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Folding and Quality Control of Glycoproteins☆

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Glossary

Calnexin/Calreticulin ER resident molecular chaperones/lectins that specifically bind to mono-glucosylated oligosaccharides.
Cargo receptor A receptor for a cargo protein, which is destined to be concentrated in a budding vesicle (COP II vesicle). ERGIC-53, for example, is believed to be a cargo receptor for certain glycoproteins.
EDEM *ER degradation enhancing* α-*mannosidase like protein*. A protein that has homology with ER α-mannosidase I and is involved in glycoprotein-specific ERAD.
FBS A subunit of SCF ubiquitin ligase complex that recognizes *N*-glycosylated proteins as a subunit. Recent evidence also suggests that one of the FBS proteins is also involved in the induction of lysophagy, i.e. the clearance of damaged lysosomes.
OST An oligosaccharyltransferase that is involved in the transfer of oligosaccharides to the asparagine unit in a sequon (in eukaryotes, Asn-X-Ser/Thr where X can be any amino acid except for Pro) of proteins. The catalytic subunit (Stt3 in eukaryotes, PglB in bacteria and AglB in archaea) is conserved in all domains of life.
NGLY1 Cytosolic deglycosylating enzymes that act on *N*-glycans. Mutations in the *NGLY1* gene results in human genetic disorder referred to as an NGLY1 deficiency.
Proteasome A gigantic protein complex in the cytosol. This complex is involved in various cellular processes, but one of the major functions is to clear aberrant, non-functional proteins.
Retrotranslocon Retrotranslocation channels that translocate aberrant proteins from the lumen of the ER to the cytosol. The molecular nature of the protein channel for this process still remains to be revealed, but HRD1 is considered to be the strong candidate for ERAD-L substrates.
UGGT A glucosyltransferase that is located in the lumen of the ER. It only acts on glycoprotein substrates that are partially unfolded, thereby serving as a "folding sensor" in the ER quality control system.

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Abbreviations

AAT	α1-antitrypsin
Aβ	Amyloid-β
AD	Alzheimer's disease
APP	Amyloid precursor proteins
BiP	Binding immunoglobulin protein
CAZy	Carbohydrate-active enzyme
CDG	Congenital disorders for glycosylation
CNX	Calnexin
CRD	Carbohydrate recognition domain
CRT	Calreticulin
EDEM	ER degradation enhancing mannosidase-like protein
EGAD	Endosome and Golgi-associated degradation
ENGase	Endo-β-*N*-acetylglucosaminidase
ER	Endoplasmic reticulum;
ERGIC	ER-Golgi intermediate compartment
ERAD	ER-associated degradation
ERQC	ER quality control
FBS	The F-box protein that recognizes sugars
FDA	Food and drug administration
FRET	Fluorescence resonance energy transfer
IRF3	Interferon-regulatory factor 3
KO	Knockout
MHC	Major histocompatibility complex
MRH	Mannose-6-phosphate receptor homologue
NMDA	*N*-methyl-D-aspartate
NMR	Nuclear magnetic resonance
NR	Nicotinamide riboside
NRF	Nuclear factor (erythroid-derived 2)-related factor
OST	Oligosaccharyltransferase
PAW	PNGase and other worm
PDI	Protein disulfide isomerase
PGC	Proliferator-activated receptor-γ cofactor
PNGase	Peptide:*N*-glycanase
PUB	PNGase and UBA or UBX-containing proteins
RING	Really interesting new gene
UBA domain	Ubiquitin-associated domain
UBX domain	A domain found in ubiquitin-regulatory proteins
UGGT	UDP-Glc:glycoprotein glucosyltransferase
UPR	Unfolded protein response

1 Introduction

1.1 ERQC, ERAD and ER-phagy

The endoplasmic reticulum (ER) is the site of synthesis of soluble and/or membrane proteins that pass through the secretory pathway in eukaryotes. These newly synthesized proteins are incorporated into the lumen of the ER via a "translocon", a protein-conducting channel.[1–4] The proteins undergo various co- and posttranslational modifications in the lumen of the ER, including cleavage of the signal sequence, *N*-glycosylation, glycosylphosphatidylinositol (GPI)-anchoring, and disulfide-bond formation/isomerization. These modifications lead to the formation of a functional conformation (folding), oligomerization and subunit formation. Once these proteins achieve the correctly folded state, they exit the ER and are delivered to their respective destinations. However, cells must ensure that only functional proteins leave the ER, since the uncontrolled exit of proteins from the ER could pose various serious problems for cells. To address this issue, eukaryotic cells carry out a process called "ER quality control" (ERQC),[5–11] in which protein folding and assembly are monitored, and the transport of immature proteins is prevented. There are, however, significant numbers of proteins that consistently fail to form the correct structure (folding or subunit formation), and it has been estimated that as much as 30% of proteins fail to acquire correct folding and undergo proteasomal degradation.[12] In the ERQC

system, unfolded proteins are assisted by various lumenal chaperones to undergo "functional" folding. However, some proteins still fail to achieve functional folding due to, for example, genetic mutations of the proteins. Such proteins are not only non-functional but may also be toxic to cells, since they can form aggregates inside or outside of the cells. To avoid this, cells have evolved a machinery that permits such unwanted proteins to be eliminated, by "dislocating" or "retro-translocating" proteins from the ER lumen into the cytosol, where the proteasome plays a central role in their degradation.[13] Such a degradation system is often referred to as "ER-associated degradation" (ERAD).[11,13–16] These pathways can also have other functions in addition to the quality control of proteins, such as the maintenance of cellular homeostasis.[17] More recent emerging evidence suggests that an alternative route exists for eliminating ER-resident defective proteins via an autophagy-lysosomal system, which is referred to as ER-phagy.[15,18–20] While the issue of how such proteins are identified for degradation remains unclear, ER-phagy appears to process large protein aggregates that are not handled by ERAD. The basic machinery for both ERAD and ER-phagy are well conserved across eukaryotic evolution.[15] In this chapter, we mainly discuss ERQC and ERAD, in which glycan structures are known to play pivotal roles in dictating the folding status of the client proteins.

1.2 *N*-glycosylation: Not simply an accessory

Glycans, including *N*-linked or asparagine-linked glycans, that are attached to proteins are known to have quite diverse functions.[21] The presence of an *N*-glycan can affect the physicochemical properties of a protein such as stability/solubility, as well as its physiological properties, such as interactions with receptors or other biological activities. It has been predicted that more than 50% of all eukaryotic proteins are actually glycoproteins, and that 90% of these molecules are *N*-glycosylated.[22] The biosynthesis of *N*-glycans begins on the cytoplasmic face of the ER, and is completed in the lumen (Fig. 1). This process is relatively well conserved throughout eukaryotes,[24] but there are species with a less complex biosynthetic pathway as the result of the secondary loss of *ALG* genes, in which encoding enzymes add glycans that are attached to dolichol, a precursor for *N*-glycans.[25] In the case of mammalian cells, the final product on dolichol is $Glc_3Man_9GlcNAc_2$ (Fig. 1). Once the completed glycans are transferred to nascent polypeptide

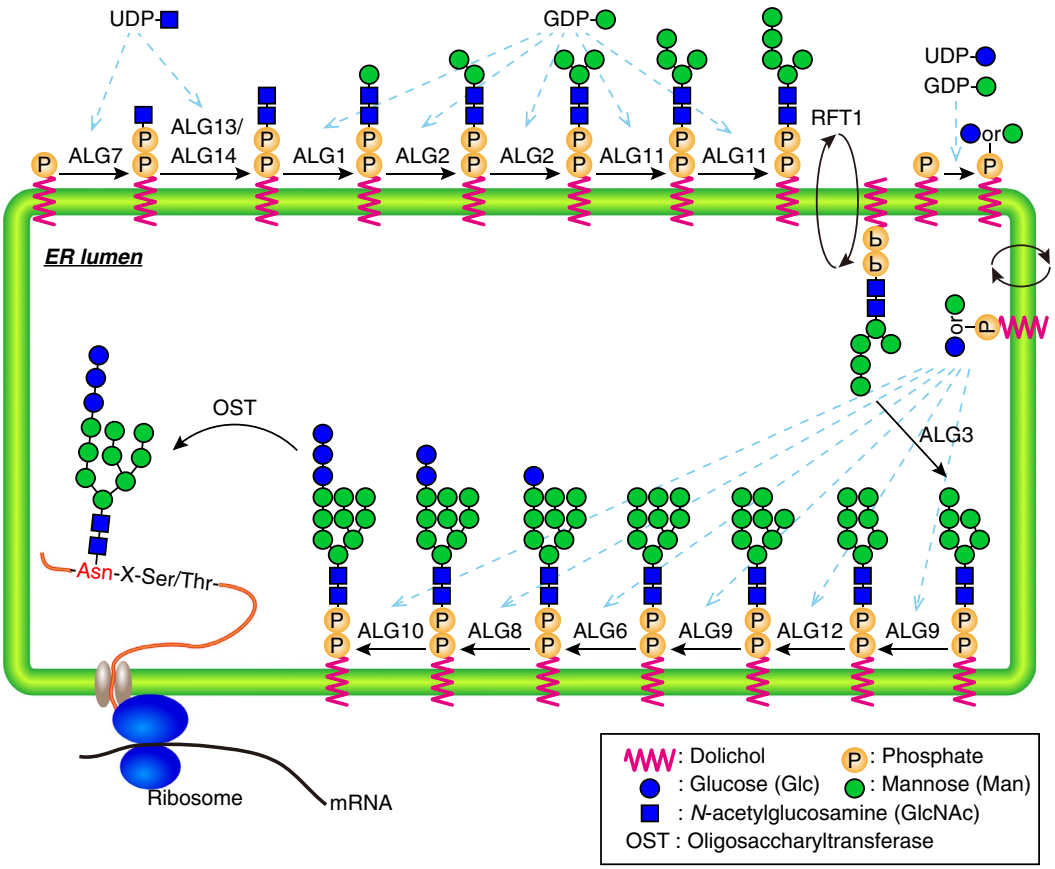

Fig. 1 Biosynthesis of Dolichylpyrophosphoryloligosaccharides in mammalian cells. Synthesis starts on the cytoplasmic face where GlcNAc-1-phosphate is transferred from UDP-GlcNAc to dolichylphosphate (Dol-P). This reaction is followed by the addition of a GlcNAc as well as five mannose units, all of which use nucleotide sugars (UDP-GlcNAc or GDP-Man) as donor substrates. The $Man_5GlcNAc_2$ oligosaccharides are then translocated (flipped) to the lumen of the ER.[23] On the lumenal side, the lipid linked $Man_5GlcNAc_2$ is further elongated by the addition of four mannose and three glucose units, all of which involve lipids donors (Dol-P-Man or Dol-P-Glc) instead of nucleotide sugars as donor substrates. Only after the biosynthesis of Dol-PP-oligosaccharide is complete, is the efficient transfer of the oligosaccharides onto a nascent polypeptide chain achieved by oligosaccharyl transferase (OST).

chains in the ER, they are rapidly trimmed, and as the proteins proceed through the Golgi complex, the structures of glycans are drastically remodeled, resulting in the formation of a variety of different oligosaccharide structures. For example, proteins that are secreted in mammalian cells normally contain sialic acid-containing, "complex-type" glycans, that are completely different from the original, high-mannose type structures that are usually considered to be an "ER" form. The question of why eukaryotic cells have evolved such a seemingly laborious system remains an enigma. However, recent studies have shown that *N*-glycan structures on glycoproteins play pivotal roles in the ERQC/ERAD system,[26–34] i.e. a specific structure is not a mere biosynthetic intermediate but represents a specific function.

