{4}. Activated PERK phosphorylates and inhibits the translation initiation factor eIF2α, thereby inhibiting translation initiation\textsuperscript{4}. By controlling translation and reducing the influx of cargo, ER chaperones and quality control factors are free to focus their attention on the current aberrations without adding additional client proteins to the ER.

Activation of b-ZIP transcription factors in the IRE1 and ATF6 adaptive signaling arms of the UPR upregulates UPR target genes that optimize the ER folding environment and enhance the efficient clearance of defective proteins through ERAD\textsuperscript{4,4}. IRE1 induction leads to the increased transcription of genes encoding proteins involved in lipid synthesis, ER import, glycosylation, anterograde trafficking. ERAD (Fig. 3) and the hexosamine biosynthetic pathway that synthesizes the N-glycan precursor GlcNAc\textsuperscript{4,4,7,23}. Augmentation of the ER volume dilutes defective proteins, whereas increasing the level of ERAD machinery facilitates rapid, efficient and less discriminating turnover of non-native cargo. A recent study has found that an increase in the hexosamine biosynthetic pathway enhanced ERAD, proteasomal activity and autophagy and resulted in an extension in the life span in Caenorhabditis elegans, providing an additional link between stress and the glyco-proteostasis network\textsuperscript{23}. ATF6 activation induces the transcription of key chaperones, cochaperones and folding enzymes involved in protein maturation and quality control, including BiP and GRP94 (ref. 72). Together, the IRE1 and ATF6 transcription factors, emerging from the adaptive arms of the UPR, remodel the ER in an attempt to alleviate the stress created by the accumulation of defective client proteins.

Once the stress is diminished, the ER environment needs to be restored to its normal operational state for optimal protein production, a sort of organellar ‘rebooting.’ Raised levels of ERAD components can interfere with efficient protein maturation by prematurely targeting folding intermediates for degradation. The proper ER balance is reestablished through a process termed ERAD tuning\textsuperscript{4,4}. Many of the ERAD components are themselves subjected to rapid turnover, either through ERAD or autophagy. ERAD machinery is stabilized by the presence of misfolded cargo, as non-native proteins appear to serve as better targets for ubiquitination and destruction. Once the misfolded proteins are cleared, the rapid degradation of ERAD machinery helps to reinstate the proper ER balance.

If the misfolded protein load is not adequately cleared after prolonged UPR activation, a cell death program is initiated as an organismal defense mechanism against the accumulation of rogue cells and toxic misfolded or misassembled proteins\textsuperscript{4,4}. Persistent PERK activation starts one of the cascades leading to cell death, thus providing a mechanism to destroy cells that were unable to be rescued by UPR activation.

Proteostasis networks can also be regulated using mechanisms beyond alterations in transcript or protein levels. An example of this added layer of complexity is the tightly controlled activity and localization of calnexin (Fig. 4). Although calnexin is modestly upregulated by UPR activation, its role in glycoproteostasis is further controlled by cofactors, cochaperones, post-translational modifications and its localization within the ER. The ER is a large organelle composed of a continuous membrane that is divided into a number of functional subcompartments\textsuperscript{23}. Post-translational modification of the C-terminal cytoplasmic tail of calnexin directs its localization within the ER. Calnexin phosphorylation supports its increased association with ribosome translocons, positioning the chaperone to aid in early glycoprotein maturation events\textsuperscript{23}. Palmitoylation enriches calnexin localization to ribosome translocons as well as to the mitochondria-associated membranes through its increased association with the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase calcium pump\textsuperscript{23}. The substrate binding activity of calnexin...
is regulated by calcium binding79. The activity of calnexin is also influenced by cochaperones or foldases. The localization of UGT1 to pre-Golgi intermediates by immunoelectron microscopy, combined with the observation that UGT1 does not modify ribosome- 
aressed nascent chains, suggests that UGT1 modification and the rebinding of calnexin to reglucosylated substrates occurs near ER exit sites78,79. ERp57 association with calreticulin has recently been shown to support the closing of the P-domain on the substrate, perhaps enhancing its ability to aid in the folding process80. Calnexin, which has a longer P-domain, can be expected to function through a similar mechanism. Interestingly, stress treatments decrease calnexin palmitoylation levels and support the reorganization of calnexin and calreticulin into the ERQC77,81, a quality control compartment that is enriched for ERAD machinery such as ER ManI/Man1B1, EDEM1, Derlin-1 and OS-9 (ref. 81). The lack of UGT1 and ERp57 in the ERQC suggests that the localization of calnexin into the ERQC might be a mechanism for ERAD substrate delivery to a dislocation and ubiquitination center, as calnexin substrate binding is expected to be weaker in the absence of ERp57 and UGT1.

**Defects in glycoproteostasis are linked to pathology**

As N-glycoproteins are critical for many important physiological processes, defects in glycoproteostasis are associated with many diseases. For example, insertions or deletions in the calreticulin gene are found in patients suffering from chronic myeloid leukemia82. Mutations in the ER exit lectin ERGIC-53 are associated with blood coagulation diseases caused by deficiencies in factors V and VIII53, and defects in UPR activation controlling secretory pathway proteostasis are linked to many maladies, including bipolar disease and diabetes83. The impaired maturation of glycoconjugates is also associated with a long list of genetic disorders called congenital disorders of glycosylation that display a wide array of symptoms owing to the vast spectrum of proteins affected by glycosylation abnormalities84. Furthermore, mutations in N-glycoproteins commonly lead to misfolding, resulting in loss of function due to enhanced ERAD clearance, cystic fibrosis and many lysosomal storage diseases are caused by such losses of function.

There are several traditional pharmacologic approaches that can be used to avoid disease-associated glycoproteostasis challenges (Table 1). For example, the targeting of viral glycoproteins for destruction has been explored as an antiviral therapy. As membrane envelopes of viruses such as influenza and HIV are comprised largely of glycoproteins, derailment of N-glycoprotein maturation by inosinsugar inhibition of glycan-processing enzymes has been used as a strategy for development of antiviral drugs83,84. Glycoproteins such as hemagglutinin and neuraminidase for influenza and gp160 for HIV are essential for the viral life cycle, and the usurping of the ER for the robust production of these complicated glycoproteins taxes ER glycoproteostasis capacity. Glucosidase inhibitors such as N-butyl-deoxynojirimycin (NB-DNJ, also known as miglustat or by the trade name Zavesca) have been explored as possible antiviral therapies as the accumulation of triglucosylated core glycans prevents the viral glycoproteins from availing themselves of the lectin chaperones calnexin and calreticulin for folding assistance83 (Fig. 3). Glucosidase inhibition, which is surprisingly well tolerated in mammals85,86, results in viral glycoprotein misfolding and the clearance through the ERAD pathway, thereby reducing the titer of infectious viral particles.

Genetic or pharmacologic adaptation of the glycoproteostasis network is being aggressively explored as a strategy to provide effective therapies for a variety of human maladies, wherein defects are rooted in glycoproteostasis. Here, we will focus on efforts launched to develop small-molecule proteostasis regulators that adapt or preemptively prepare the ER for an insult or stress, boosting the efficiency of N-glycoprotein maturation. Some proteostasis regulators being studied increase the concentration of calcium in the ER, whereas others act as arm-selective UPR activators. Both strategies help to optimize the ER for maturation of properly folded substrates and the clearance of defective client proteins.

The ER is a site for Ca2+ storage and regulation87, and therefore it is not surprising that many ER glycoproteostasis network components are calcium-binding proteins, including calnexin and calreticulin. As the ER regulates glycoproteostasis and calcium homeostasis, it is likely that their regulation is interdependent. Thus, it may be possible to achieve synergy in the rescue of glyco- proteostasis by also altering ER Ca2+ levels. In fact, the influence that ER Ca2+ levels have on N-glycoprotein maturation seems to be protein specific. Inhibiting sarcoplasmin reticulum Ca2+-ATPase ER calcium influx channels has been found to increase the proper trafficking of the mutant in CFTR (AF508) predominantly associated with cystic fibrosis88. The issue for CFTR appears to be overzealous quality control that retains a mutant N-glycoprotein with partial chloride channel activity in the ER, directing it for ERAD. ER retention of CFTR seems to be relaxed by a decrease in ER Ca2+ levels. Thus, decreasing ER Ca2+ levels could serve to get more CFTR to the plasma membrane, thereby enhancing the efficacy of ivacaftor (trade name Kalydeco), a channel potentiator introduced by Vertex Pharmaceuticals89–91. Depletion of ER calcium has been shown to disrupt the ER proteostasis network by permitting the release of chaperones from the ER82. In contrast, the N-glycosylated mutant lysosomal enzymes associated with lysosomal storage disease need more attention from calnexin and calreticulin to fold properly, and increased ER Ca2+ levels provide an environment that allows more efficient folding and trafficking of these misfolding-prone enzymes. Thus, the Ca2+ channel blockers diltiazem and verapamil, which inhibit the ER ryanodine receptor calcium efflux channels and other targets, have been used to improve the cellular trafficking and activity of N-glycosylated mutant lysosomal enzymes associated with lysosomal storage diseases92–94. Altering ER Ca2+ levels in concert with direct perturbation of glycoproteostasis machinery is a promising therapeutic strategy, particularly as we learn more about how the mutant lysosomal enzyme–glycoproteostasis network component interactions change upon perturbation.