2 *N*-glycan dependent quality control—Early events

2.1 Oligosaccharyltransferase (OST)

Most eukaryotic cells synthesize *N*-linked glycans by an *en bloc* transfer of the $Glc_3Man_9GlcNAc_2$ oligosaccharide (Fig. 2; some structural variation occurs depending upon the species[25]), which is initially constructed on dolichol pyrophosphate, onto the Asn-X-Ser/Thr site (where X can be any amino acid except proline) of the nascent polypeptide in the ER lumen (Fig. 1). This enzyme activity is mediated by the action of an oligosaccharyltransferase (OST).[35–39] OST is a membrane protein complex, comprised of at least eight different subunits.[39] In *S. cerevisiae*, Subcomplex 1 contains Ost1p and Ost5p; Subcomplex 2 contains Stt3p, Ost4p and either Ostp3p or Ost6p; and Subcomplex 3 contains Swp1p, Wbp1p and Ost2p, five of which (Ost1p, Stt3p, Swp1, Wbp1 and Ost2p) are essential proteins.[39] Ost3p/Ost6p are paralogues that define distinct OST isoforms.[40–43] Several studies have shown that Stt3p is the central subunit involved in the transfer of the glycan to the polypeptide.[41,44,45] Furthermore, Stt3p and its bacterial or archaeal homologues, PglB or AglB respectively, share an unexpectedly high structural similarity, and can exert OST activity by itself,[46–51] strongly indicating that Stt3p is the catalytic subunit of OST. In multicellular plants and metazoans, there are two STT3 paralogues, i.e. STT3A and STT3B.[41,52,53] In mammalian cells, most of the subunits have yeast homologues; Subcomplex 1 contains RPN1 (Ost1p) and TMEM258 (Ost5p), Subcomplex 3 contains RPN2 (Swp1p), OST48 (Wbp1p) and DAD1 (Ost2p), while there are two types of Subcomplex 2—one containing STT3A (Stt3p) with OST4 (Ost4p) and mammalian-specific subunits, DC2 and KCP2; while the other contains STT3B (Stt3p) and MugT1 or TUSC3 (Ost3p/Ost6p) (reviewed in ref.[39]). It has been suggested that in mammalian cells, the STT3A complex is associated with the translocation channel and is involved in cotranslational

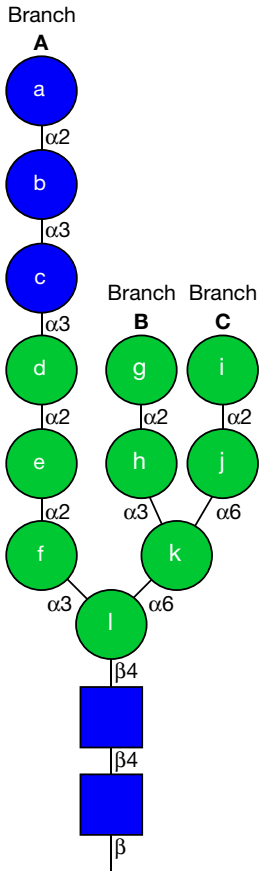

Fig. 2 Schematic representation of the transfer of a Glc3Man9GlcNAc2—oligosaccharide onto the nascent polypeptide chains in the ER of mammalian cells.

N-glycosylation, while the STT3B complex mediates the co- and posttranslational *N*-glycosylation of acceptor sites that are skipped by the STT3A complex.[54–59] MagT1 and TUSC3, and their yeast counterparts Ost3p and Ost6p serve as an oxidoreductase and are involved in the efficient *N*-glycosylation of a subset of acceptor sites.[52,60,61] The Ost6p or TUSC3 protein has also been structurally characterized and the results indicate that they form a typical thioredoxin fold with a conserved active site CXXC motif.[60,62]

The 3D X-ray crystallography structure of PglB or AglB,[63–68] as well as the structures of yeast OST complex[69,70] or a mammalian STT3A complex bound to the protein translocation channel,[71] determined by cryo-electron microscopy, provide us with various insights into the structural organization, recognition of substrates and the catalytic mechanism (reviewed in ref.[39]).

The functional importance of OST is exemplified by the recent finding of human congenital disorders for glycosylation (CDG)[72] (OST48 (DDOST),[73] TUSC3,[74–77] MagT1,[78–81] RPN2,[82] STT3A[77,83,84] and STT3B[83]), in which mutations in various OST subunits were identified. Moreover, mutations in SSR3/4, subunits of the TRAP complex, were found to be associated with the hypoglycosylation of proteins, similar to CDGs,[85] presumably because the TRAP complex interacts with the STT3A complex.[86,87] The fact that mutations of STT3A and STT3B both cause CDG clearly indicates that they have non-redundant functions with different client proteins.

Following translocation to the ER and *N*-glycosylation, the glycoproteins, with the aid of ER lumenal chaperones, begin the process of co-translational folding. For polytopic membrane proteins, the folding process also involves their exit from the translocon complex into the lipid bilayer in an appropriate architecture. This process continues, even after the translation of the protein is complete. In some cases, the presence of a glycan itself is required for the proper folding of a protein, an example of which is impaired secretion exerted by tunicamycin, an inhibitor of *N*-glycosylation, through improper or impaired folding. However, there are also many examples in which the functions of proteins remain unaffected, indicating that the effect of *N*-glycosylation varies among proteins (reviewed in ref.[88–90]).

2.2 α-Glucosidases I/II and Malectin

The first glycan processing event that occurs during the early stage of folding is the trimming of peripheral glucose units via the action of α-glucosidases I and II (Fig. 3). The biological importance of such a deglucosylation event was first noticed by the observation that an inhibitor of these glucosidases led to the rapid degradation of certain glycoproteins.[91–94] In this connection, it is interesting to note that upon incubation with a glucosidase inhibitor, temporal formation of aggregates is observed in the ER.[95] The aggregates are insoluble in an SDS-polyacrylamide gel, raising the possibility that the rapid "clearance" of proteins may, at least in some cases, not be due to rapid degradation but rather to aggregate formation. Indeed, the glycoprotein in the "controlled" aggregate was ultimately degraded by proteasome activity, indicating that the aggregate had slowly dissolved and was then retrotranslocated into the cytosol in a regulated manner. The molecular mechanism by which this aggregate is formed remains unclear at this time, but lumenal chaperones such as BiP (GRP78) or the protein disulfide isomerase (PDI), but not calnexin (CNX), have been observed to be present in the aggregate, suggesting the involvement of these chaperones in the formation of this ER aggregate.[95]

α-Glucosidase I is a type II membrane protein that removes the outermost α1,2-linked Glc residue (residue a in Fig. 2) from a glycoprotein.[96,97] This enzyme belongs to glycoside hydrolase (GH) family 63 in the CAZy database.[98] A defect in glucosidase I has been reported in a neonate with severe hypotonia and dysmorphic features[99,100] (MOGS-CDG or CDG-IIb). The patients also showed an increased resistance to certain types of viral infections, which is believed to be the result of impaired *N*-glycan processing.[101,102] The disruption of the orthologue gene (gsc-1) in *Arabidopsis thaliana* results in pleiotropic defects, including abnormally shrunken seeds.[103] The *Saccharomyces cerevisiae* α-glucosidase I orthologue (*GLS1/CHW41*) is not essential for normal growth,[104,105] but a defect results in reduced levels of β-1,6-glucans in the cell walls,[105] which causes hypersensitivity to the drug Calcofluor white.[97,105] The 3D structure of the catalytic domain of yeast Gls1 has been solved by X-ray crystallography.[106] The *GLS1* gene is also allelic to the *DER7* gene, which is required for the degradation of misfolded glycoproteins.[107] In sharp contrast, deletion of the *gls1* gene in *Schizosaccharomyces pombe* results in lethality[108] or severe growth phenotypes.[109] This enzyme activity, as well as the synthesis of dolichol-P-Glc,[110,111] are defective in trypanosomatid protozoa.[112] Therefore, unglucosylated oligosaccharides are transferred by OST in these species.[111,113] However, they appear to contain other components (α-glucosidase II, calreticulin (CRT), and glucosyltransferase) that are required for the CNX/CRT cycle[114–119] (see below).

Malectin is an ER-resident lectin that is conserved in the animal kingdom, and specifically binds to $Glc_2Man_9GlcNAc_2$ glycans.[120–122] Malectin binds to client proteins in both an *N*-glycan- and in a folding status-dependent fashion, thereby preventing their secretion.[122] The expression of malectin is induced upon ER stress, and it binds to substrates without affecting their entry to the CNX cycle system (see below).[123] It was therefore proposed that the binding of malectin to its substrates may be activated upon ER stress to inhibit the secretion of defective proteins under conditions in which ER quality control systems are compromised.[123] Malectin also forms a complex with ribophorin I,[124] and its association with OST was subsequently confirmed by a proteomics analysis of OST-associating proteins.[52] The association of malectin with ribophorin appears crucial for association with misfolded glycoproteins and, accordingly, the prevention of their secretion.[124,125] It was subsequently found that malectin is also involved in the human cytomegalovirus product (US2)-mediated rapid degradation of major histocompatibility complex (MHC) class I molecules.[126] On the other hand, it has been suggested that malectin is not involved in the degradation of a glycoprotein ERAD substrate.[126] The functional importance of malectin in the glycoprotein ERAD process remains ambiguous.