Small-molecule stress-responsive signaling pathway activators can serve as proteostasis regulators to correct the trafficking of mutant N-glycoproteins associated with a number of diseases. Activation of the UPR using proteasome inhibitors such as MG-132 has been used to correct the trafficking of N-glycosylated mutant lysosomal enzymes associated with Gaucher and Tay-Sachs diseases95. Interestingly, enhanced glucocerebrosidase trafficking in MG-132–treated fibroblasts derived from patients with Gaucher disease was associated with downregulation of the expression level of the peptidyl proline isomerase FKBP10, suggesting that altering the finely choreographed interactions in the ER can be used to enhance mutant N-glycoprotein folding96. The histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) has been used to correct the cellular trafficking of mutant α-1-antitrypsin and GABA_A receptors associated with epilepsy97,98. Although the mechanism for SAHA enhancement is uncertain, it appears to involve, in part, HDAC7 silencing and a calnexin-sensitive pathway.

**Summary and future directions**

The importance of the intrinsic and extrinsic effects of N-glycosylation on glycoprotein folding is underscored by the nearly universal conservation of protein N-glycosylation across eukaryotes99,100. Maintaining an effective glycoproteostasis network is pivotal for cellular proliferation and normal cell function. A number of mechanisms, involving ER, Golgi and cytoplasmic
components exist to keep the glycoproteostasis network functioning. ER stress is one of the major factors contributing to the loss of glycoproteostasis, as glycoproteins are synthesized, modified and folded in the ER.

The activation of UPR stress sensors is one of the first steps to alleviate the accumulation of misfolded or misassembled glycoproteins. Once the UPR is activated, a number of lectin chaperones, chaperones and glycan-modifying enzymes are upregulated to rebalance ER proteostasis. Stress also contributes to the post-translational modification and altered localization of calnexin in the ER. Further investigations are required to determine how the organization of other components of the glycoproteostasis network is regulated by stress in the ER. Current efforts are also under way to develop methodologies to activate the individual arms of the UPR, the hypothesis being that more targeted therapies could be tailored to individual diseases while minimizing potential side effects.

### Table 1 | Compounds tested as chemical regulators of glycoproteostasis.

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castanospermine</td>
<td><img src="image1" alt="Structure" /></td>
<td>α- and β-glucosidase inhibitor</td>
</tr>
<tr>
<td>1-Deoxynojirimycin</td>
<td><img src="image2" alt="Structure" /></td>
<td>α1,2-Mannosidase inhibitor</td>
</tr>
<tr>
<td>1-Deoxynojirimycin derivatives: N-butyl-deoxynojirimycin (miglustat, trade name Zavesca), N-9-methoxynonyl-DNJ, UV-4</td>
<td><img src="image3" alt="Structure" /></td>
<td>α-Glucosidase inhibitor</td>
</tr>
<tr>
<td>Diltiazem</td>
<td><img src="image4" alt="Structure" /></td>
<td>Calcium channel blocker</td>
</tr>
<tr>
<td>Kifunensine</td>
<td><img src="image5" alt="Structure" /></td>
<td>Class I glycoprotein processing α-mannosidase inhibitor</td>
</tr>
<tr>
<td>MG-132</td>
<td><img src="image6" alt="Structure" /></td>
<td>Reversible proteasome inhibitor</td>
</tr>
<tr>
<td>Suberoylanilide hydroxamic acid</td>
<td><img src="image7" alt="Structure" /></td>
<td>Histone deacetylase inhibitor (zinc ion chelator)</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td><img src="image8" alt="Structure" /></td>
<td>Blocks N-linked glycosylation by inhibiting transfer of N-acetylglucosamine-1-phosphate from UDP-N-acetylglucosamine to dolichol phosphate</td>
</tr>
<tr>
<td>Verapamil</td>
<td><img src="image9" alt="Structure" /></td>
<td>Calcium channel blocker</td>
</tr>
</tbody>
</table>
knowledge surrounding the parts list and the systems functions of the glycoproteostasis network and its regulation will lead to more sophisticated therapeutic strategies as well as contribute to our understanding of how glycoproteostasis is achieved.

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6. Freeze, H.H., Chong, J.X., Bamshad, M.J. & Ng, B.G. Solving glycosylation problems by mass spectrometry: sophisticated therapeutic strategies as well as contribute to our understanding of how glycoproteostasis is achieved.


29. Additional helpful survey of structural environment of N-linked glycans.


33. This article introduced the enhanced aromatic sequon, an N-glycosylated structural module that stabilizes the proteins in which it is found and appears to be glycosylated with unusually high efficiency by OST.


47. Describes a detailed model for the molecular choreography for nascent glycoprotein maturation and its interaction with ER lectin chaperones.


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Competing financial interests
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