α-Glucosidase II is a soluble ER lumenal enzyme that functions to cleave inner α1,3-linked glucoses (residues b, c in Fig. 2), and is composed of two subunits, α and β.[127,128] The sequence in the C-terminal half of the α chain contains a catalytic subunit that

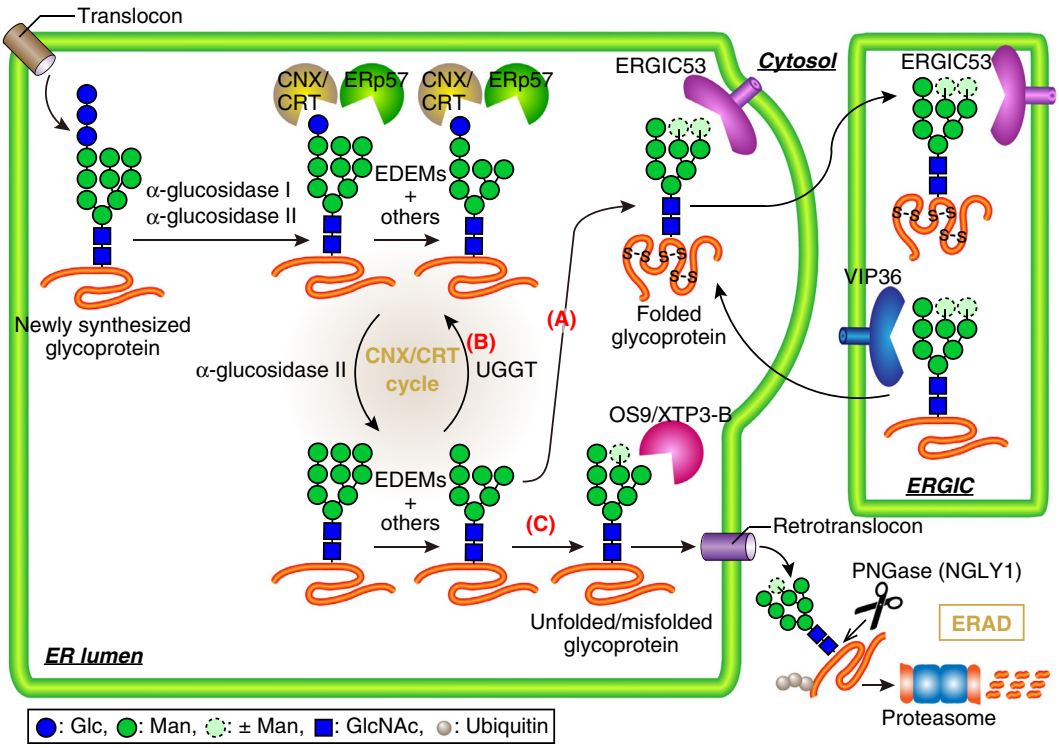

Fig. 3 Glycoprotein ERQC/ERAD system in mammalian cells. Immediately after transfer of the *N*-glycan to the nascent polypeptide by OST, the non-reducing end Glc is removed by the action of α-glucosidase I. That reaction is followed by the action of α-glucosidase II, which removes the middle Glc residue, thus generating the mono-glucosylated core glycan. Calnexin (CNX) or calreticulin (CRT), depending upon the accessibility to the location of the glycan, are associated with the mono-glucosylated glycan. This interaction affords time for the proteins to mature with the aid of various lumenal chaperones. At the same time, the CNX/CRT-binding protein, ERp57, provides assistance in forming the correct disulfide bond while being associated with CNX/CRT. When α-glucosidase II removes the final glucose, the glycoprotein dissociates from CNX/CRT. The protein then either (A) leaves the ER, if it is folded properly or forms the correct complex to acquire a functional conformation, or (B) is recognized, and reglucosylated by UDP-glucose:glycoprotein glycoprotein glucosyltransferase (UGGT), if the protein is partially malfolded, thus permitting it to reenter another CNX/CRT cycle, or (C) is retrotranslocated from the ER into the cytosol for degradation (ERAD), if the protein folding state is not recognized by UGGT. The (C) process is guided by the trimming of α1,2-linked mannose residues by EDEM1/3/MAN1B1/Golgi α-mannosidase I, and the recognition of the exposed α1,6-linked mannose by OS-9/XTP-3B. In pathway (A), lectins such as ERGIC-53 serve as a cargo receptor to facilitate the exit of glycoproteins to their respective destination. Glycoproteins without the correct processing of *N*-glycans can sometimes still exit the ER; in such cases there are several back-up systems to correct the problem. For example, VIP36 may return proteins with immature glycans back to the ER to allow them more time to fold properly, or endo-α-1,2-mannosidase (not shown in this figure) may remove the improper Glc residue(s) and the first mannose of the A branch, to assist *N*-glycans to mature correctly. In pathway (C), aberrant glycoproteins, which are disposed of into the cytosol through retrotranslocation-specific channels ("retrotranslocons"), are recognized by an SCF^{Fbs} ubiquitin ligase complex for polyubiquitination or are deglycosylated by PNGase, and the proteins are ultimately degraded by 26S proteasomes. Note that a part of this scheme is still hypothetical and not all the processes have been unequivocally confirmed. For more details, see the text.

belongs to GH family 31. This enzyme both allows substrates to enter the CNX/CRT cycle, and to exit from it (see below) (Fig. 3). The β chain of α-glucosidase II contains a C-terminal KDEL ER retrieval sequence, as well as a domain that is referred to as the MRH (mannose-6-phosphate receptor homologue) domain.[129–133] The MRH domain acts as a lectin for high mannose-type *N*-glycans and therefore facilitates the enzyme activity of the α subunit.[134–138] A co-expression study of the α and β subunits indicated that both subunits are essential for enzymatic activity, solubility and/or stability, as well as retention of the enzyme in the ER.[139,140] Inhibition or genetic disruption of α-glucosidase II results in the partial impairment of the ERQC/ERAD process, with secretion of incompletely folded proteins or the premature degradation of misfolded glycoproteins.[141–144] The disruption of the catalytic subunit in *S. pombe* results in the complete loss of α-glucosidase II activity, induction of the unfolded protein response (UPR), and accumulation of cargo proteins in the ER.[143] Mutations in the β subunit in humans are associated with autosomal dominant polycystic liver disease,[145,146] an inherited condition involving the formation of multiple cysts of biliary epithelial origin in the liver. The *S. cerevisiae* α-glucosidase II gene, *GLS2/ROT2* encodes an orthologue of the α subunit, while *GTB1* encodes the β subunit.[128,147] Mutants lacking Gls2 show no detectable growth defects but, as in the case of the *gls1* mutant, they produce reduced levels of β1,6-glucans[148] and contain elevated levels of chitin in the cell walls.[149] The lack of this gene also drastically slows the degradation of a misfolded glycoprotein.[150] This enzyme trims the second and third α1,3-linked glucose units by the step-wise trimming of glucose units, with different kinetics,[136] while this difference was not obvious in the presence of crowding agents, i.e. high concentration of proteins or polyethylene glycol.[151] The 3D X-ray structures of the catalytic α subunit in the presence of the binding domain of the β

subunit from mouse[152] or a thermophilic fungi[153] have been determined. Moreover, the 3D X-ray structure of the catalytic α subunit complexed with two different glucosyl ligands has provided the structural basis for a two-step deglucosylation, suggesting that the deglucosylation reactions do not proceed successively and, after the first glucose trimming event, the substrates must be dissociated from the enzyme.[154] This characteristic might allow substrates to have sufficient time to interact with calnexin (CNX) or calreticulin (CRT), lectins that bind to mono-glucosylated glycoproteins (see below) (Fig. 3).

Glucosidase inhibitors such as castanospermine or deoxynojirimycin inhibit the activity of ER α-glucosidases. Addition of these inhibitors co-translationally prevents the transient association of CNX/CRT with glycoproteins, while addition post-translationally prolongs the association (see below). Glucosidase inhibitors are considered to be attractive antiviral drugs against several viruses that presumably depend on the CNX/CRT cycle for maturation[155–158] (see below).

2.3 Calnexin (CNX)/Calreticulin (CRT) Cycle

After rapid deglucosylation in the lumen of the ER, the nascent *N*-glycosylated polypeptide encounters the lectin chaperones CNX and CRT (Fig. 3). Both proteins are monomeric, calcium-binding proteins that reside mainly in the ER. CNX is a type I membrane protein, and CRT is a soluble paralogue. CRT is a multifunctional protein that is found in several locations (the ER, nucleus, cytosol, secretory granules and the plasma membrane). While CRT shares a common function with CNX on glycoprotein folding or calcium homeostasis, it also has other functions that include the trafficking of nuclear receptors, mRNA stability, complement activation and inhibition of angiogenesis.[159,160] CRT is retained in the ER through a KDEL-like C-terminal signal, while CNX shows an ER-localization signal on its cytosolic tail.[28]

Several studies have described transient interactions of CNX and membrane/soluble proteins in the ER.[161–164] Both CNX and CRT were subsequently shown to selectively bind to mono-glucosylated glycans, leading to the hypothesis of the so-called "CNX/CRT cycle"[93,165–170] (Fig. 3). CNX as well as CRT interact with their substrates co-translationally as the protein is translocated into the ER through the translocon[171–174] (Fig. 3). CRT binds to mono-glucosylated glycans with micromolar affinity.[175,176] The innermost Glc residue (residue c in Fig. 2) as well as the three adjacent Man residues (residues d, e, f in Fig. 2) contribute to the binding.[176,177] Since CNX is a membrane protein and CRT is a soluble form, together they appear to cover different *N*-glycans on a given protein, based on the accessibility of these glycans.[178,179] It is generally thought that CNX binds to membrane-proximal glycans more strongly, while the CRT binds to glycan structures that are deeper in the lumen.[180]

Structural analysis of the ectodomain of CNX indicated that CNX is mainly comprised of a few distinct domains.[181] The first N-terminal domain forms a globular β-sandwich structure, which is a feature of the carbohydrate-binding domain of leguminous lectins, and is followed by the P-domain (proline-rich domain), which forms a long hairpin structure and extends away from the lectin domain and, finally, an acidic C-terminal domain. The NMR structure of the CRT P-domain,[182] and the 3D X-ray structures of CRT with a truncated P-domain[183] and the CRT lectin domain have been determined.[184,185] The crystal structures of CRT with a glycan ligand confirmed the binding mode of CRT to the mono-glucosylated glycans.[184]

Generally, the role of CNX/CRT as (glycoprotein-specific) chaperones is to prevent substrates from undergoing aggregation, thereby increasing the efficiency of glycoprotein folding.[186,187] There is a still controversial issue, however, regarding the involvement of protein-protein interactions in the binding of CNX/CRT to the substrate. The first model, called the "dual-binding model",[188] proposes that there are two binding modes between CNX/CRT and the substrates, namely, lectin-oligosaccharide interactions and protein-protein interactions. Thus, CNX/CRT function as typical molecular chaperones, preventing aggregation by shielding hydrophobic segments from exposure. This model is supported by the finding that CNX co-immunoprecipitates with some incompletely folded proteins.[189–191] Moreover, *in vitro* experiments suggest that CNX/CRT can suppress the aggregation of certain denatured proteins in a glycan-independent[187,192–194] or -dependent manner.[194–196] In addition, a lectin-deficient mutant of CNX retains its association with substrates and its chaperone functions.[197] It has also been reported that the addition of ATP causes a change in the properties of CNX/CRT.[192,193,198,199] Surprisingly, ATP-hydrolysis activity is observed in CNX/CRT.[192,193] Moreover, CNX chaperone activity is dependent on its conformation state, and polypeptide binding (chaperone) activity is enhanced under conditions that induce CNX dimerization (oligomerization), while its oligosaccharide-binding activity is enhanced under conditions that enhance the structural stability of CNX monomers.[200] These results indicate that a specific conformation of CNX is required for its chaperone activity.

On the other hand, the "lectin-only model"[201] proposes that CNX/CRT associates with glycoprotein substrates solely through lectin-oligosaccharide interactions. This model is supported by the observations that many proteins do not bind to CNX/CRT when glucose trimming is impaired.[164,165,169,202] Furthermore, the binding of CNX to monoglucosylated model glycoproteins is independent of protein conformation, indicating that the CNX or CRT cannot distinguish between protein conformers.[129,175,176,203] X-ray analysis of the CNX ectodomain failed to reveal any obvious binding sites for hydrophobic peptides.[181] Moreover, preventing the dissociation of CNX/CRT from substrates by the post-translational addition of a glucosidase inhibitor does not ensure that the substrate undergoes folding, oxidation and oligomerization, indicating that the folding event occurs after the release of the molecule from the lectin chaperones.[167] Therefore, in this model, CNX and CRT act as chaperones exclusively through lectin-glycan interactions, slowing the folding process and increasing the efficiency of the maturation process.

The amino acids that are important for the peptide binding site in CRT have been identified.[204] Extensive analyses of the functionality of various CRT mutants demonstrated that lectin-deficient mutants showed a profound impairment of CRT functions (biogenesis of MHC class I molecules, the solubility of mutant forms of α1-antitrypsin (AAT) and interactions with newly

synthesized glycoproteins). In sharp contrast, peptide binding-deficient mutants had little effect, clearly indicating that the lectin-based function of CRT is of major importance for the chaperone functions of CRT in the ER.[204]

CRT-deficient mice are embryonically lethal,[205,206] and somatic mutations in the CRT gene (*CARL*) have been identified in myeloproliferative neoplasms.[207,208] On the other hand, despite an early report showing early postnatal death,[209] CNX-deficient mice are viable.[210] These mice develop myelinopathy, while other systems including immune function or reproduction are not affected. CRT KO cells are viable but Ca^{2+}-homeostasis is impaired.[211] In these cells, the folding of the bradykinin receptor, which initiates the release of inositol 1,4,5-triphosphate-induced Ca^{2+}, is altered. The amino acid residues that are essential for the correct folding of bradykinin have been identified.[212,213] It is interesting to note that some mutants that abrogate binding to ERp57 (see below) still retain full chaperone activity towards the bradykinin receptor, indicating that this defect may not be directly related to the CNX/CRT cycle.

Mutations in the CNX orthologue in *Drosophila melanogaster* (calnexin99A) results in severe defects in rhodopsin expression and also impairs the ability to control cytosolic Ca^{2+}-levels.[214] It was suggested that defects in both rhodopsin maturation and Ca^{2+}-toxicity contribute to the retinal degeneration in these mutants. In *A. thaliana*, there are three CRT and two CNX genes, and knocking out all five of these genes resulted in a lethal condition for this plant.[215] The CNX orthologue in *S. pombe* is essential for its viability.[216,217] Interestingly, the CNX mutant in fission yeast that are devoid of chaperone activity or lectin activity still confers viability, suggesting that CNX in *S. pombe* has an essential role, which is, in fact, independent of its lectin/chaperone activity.[218,219] It has been speculated that the essential function of calnexin in this yeast is in the binding of BiP and/or the scaffolding of other ERQC components.[217,219] In contrast, the sole CNX orthologue (Cne1) in *S. cerevisiae* is not essential.[220] Although this protein appears to be dispensable for the degradation of some ERAD substrates in budding yeast,[221] evidence shows that CNX is required for the degradation of non-glycosylated/underglycosylated proteins.[222,223] The binding activity of Cne1 towards mono-glucosylated glycans has also been reported.[195] Functional interaction of this protein has been demonstrated with ER chaperones such as Kar2/BiP,[222] PDIs[224] as well as Pbn1, an essential ER membrane protein that is required for proper folding and/or the stability of a subset of proteins.[225] The precise role of Cne1 in this yeast remains relatively unclear.

2.4 Interaction of CNX/CRT with folding-assisting proteins

Most proteins in the ER must acquire the correct disulfide bonds through the action of various oxidoreductases that are characterized by the presence of so-called "thioredoxin domains." Some thioredoxin domains possess CXXC motifs that serve as catalytically active sites for enzyme activity. PDI (also known as PDIA2) is the most abundant oxidoreductase in the ER of mammalian cells, with its thioredoxin domains organized by a, b, b′ and a′, with a and a′ having the characteristic CXXC active sites. ERp57 (also known as PDIA3) possesses the same thioredoxin domain organization and is thereby considered to be a close paralogue of PDI. While PDI contains an acidic C-terminus with a KDEL ER retention signal, ERp57 contains a lysine-rich basic tail at the C-terminus with a QDEL retention signal. ERp57 forms a complex with CNX/CRT, and can specifically interact with glycoproteins to promote the formation of the correct disulfide bonds[226–231] (Fig. 3). ERp57 binds to the acidic tip of the P-domain of CNX/CRT mainly through the basic tail and the C-terminal domain including the basic region,[232–235] and this interaction determines the substrate specificity of ERp57.[236,237]

Deletion of ERp57 results in an embryonic lethal phenotype in mice.[238] Interestingly, in ERp57-deficient cells a short-lived interaction of MHC class I molecules with the peptide-loading complex was observed, indicating that ERp57 is required for the assembly of the stable peptide-loading complex.

CNX and CRT can also bind to another PDI family of proteins referred to as ERp29 (also known as PDIA9).[239–241] ERp29 is a chaperone that is involved in the folding/secretion of various client proteins[242,243] but lacks an active thioredoxin motif, suggesting that this protein does not function as a PDI.[243] The 3D structure of the CRT P domain and a C-terminal domain of ERp29 was recently determined by X-ray crystallography.[244]

CNX/CRT also interacts with cyclophilin B (CypB), the ER resident peptidyl prolyl *cis-trans*-isomerase.[235,245,246] A region of charged residues in CypB is responsible for this interaction with the tip of the P domain.[235]

2.5 UDP-Glucose:Glycoprotein glucosyltransferase (UGGT): A folding sensor

The reason why the CNX/CRT folding recognition system is called a "cycle" is because it is connected to a system that monitors the folding state of proteins and can resubmit unfolded proteins for additional folding attempts. Folded or unfolded client proteins are stochastically deglucosylated by α-glucosidase II, and immature unfolded deglucosylated proteins are detected, reglucosylated and sent back to the CNX/CRT-bound state. Reglucosylation is achieved by the action of an enzyme termed UDP-Glc:glycoprotein glucosyltransferase (UGGT) (Fig. 3). UGGT is a large, soluble protein with a C-terminal KDEL-like ER-retrieval sequence.[247–254] The catalytic activity of the enzyme resides in the C-terminal domain (~20% of the protein), which belongs to glycosyltransferase (GT) family 24, according to the CAZy database.[98] UGGT preferentially recognizes partially structured (molten globule-like) proteins, but is inactive on fully folded or completely unfolded proteins.[251,255–259] This substrate specificity permits UGGT to play an important role in the sorting of glycoprotein substrates in three ways; folded and unglucosylated proteins exit the ER through the secretory pathway; reglucosylated partially folded proteins reenter the CNX/CRT cycle for additional folding attempts, and terminally misfolded proteins (which are not substrates for UGGT) are extracted and processed by the ERAD system. Proteins that are removed from the CNX/CRT cycle can again interact with BiP or PDI, and these interactions appear to be a prerequisite for ERAD.[260,261] The interplay of the CNX/CRT cycles with the other chaperone system (BiP) is also observed at the early stage of tyrosinase maturation,

where BiP binds rapidly to the substrate that is passed off to the CNX/CRT system once it undergoes co-translational *N*-glycosylation.[262]

Biochemical studies indicate that UGGT may recognize[1] the innermost GlcNAc residue on the *N*-glycan and[2] exposed hydrophobic regions on the substrate protein.[258,263,264] It should be noted that UGGT can also glucosylate well folded, yet unassembled proteins,[265] glycopeptides with hydrophobic segments,[266] or even hydrophobic non-proteinaceous aglycones.[267,268] Most recent studies indicate that UGGT can sense even local folding defects in an otherwise correctly folded protein.[259] In this case, glycans attached to the polypeptide within the misfolded sites are glucosylated, suggesting a close link between the unfolded domain and recognition by UGGT. On the other hand, a crystal structure analysis of a model substrate for UGGT showed that UGGT can modify *N*-linked glycans that are positioned at least 40 Å from localized region of disorder.[269] It is also interesting to note that this substrate, an exo-(1,3)-β-glucanase, is enzymatically active, although it is efficiently recognized by UGGT. These collective results indicate that UGGT can recognize subtle conformational differences in a glycoprotein substrate.

There are two paralogues of UGGT in mammalian cells, UGGT1 and UGGT2. Initial studies indicated that UGGT2 was not enzymatically active,[252] but it was subsequently shown that both isoforms of human UGGT are enzymatically active.[270] Knockdown of the two isoforms in *Caenorhabditis elegans* resulted in distinct phenotypes, suggesting that both proteins play distinct roles.[271]

The targeted deletion of the UGGT1 gene *(UGT1)* results in an embryonic lethal phenotype but the phenotype is variable with some mice surviving until birth.[272] However, mouse embryonic fibroblasts (MEF) of UGGT-deleted cells did not exhibit any significant growth or morphological defects.[273] The deletion of this gene leads to the accelerated release of ERQC substrates from the CNX/CRT cycle, but the release is only observed after a long lag phase, suggesting that the first release from CNX/CRT that initiates the cycling of misfolded glycoproteins requires persistent glycoprotein misfolding.[273] This UGGT activity is essential in *S. pombe* under conditions of stress,[274,275] while there is no detectable UGGT activity in *S. cerevisiae*.[249] An immunolocalization study of UGGT, α-glucosidase II and CRT showed that the localization of these proteins are not restricted to the ER but they are also found in ER-Golgi intermediate compartments (ERGIC),[276] suggesting that post-ER compartments may also be involved in the ERQC of glycoproteins.

The selenium containing, thioredoxin-like protein Sep15 associates with UGGT proteins.[270,277,278] This interaction involves 1:1 stoichiometry,[278] and it has been suggested that Sep15 can enhance the activity of both UGGT1 and UGGT2.[270] Sep15-knockout mice develop cataracts early in life[279] and a higher interferon-γ level[280] in serum was observed. The issue of how these phenotypes are related to UGGT activity remain elusive.

Several studies have been carried out to investigate the structures of UGGT from different sources[281–284] (reviewed in ref.[31]). In the UGGT structures, surface-exposed hydrophobic regions are observed around the active site, implying that the C-terminal catalytic domain may be involved in sensing substrate folding. This hypothesis has experimental support—a $Man_9GlcNAc_2$-probe with a hydrophobic aglycon selectively binds to the amino acid residue near the hydrophobic patches.[285] It has been suggested that the C-terminal catalytic domain is sufficient for producing a functional UGGT enzyme.[286] While the presence of an N-terminal domain of UGGT1 results in enhancement in activity, this is not the case for UGGT2, suggesting that the contribution of the N-terminal domain may be distinct between these two paralogues.

2.6 Is CNX/CRT cycle required for the general folding of glycoproteins?

It is interesting to note that, although the components of the CNT/CRT cycle (CNX/CRT/UGGT/ERp57) are all essential for the normal development of mice, cells that are deficient in these genes are normal at the cellular level.[205,206,209,238,273,287] In fact, to date, only a subset of glycoproteins are strongly dependent on the CNX/CRT cycle system.[238,261,287–290] The deletion of these genes does not elicit an ER stress/unfolded protein response (UPR) in cells.[261,273,287] ERp57-null cells showed an unperturbed maturation/transport of several cell surface glycoproteins containing disulfide bonds.[289] Moreover, the conditional deletion of ERp57 in B lymphocytes/plasma cells showed no defect in the production of immunoglobulin chains, the most abundant *N*-glycosylated/disulfide-bonded glycoproteins in these cells.[289] These studies have led to the hypothesis that the importance of the CNX/CRT cycle is strictly confined to a subset of substrates, most notably the biogenesis of MHC class I peptide-loading complexes.[291–293] In the case of ERp57, in its absence, other PDI proteins take over for the productive maturation of most proteins.[287] ERp72, an ER-resident oxidoreductase, has been identified as one of the back-up proteins that functions in the absence of ERp57.[287]

In vivo genetic evidence suggests that mannose-trimming results in negligible effects on UGGT activity, while α-glucosidase II is significantly reduced.[294] This contrasts with the results of *in vitro* studies, which indicate that the activities of both UGGT and α-glucosidase II are reduced by mannose-trimming.[267,295] The reason for this discrepancy remains unclear. It is also interesting to note that, while UGGT delays the secretion of immature glycoproteins, it does not affect the processing of misfolded proteins by ERAD.[296] This observation clearly indicates that the ERAD machinery is very effective in releasing client proteins from the CNX/CRT cycle.

3 Effect of man trimming on the degradation and transport of glycoproteins

3.1 Mns1/MAN1B1

Cells must consistently eliminate misfolded glycoproteins by allowing them to exit from the CNX/CRT cycles and undergo degradation through the ERAD system. This can be achieved by UGGT failing to recognize them or, otherwise, a mechanism

must exist by which cells sense that glycoproteins have remained too long in the ER. As a general rule, mannose trimming is considered as an indication of prolonged retention in the ER, and therefore acts as a "molecular clock" for assigning the carrier protein for degradation, the so-called mannose timer model.[150,297] This idea originally arose from observations that upon treatment with an α-mannosidase inhibitor such as kifunensine, some ERAD substrates become quite stable in cells.[92,94,298–301] These phenomena can be attributed to the enhanced efficiency of the CNX/CRT cycle, since demannosylation appears to cause a reduced efficiency for both α-glucosidase II and UGGT.[255,267,302] Moreover, trimming of α1,2-linked Man at the B and C branches (residues g and i in Fig. 2) makes the resulting mono-glucosylated glycans weaker ligands for CNX/CRT.[303–305] Mns1 is the sole ER α1,2-mannosidase in *S. cerevisiae*.[306,307] Interestingly, stabilization of ERAD substrates can also be seen in *MNS1*-deleted cells of *S. cerevisiae*, although this organism does not possess a functional CNX/CRT cycle.[150,221] There is also a glycoprotein substrate, for which Mns1 is not required for efficient degradation in this yeast.[308] Mns1 belongs to GH family 47, is a type II membrane protein that does not contain an ER retention signal, and requires the Rer1 protein for proper localization to the ER.[309] It nearly exclusively trims the outermost α1,2-linked mannose in the B branch (residue g in Fig. 2) and $Man_8GlcNAc_2$ (denoted as isomer B^{24}) is believed to be the product of the last trimming reaction for normal glycoproteins in this yeast. Therefore, as a system that is independent of a CNX/CRT system, the involvement of a putative "Man8-conformer B-specific lectin" in the degradation of aberrant glycoproteins has been proposed by Jakob et al.[150] In this hypothesis, a lectin recognizes misfolded glycoproteins with a trimmed mannose and facilitates the degradation of the substrates (see below).

The orthologue of ER α-mannosidase I in *S. pombe* exhibits only feeble enzyme activity, but the disruption of the gene nevertheless significantly impairs the degradation of misfolded proteins,[310] raising the possibility that the role of this enzyme might not be dependent on its enzymatic activity.

MAN1B1 is generally regarded as an orthologue of Mns1 in mammalian cells.[311,312] This enzyme, a short-lived protein that is degraded in lysosomes,[313] was initially thought to play a role similar to that of Mns1, i.e. Man9-to-Man8B conversion, but this conclusion has now been questioned (see below). Nevertheless, a large body of evidence suggests that MAN1B1 is involved in the mannose-trimming of ERAD substrates. Indeed, overexpression of MAN1B1 can enhance the degradation of glycoprotein ERAD substrates,[314,315] whereas knockdown of MAN1B1 inhibits mannose processing.[316] More recent evidence also suggests that Golgi α-mannosidase I might also be involved in the mannose trimming of ERAD substrates.[317,318] It was also suggested that mannose trimming on ERAD substrates in mammalian cells normally proceeds beyond Man8, and possibly as far as $Man_5GlcNAc_2$ in mammalian cells.[314,319–322] Further mannose trimming can occur, at least in mammals, by this MAN1B1 enzyme in a specialized compartment, which is referred to as the ER-derived quality control compartment (ERQC),[10,323,324] where this enzyme is highly concentrated.[316,325] Indeed, in *in vitro* experiments, this enzyme cleaved all α1,2-linked Man units.[326,327]

Assuming that Man trimming occurs only at α1,2-linked Man residues, $Man_8GlcNAc_2$ or $Man_7GlcNAc_2$ (if residue **i** in Fig. 2 is further trimmed) can still be reglucosylated by UGGT (Figs. 2 and 3). However, the trimming of α1,2-linked Man at the A branch (residue d in Fig. 2) will generate oligosaccharide structures that are no longer substrates for UGGT, thus eliminating the substrates from the CNX/CRT cycles (Figs. 2 and 3). Thus, the trimming of Man a may be the decisive point for the protein to enter the ERAD route. It should also be noted that this Man is also effectively removed (when glucosylated) by endo-α-1,2-mannosidase, an enzyme located in the post-ER compartment that probably plays a role in backing up the premature exit of glucosylated proteins to ensure that the processing of mature oligosaccharides (complex-type *N*-glycans) continues[276,328–334] (Fig. 3). Therefore, endo-α-1,2-mannosidase cleavage may also be a critical step for extracting substrates from CNX/CRT cycle.[335]

Interestingly, MAN1B1 has recently been reported to be mainly localized in the Golgi apparatus,[336,337] while others claimed that this is an experimental artifact.[325] MNS3, an *A. thaliana* orthologue of MAN1N1, is also localized in the *cis*-Golgi.[338] Regarding the functional importance of MAN1B1 in the Golgi, a model has been proposed in which misfolded glycoproteins that escape the ER and reach the Golgi meet MAN1B1, and are then demannosylated and sent back to the ER for degradation.[339] Moreover, mutations in the *MAN1B1* gene were revealed to cause a type 2 congenital disorder of glycosylation (MAN1B1-CDG[337,340–344];). This disorder is characterized by symptoms that can include intellectual disability, delayed motor movement, facial dysmorphism and truncal obesity. In patient-derived cells, altered Golgi morphology was apparent, further indicating the existence of a link between MAN1B1 and Golgi functionality. For this reason, it may be necessary to revisit the nomenclature of MAN1B1, as the name "ER α-mannosidase I" is confusing for a Golgi-localized protein. More recently, it was proposed that MAN1B1 has a non-enzymatic role in post-ER quality control of proteins[339,345]; this interesting hypothesis should be explored in future studies. It should also be noted that a non-enzymatic function of MAN1B1 has also been suggested to be involved in the development of liver disease or liver carcinogenesis.[346]

3.2 EDEM, a membrane protein that enhances glycoprotein ERAD

Mammalian EDEM1 (*ER degradation enhancing α-mannosidase like proteins*) and yeast Htm1/Mnl1 proteins were first identified as homologues of Mns1/MAN1B1 that are required for glycoprotein ERAD.[347–349] There are two more EDEM paralogues in mammalian cells, EDEM2/3, which are also inducible by ER stress.[322,350] On the other hand, although Mnl2, a paralogue of Htm1/Mnl1, has been reported to play a role in the ERAD process,[351] the issue of whether this protein has α-mannosidase activity remains to be clarified. EDEMs belong to the GH family 47, and EDEM1 was initially reported to be a type 2 membrane protein in COS cells,[352] but exists in the form of a soluble ER protein in other cell lines.[350,353] On the other hand, it is thought that EDEM2/3 are soluble proteins that are localized in the ER.[322,354] Likewise, Htm1 was originally predicted to be a type 2 membrane protein localized in the ER,[348,349] while it was subsequently found to be a soluble ER protein.[355] EDEM was originally discovered as a UPR-inducible gene,

implying a role in tolerance against the ER stress.[347] It is interesting to note, however, that *HTM1* is not inducible by UPR in *S. cerevisiae*.[356]

With regard to function, EDEM proteins were initially assumed to be a "degradation lectin"[150] that may recognize Man8B glycans,[347–349] but the overexpression of EDEM proteins was later found to result in the trimming of mannoses,[322,357,358] implying that they may also function as α-mannosidases. Consistent with this observation, structural modeling showed no discernable difference in the presence and position of catalytic residues between EDEMs and the MAN1B1, the other α-mannosidase belonging to GH family 47.[359] The Glu147-to-Gln mutation, which abrogates the enzymatic activity of EDEM3, compromises ERAD-enhancing activity, suggesting the existence of a close relationship between its enzymatic activity and function in the ERAD process.[322] Moreover, the α-mannosidase activity of Htm1 can process mannose from the C branch *in vivo* (residue i in Fig. 2).[360,361] This reaction results in a specific α1-6-linked mannose to be exposed (residue j in Fig. 2), which is recognized by a "*bona fide* degradation lectin", i.e. ER lectins that contain an MRH-domain (OS-9/XTP3-B in mammalian cells, Yos9 in yeast) (see below) (Fig. 3). Moreover, a comprehensive analysis of EDEM/Man1B1-knockout chicken DT40 cells or human HCT116 cells clearly showed that, contrary to previous assumptions, EDEM2 is mainly involved in the conversion of Man9 to Man8B, while Man8B to Man7-5 glycans are mainly formed mainly by EDEM3 and partly by EDEM1.[362] These results indicate that all EDEMs possess α-mannosidase activity, but the specificity appears to be different among the paralogues.

EDEMs or Htm1 is required for the efficient degradation of *N*-glycoproteins, but not for non-glycosylated proteins.[223,314,347–349,352,363–367] As its name implies, the overexpression of EDEM enhances the turnover of misfolded glycoproteins presumably by, in the case of mammals, removing these substrates from the CNX/CRT cycle.[347,368] Moreover, the downregulation/knockout of this gene[362,368] or the deletion of *HTM1/MNL1* in *S. cerevisiae*[348,349] inhibits the ERAD of glycoproteins. It should be noted that, although the glycan-dependency of EDEM-mediated degradation is apparent, not all *N*-glycoproteins are degraded via Htm1 in *S. cerevisiae*.[365,366] Abolishing the ER stress-induced induction of EDEM also inhibits the ERAD glycoprotein.[363,369] The upregulation of EDEM, but not CNX, rescues the EDEM downregulation-induced disulfide-bonded formation of β-secretase aggregates in the ER, thus restoring folding efficiency and sustaining secretion capacity at a level comparable to those of wild type cells.[363] Therefore, the level of ERAD machinery involved in EDEM plays an important role in maintaining the functional folding environment in the ER.

Despite the wealth of *in cellulo* evidence, initial attempts to detect *in vitro* α-mannosidase activity in EDEM proteins were not successful.[347,354] It has been, however, suggested that the interaction of EDEM proteins with oxidoreductases is key for its enzyme activity; the first evidence of such interactions was reported in a yeast Htm1-Pdi1 complex. Htm1 forms a complex with Pdi1 through the formation of an intermolecular disulfide bond[355,361] and the enzyme activity of the Htm1/Mnl1-Pdi1 complex was subsequently confirmed *in vitro*[370–372] as well as *in vivo*.[372]

In a search to understand their enigmatic function, PDI-binding proteins were analyzed for their binding to EDEM proteins in mammalian cells. Comprehensive genetic analyses led to identification of TXNDC11, a type II transmembrane protein with 5 thioredoxin-like domains as a protein involved in glycoprotein ERAD.[373] Moreover, immunoprecipitation of this protein identified EDEM proteins as binding partners.[373] Indeed, interactions of EDEM1 or EDEM2 to TXNDC11 was observed.[374] Moreover, another PDI-like protein, ERp46 was found to associate with EDEM3.[375] EDEM3 forms disulfide bonds with ERp46 and the resulting EDEM3-ERp46 protein complex possesses *in vitro* α-mannosidase activity.[375] Finally, EDEM2 proteins form a stable disulfide bond with TXNDC11 and catalyze the conversion of Man9 to Man8B, removing the middle mannose residue (residue g in Fig. 2).[376] These collective results suggest that EDEM proteins in mammalian cells also have PDI-like proteins as binding partners for exerting α-mannosidase activity. It has also been suggested that the enzyme activity of the EDEM-PDI-like protein complex appears to be modulated by the folding state of the substrates *in vitro*.[371,374]

In mammalian cells, not all EDEM orthologues possess the classical sequences required for ER retention, and the mechanism by which these proteins are localized in the ER remains undetermined. It was also suggested that EDEM proteins can be also concentrated in vesicles lacking the COPII coat,[353] later termed as LC3-I-positive "EDEMosomes".[377,378] It should also be noted that EDEMs associate with ER resident proteins such as CNX,[352,368] MAN1B1,[379] ERdj5[380] or SEL1L[381]; such protein-protein interactions may allow EDEMs to remain in the ER or EDEMosomes.

There are various mannosidase inhibitors with different specificity towards ER-localized α1,2-mannosidase available.[382] These inhibitors not only clarify the possible two independent trimming pathways in the ER, but also provides a tool to understand the glycan structure-specific signals critical for glycoprotein quality control. Obvious targets for these inhibitors are MAN1B1 and EDEM proteins, although involvement of Golgi α-mannosidases I is also possible.

3.3 OS-9, a *bona fide* "Degradation Lectin"

OS-9 or XTP3-B in mammalian cells, and Yos9 in *S. cerevisiae* have been identified as a new player that is involved in the recognition of misfolded glycoproteins in the ERAD process. The human OS-9 protein was originally identified as a protein that was upregulated in osteosarcomas.[383] This protein is highly conserved in eukaryotes.[384,385] XTP3-B (XTP3-transactivated gene B protein)/Erlectin is a paralogue of OS-9.[386]

One of the characteristics of this protein is that it contains an N-terminal MRH lectin domain.[133] The yeast orthologue of the OS-9 protein, Yos9, is a 75 kDa soluble ER protein associated with the lumenal side of the ER membrane.[387] It was initially shown that Yos9 is involved in the maturation and transport of GPI-anchored proteins.[387]

The first indication that Yos9 is involved in glycoprotein ERAD came from a genome-wide screen exploiting the degradation of a glycoprotein ERAD substrate.[388] The lack of this protein, as is the case with Htm1/Mnl1, only affects the degradation of *N*-glycosylated proteins, but not those with a non-glycosylated substrate.[388–391] There is no obvious additive effect between *YOS9*-deletion and *HTM1* (EDEM)-deletion[388,390,391] or *MNS1* (ER α-mannosidase I)-deletion,[389] implying that these components are involved in the same pathway. Yos9 forms a complex with glycoprotein substrates.[389–391] Mutations in the MRH domain, which are predicted to cause loss of carbohydrate-binding activity, also abolish the glycoprotein ERAD function, suggesting the functional importance of this domain.[389,391] Yos9/OS-9 also binds directly with Hrd3/SEL1L,[392,393] and therefore is a component of the HRD1 retrotranslocation channel[394,395]

Yeast Yos9 and OS-9 recombinant proteins were found to bind to *N*-glycans when the α1,2-linked Man in the C branch was removed (residue **i** in Fig. 3),[360,396,397] clearly indicating that the binding mechanism is conserved from yeast to mammalian cells. The MRH domain for human OS-9 or yeast Yos9 has been structurally characterized and the results indicate that the binding mode of this domain includes the exposed α1,6-linked Man residue (residue j in Fig. 2).[398,399] Therefore, mechanisms have been suggested whereby the α1,2-linked Man in branch C (residue i in Fig. 2) is removed by the action of the Htm1 protein (or EDEM1/3), which exposes the recognition residue for Yos9 (or OS-9/XTP3-B) (Fig. 3).[360,361] It has been suggested that the MRH domain is critical for not only binding to glycoprotein substrates[396,397,400] but also binding to SEL1L, presumably through their *N*-glycans.[401] It has been proposed that the Yos9 protein has a role in the degradation of a non-glycosylated ERAD substrate, and its effect was MRH domain-independent.[402,403] On the other hand, XTP3-B, but not OS-9, inhibits the ERAD of non-glycosylated substrates,[404] while OS-9 was required for the ERAD of a non-glycosylated protein.[405] OS-9 and XTP3-B can compete for binding to SEL1L possibly through their lectin activity, suggesting that a lectin occupying the binding site on SEL1L may influence the turnover of non-glycosylated substrates. OS-9 binds to GRP94, an ER resident molecular chaperone,[406] whereas it has also been reported that a hyperglycosylated GRP94 may be simply recognized by OS-9 as a misfolded substrate.[407] Interestingly, while hyperglycosylated GRP94 is rapidly degraded, lysosomes, but not proteasome (ERAD), appears to be involved in the degradation.[407]

XTP3-B contains two MRH domains and only the C-terminal MRH domain appears to exhibit lectin activity.[408,409] OS-9 and XTP3-B are considered to be functionally redundant,[401,404,410,411] while such redundancy has not been observed for a subset of ERAD substrates.[400,404,405,409,412] These lectins play redundant roles in maintaining the stability of SEL1L proteins while MRH glycan binding is not essential for its stabilizing effect.[404] Mannose-trimming appears to be critical for XTP3-B binding to the glycoprotein substrates,[413] while binding to untrimmed, $Man_9GlcNAC_2$ glycans has also been suggested.[409]

3.4 Lectin cargo receptors: Glyco-specific transport

Once glycoproteins acquire the correct folding state and escape the ERQC, yet another class of lectins is believed to be involved in their selective anterograde trafficking. Among these, ERGIC-53/LMAN1 is the best-characterized example.[305,414–418] This protein is a ubiquitously expressed, non-glycosylated type I membrane protein of 53 kDa, which constitutively recycles between the ER and ER-Golgi intermediate compartment (ERGIC).[414–416,419] Interestingly, this protein changes its subcellular location to the Golgi under conditions of ER stress.[420] The protein was originally identified as an antigen for the Golgi-rich fraction.[421–425] It was later found to be identical to MR60, an intracellular protein isolated by mannose-affinity column chromatography.[426,427] A remarkable feature of ERGIC-53 is that it contains a ~200-residue segment in the lumenal domain, which shares a homology with the carbohydrate recognition domain (CRD) of leguminous plant lectins (L-type lectin).[428] ERGIC-53 has complex machinery involving COPI/II to permit it to recycle between ER and ERGIC, and all domains (cytoplasmic, transmembrane and lumenal) appear to contribute to its correct localization.[415]

An examination of the sequence database led to the identification of additional proteins with homology to ERGIC-53.[429,430] The homologues have been classified into three main phylogenic groups: the ERGIC-53/ERGL; VIP36/VIPL; and a group of fungal orthologues.[429] ERGL/LMAN1L is a highly homologous paralogue of ERGIC-53, and is expressed at high levels in normal and neoplastic prostate.[431] VIP36/LMAN2 is another membrane-bound animal L-type lectin that is concentrated in the *cis*-Golgi/early secretory pathway,[428,432,433] cycles between the Golgi and the ER,[434] but contains complex-type glycosylation,[435] suggesting that it can also reach the medial/trans Golgi. On the other hand, another closely-related L-type lectin, VIPL/LMAN2L is localized mainly in the ER.[429]

The initial indication of the importance of ERGIC-53 in the export of glycoproteins arose from a study of a congenital sucrase-isomaltase (SI) deficiency patient.[436] In this patient a point mutation in SI leads to the accumulation of the enzyme in the ERGIC and Golgi, and the entire Golgi labeled positive for ERGIC-53. These data are consistent with a prolonged association of the mutant SI with ERGIC-53. Moreover, the genetic disorder in ERGIC-53 has been found to cause an autosomal recessive bleeding disorder leading to a deficiency in blood coagulation factors V and VIII.[437–440] In these patients, the plasma levels of the two factors are reduced to 5–30% of the normal level. Indeed, ERGIC-53 was found to be required for the efficient transport of these proteins.[441] MCFD2 (multiple coagulation factor deficiency 2) has also been identified as another protein in which mutations are observed in individuals with similar bleeding disorders but with no mutations in the ERGIC-53 gene.[442] MCFD2 and ERGIC-53 form a stable, Ca^{2+}-dependent complex that likely serves as a cargo receptor for the efficient ER-to-Golgi transport of factors V and VIII[443,444] (Fig. 3).

ERGIC-53 forms a hexameric transmembrane protein in two forms; one being a disulfide-linked complex and the other a non-covalent complex made up of two disulfide-linked dimers.[445,446] There are other proteins that require functional ERGIC-53 for efficient trafficking/ER export. Those proteins include cathepsin Z,[447–449] cathepsin C,[450] nicastrin,[451] immunoglobulin M,[452–454]

AAT,[455] fibroblast growth factor receptor 3,[456] sulfatase modifying factor 1,[457] Mac-2BP,[458] matrix metalloproteinase-9[459] and several members of the Cys-loop superfamily of neuroreceptors.[460] MCFD2 is dispensable for the transport of cathepsin C or Z,[461] while it is required for the secretion of Mac-2BP.[458] On the other hand, AAT[462] or guanylyl cyclase C[463] have been identified as client proteins for VIP36 and its interaction is through the *N*-glycans of the client proteins. Moreover, an interaction of VIP36 with the ER molecular chaperone BiP has also been observed; in this case the interaction appears to be glycan-independent.[464] VIP36 has also been implicated in the exosomal release of GPRC5B[465] or pathological prion formation of the cellular prion protein,[466] although the molecular mechanisms responsible for these phenomena remain elusive.

More recently, ERGIC-53 was reported to be critical for the production of the infectious particles of virus including arenavirus, coronavirus and filovirus.[467] Intriguingly, the interaction between virus glycoproteins and ERGIC-53 appears to be through a carbohydrate recognition domain (CRD) but in a lectin-independent manner, since CRD mutants defective in mannose binding are still able to bind to viral glycoproteins.[467]

Binding of ERGIC-53 to Man is pH and Ca^{2+}-dependent.[468] The ERGIC is predicted to have a lower Ca^{2+}-concentration and pH than the ER, conditions under which the binding of ERGIC-53 to Man is impaired.[468] Therefore, the following model has been proposed as a mechanism underlying reversible lectin-cargo interactions[1]: ERGIC-53 proteins encounters the cargo at the ER (where a high affinity is retained) and[2] releases them at the ERGIC (where a lower affinity is expected) (Fig. 3).[468] In this model, a conserved His residue is predicted to serve as a pH/Ca^{2+} sensor, since it can be protonated at the ERGIC at a lower pH, leading to the loss of Ca^{2+}, triggering the release of its cargo.[468]

A more recent study revealed that ERGIC-53 appears to recognize a conformation-based motif (a high mannose glycan associated with a surface-exposed peptide β-hairpin loop), which only appears when the substrate is completely folded.[449] These results imply the existence of a "quality control" mechanism in which only folded proteins are captured by ERGIC-53.[449]

The structures of ERGIC-53 CRD, with or without MCFD2 or carbohydrate ligands have been determined.[469–474] MCFD2 but not the ERGIC-53 CRD undergoes conformational changes upon their interaction, suggesting cooperative interplay between ERGIC-53 and MCFD2 in interactions with factors V and VIII.[471,474] In a 3D structure of the complex of ERGIC-53 CRD and α1,2-mannotriose, the CRD interacts with the glycans in two modes. In one, the 3-OH group of the non-reducing terminal Man is oriented outward with respect to the sugar binding pocket, allowing for substitution with a Glcα1-3 linkage without steric hindrance[473]; this binding mode was not employed by VIP36, as revealed by a structural analysis with the same ligand.[475] This is consistent with the finding that ERGIC-53, but not VIP36 or VIPL, binds with similar affinity to mono-glucosylated and non-glucosylated high mannose-type *N*-glycans.[476]

Similar to ERGIC-53, VIP36 has an affinity for glycoproteins that contain high mannose-type glycans.[477,478] Detailed analysis of the carbohydrate-binding specificity showed that VIP36 has a high affinity for high mannose type bearing Manα1-2Manα1-2Man branches (residues d, e, f in Fig. 2).[479] The binding is optimal at a pH of around 6.5 but at higher pH (i.e. pH at the ER) its affinity is reduced, indicating that VIP36, in sharp contrast to the case of ERGIC-53, may function as a "retrieving lectin," capturing proteins the contain immaturely processed glycoproteins that have erroneously escaped from the ER, and redirect them to the ER (Fig. 3).[479] Moreover, a comprehensive, comparative analysis of glycan binding specificity between CRD of ERGIC-53, VIPL and VIP36 was carried out.[476] Different pH profiles have been observed for the binding of $Man_9GlcNAc_2$-glycans to VIPL and VIP36; VIP36 CRD exhibits a bell-shaped pH dependence with an optimal pH ~6.5, whereas the affinity of VIPL CRD, consistent with the previous cell-binding assay,[480] steadily increased with increasing pH from 5.5 to 7.5.[479] The ERGIC-53 CRD binds to high mannose-type glycans with low affinity and broad specificity. This low affinity may be compromised by the formation of a hexameric structure, protein-protein interactions or through complex formation with MCFD2.

There is much less information available regarding the functional role of VIPL. Overexpression of VIPL interferes with ERGIC-53 cycling and redistributes this protein in the ER, suggesting that it may somehow regulate ERGIC-53 function.[429,430] The knockdown of VIPL gene expression also revealed that the export of a subset of proteins is retarded.[429] The *LMAN2L* (VIPL) allele is reported to be associated with bipolar disorder,[481,482] or schizophrenia.[482] Missense autosomal recessive[483] or dominant mutations[484] in the *LMAN2L* gene have been reported in a family with intellectual disability and reoccurring epilepsy. These results suggest the importance of the VIPL protein in normal brain development/functions.

Mice with the *LMAN1* (ERGIC-53) gene knocked out have been generated and found to exhibit a strain-specific partial lethal phenotype, decreased levels of plasma factors V and VIII and decreased levels of platelet factor V, with milder phenotypes than in human subjects.[485,486] Moreover, hepatocytes of *Lman1*-KO mice show an accumulation of AAT,[485] a known cargo for ERGIC-53.[455] Moreover, studies of *Mcfd2* double KO mice revealed that they exhibit normal survival in different genetic backgrounds, clearly indicating the distinct function of MCFD2 and LMAN1 *in vivo*.[487] Surprisingly, decreased plasma levels of AAT were observed for all male mice for *Lman1*, *Mcfd2*, *and Lman1 Mcfd2* double KO mice, with ER accumulation of this protein in hepatocytes, indicating that both LMAN1 and MCFD2 are important for AAT secretion.[487] Levels of factors V and VIII in *Lman1 Mcfd2* double KO mice are higher than that of *Mcfd2* KO mice, suggesting the existence of an alternative pathway for secretion of factors V and VIII in double KO mice.[487]

The third class of this lectin family contains Emp46 and Emp47 in *S. cerevisiae*.[488–491] Emp46 and Emp47 are homologous membrane proteins found in the early secretory pathway of *S. cerevisiae*, and deletion of these genes causes a partial defect in secretion.[489] Emp47 is required for the transport of Emp46 from the ER, forming oligomeric complexes through their coiled-coil domain, but the converse is not true. Moreover, Emp46-Emp47 interactions were not detected in the Golgi, suggesting that Emp47 acts as a receptor for Emp46, escorting it from the ER to the Golgi.[490] The 3D structures of CRDs of Emp46 and Emp47 has been determined,[492] and show a β-sandwich folding similar to that of ERGIC-53.[469] In contract to the CRD of ERGIC-53 that binds Ca^{2+}

ions, the CRD of Emp46 instead binds K^+ ions.[492] The binding of K^+ ions appears to be essential for the function of Emp46 as a Y131F mutant, which cannot bind K^+ ions, failed to rescue the growth phenotype of an *emp46Δ emp47Δ* strain at non-permissive temperatures (37 °C).[492] The pH-dependent assembly of the coiled-coil segment of Emp46/Emp47 has also been examined, and the results suggest that a glutamate residue in the coiled-coil domain of Emp46 plays a key role in the pH sensitivity of hetero-complex formation.[493] Through an analysis of various mutants, the dissociation of a hetero-complex at low pH is thought to be caused by the stabilization of the Emp46 coiled-coil domain itself.[494]

It should be noted that, although ERGIC-53 is clearly required for the efficient transport of some glycoproteins, only a few glycoproteins appear to be affected and even so, the effect is only partial. How this selectivity/incompleteness is achieved is an open question.

3.5 Is vesicular transport required for efficient glycoprotein ERAD?

Another interesting question concerning glycoprotein ERAD is the requirement for protein trafficking. In *S. cerevisiae*, some soluble glycoprotein ERAD substrates require functional vesicular transport between the ER and Golgi apparatus for their efficient degradation.[367,495–497] Consistent with these observations, soluble misfolded glycoproteins can be packaged into COPII transport vesicles, while a membrane glycoprotein ERAD substrate is completely excluded.[495] Indeed, analyses of intracellular free *N*-glycans (predominantly representing glycans that are released by the cytoplasmic peptide:*N*-glycanase in *S. cerevisiae*) indicated that a portion of the ERAD substrates become modified by the action of Och1, a Golgi-resident mannosyltransferase, further supporting the hypothesis that some ERAD substrates travel through the Golgi before retrotranslocation to the cytosol.[498] The issue of whether a trip to the Golgi (via COPII vesicles) is actually required for the efficient degradation of soluble ERAD substrates has not been firmly established. As an alternative possibility, it has been suggested that the effect is indirect and may be due to the effect of the mislocalization of components required for the ERAD of soluble glycoproteins.[497]

A new pathway for the proteasomal degradation of membrane proteins, referred to as endosomal and Golgi-associated degradation (EGAD), has been reported.[499] In this process, substrates are exported out of the ER, and once in the Golgi and endosomes, they are polyubiquitinated and Cdc48 then extracts proteins from the membrane, a process that is coupled to proteasomal degradation.[499] Orm2, a negative regulator of sphingolipid biosynthesis, has been identified as an EGAD substrate.[499] It is therefore possible that some substrates are required to travel to Golgi or endosomes in order to be degraded by proteasomes.

4 Retrotranslocation and cytosolic events of glycoprotein ERAD

4.1 Substrate recognition and retrotranslocation

Since the discovery of the ERAD process, the issue of how the substrates are recognized and retrotranslocated into the cytosol has been a subject of intense study. Regarding substrate recognition, studies using *S. cerevisiae* have led to the conclusion that there are three major ERAD pathways (ERAD-C, ERAD-L, and ERAD-M) depending on whether the misfolded domains are localized in the cytosolic side, the lumen or within the membrane.[365,394,500] It has also been suggested that these pathways involve the action of distinct ubiquitin ligases. ERAD-L substrates use the Hrd1 RING-finger ligase that is complexed with other membrane proteins such as Hrd3, Usa1, Der1 and Yos9.[394,395,501,502] For ERAD-M substrates, they use Hrd1/Hrd3, and in some cases Usa1,[503] but not Der1.[394] ERAD-C substrates require Doa10, another RING-finger ligase,[504] while it has also been reported that this ubiquitin ligase can also recognize an intramembrane region of Sbh2, a Sec61 β-subunit homologue 2 protein.[505] Another pathway dedicated to the degradation of inner nuclear proteins exists, which utilizes a ubiquitin ligase consisting of Asi1, Asi2, and Asi3.[506,507] The Asi complex appears to recognize substrate features in the membrane. All ERAD pathways merge in the cytosolic phase, where an ATP complex consisting of the AAA+ ATPase Cdc48 (VCP or p97 in mammals), Ufd1 and Npl4[508–513] is involved. In mammalian cells, Hrd1 is related to human HRD1 and gp78[514,515] while Doa10 is related to March6/TEB4.[504]

Retrotranslocation channels (retrotranslocons) on the ER membrane continues to be a controversial issue, with Hrd1 the most likely candidate to form a protein-conducting channel for ERAD-L proteins.[13] Hrd1 is in close proximity to ERAD substrates during the retrotranslocation process.[516] Moreover, Hrd1-overexpression bypasses the requirement for other Hrd1 complex proteins[516]: Hrd3, Usa1, and Der1.[394,501,502] Hrd1 function also requires oligomerization, which is facilitated by Usa1.[503,516] *in vitro* reconstitution experiments using purified proteins have shown that Hrd1 binds to and polyubiquitinates soluble ERAD-L substrates.[517] Finally, the retro-translocation process is recapitulated with proteoliposomes that contain Hrd1 and a substrate,[518] thereby making a strong case for Hrd1 as a retrotranslocon. Hrd1 undergoes efficient auto-ubiquitination, and a model in which the auto-ubiquitination of Hrd1 triggers the opening of the channel for ERAD-L substrates has been proposed.[518] This conclusion is consistent with the observation that ubiquitination is still required when all of the lysine residues in the ERAD substrates are removed.[518–521]

The Hrd1/Hrd3 complex was recently structurally characterized by cryo-electron microscopy.[522] The 3D structure of the complex contains two symmetric Hrd1/Hrd3 complexes, and Hrd1 forms a cavity towards the cytosolic side. It is noteworthy that a similar cavity was detected in the Sec61 protein-conducting channel,[523] SecY, a bacterial Sec61 orthologue,[524] and the YidC, a transmembrane chaperone/insertase.[525] The formation of such a cavity may be a general principle of protein-conducting channels to reduce the energy needed for the movement of a polypeptide into or out of the membrane.[13] Whether other ubiquitin ligases such as Doa10 or the Asi complex also form a similar protein-conducting channels remains to be seen.

4.2 Sugar-recognizing ubiquitin ligases (FBS proteins)

Once in the cytosol, retrotranslocated proteins are ultimately degraded via the ubiquitin/proteasome-dependent pathway. Various studies have implicated the existence of a subcomplex that is specifically involved in the degradation of glycoproteins. In most ERAD substrates, polyubiquitination serves as a targeting signal, and this occurs by the concerted action of activating E1, conjugating E2 and ligating E3 enzymes.[526,527] Among the E3 ligases, Cullin-RING E3 ligases are the largest family of E3 enzymes in all eukaryotes.[528] In this category, the SKP1-CUL1-F-box protein (SCF) E3 ubiquitin ligase complexes are the best characterized ones, and are known to consist of four subunits, a scaffold protein CUL1, a RING protein RBX1, an adaptor protein SKP1, and substrate-recognizing F-box proteins. F-box proteins bear an N-terminal F-box domain that is responsible for the binding to SKP1,[529] and a diverse C-terminal substrate-binding domain. Regarding the substrate recognition domain, the 69 F-box proteins are grouped into three classes, 11 FBXW proteins, characterized by WD40 repeat domains, 21 FBXL proteins with leucine-rich repeats, and the 37 FBXO protein that does not contain any characteristic domains.[530] F-box proteins often recognize post-translational modifications of proteins such as phosphorylation,[531,532] methylation,[533] or acetylation.[534] FBS proteins are a group of proteins that recognize sugars (glycans).[535] FBS1 (FBG1/FBXO2/NFB42/OCP1), FBS2 (FBG2/FBXO6), FBS3 (FBG5/FBXO27) and FBXO17 (FBG4) have all been shown to bind to *N*-glycans.[536–542] On the other hand, another F-box protein, FBXO44 (FBG3) structurally belonging to this group, showed no detectable glycan-binding.[538,539] Consistent with this observation, recently determined crystal structures of FBXO44-SKP1 revealed that FBXO44 has different hydrogen bond networks from those of FBS1 and FBS2, which prevent the formation of glycan-binding pocket.[543,544] There are differences in the binding specificity of these enzymes; while FBS1 and FBS2 prefer to bind to high mannose-type *N*-glycans with innermost $Man_3GlcNAc_2$ cores,[538,545,546] FBS3 also binds strongly to glycoproteins that contain complex-type glycans.[538,540]

Of the FBS protein family, FBS1 is the best-characterized protein in terms of physiological functions. The expression of FBS1 is restricted, and is highly expressed in the brain and inner ear of rodents,[547,548] whereas FBS2 exhibits a wide tissue distribution.[537] SCF^{Fbs1}, as well as SCF^{Fbs2}, are known to be involved in the ERAD process, and a number of ERAD substrates that are degraded by these complexes have been identified.[413,536,537,549–555] FBS1 also binds to CHIP, a co-chaperone with ubiquitin ligase activity.[556]

NMDA receptors play an essential role in some form of synaptic plasticity, learning and memory. SCF^{Fbs1} controls the amount and localization of their specific subunits, GluN1/GlcN2A.[549,557] FBS1 ubiquitinates and reduces the level of BACE1, a key enzyme that is involved in the production of amyloid-β (Aβ), and amyloid precursor proteins (APP), thus attenuating Aβ production.[550,552] The Nicotinamide riboside (NR)-treatment of Alzheimer's disease (AD) model mouse restored cognition through the upregulation of the Proliferator-activated receptor-γ cofactor 1 (PGC)-1a and, in turn, enhanced the expression of FBS1.[558] It is therefore tempting to speculate that promoting FBS1 activity by a drug such as NR may represent a novel therapeutic strategy for the treatment of AD. In this connection, it is also noteworthy that the Nogo receptor 2 has been identified as a binding protein for FBS1.[559] Since Nogo receptors are known to bind to APP[560–562] and AD model mice lacking the Nogo receptor 2 show reduced amyloid plaque formation,[562] reducing the level of Nogo receptors by FBS1 may also be beneficial for the attenuation of the pathogenesis associated with AD. It is also noteworthy that statistically significant differences in allelic frequencies were detected in an FBXO2 variant between Han Chinese Parkinson's disease patients and controls.[563]

FBS1 was previously identified as a protein that is highly expressed in the organ of Corti and was denoted as the organ of Corti protein 1 (OCP1).[564] Another protein that is abundant in the organ of Corti, OCP2, is SKP1.[564] Mice lacking FBS1 develop age-related hearing-loss.[565] OCP1 and OCP2 form complexes that co-localize with the epithelial gap-junction system.[548] Moreover, FBS1 was found to bind with connexin 26.[566] Since mutations in the connexin 26 gene are the most common cause of hereditary deafness,[567,568] FBS1-SKP1-connexin 26 interactions may somehow be critical for the mammalian hearing system. As shown by a previous study,[569] FSB1 is mainly present as a heterodimer with SKP1 but not as a component of SCF complex in the inner-ear, and its function in inner-ear homeostasis may be distinct from the conventional SCF complex.[557] On the other hand, SCF^{Fbs1} and SFC^{Fbs2} complexes can bind to the ER through an interaction with Cdc48/p97.[570]

While the expression of FBS1 is normally restricted, it can be induced in response to some stressors. FBS1 is upregulated in livers of obese mice and disrupts glucose homeostasis through the degradation of insulin receptors.[571] FBS1 upregulation in gastric cancer[572] or colorectal cancer[573] has also been observed, and, in both cases, the level of FBS1 is correlated with the metastatic features of the cancer. Infection by the Epstein-Barr virus also activates FBS1, and induces the degradation of the EBV glycoprotein B, thus limiting the extent of viral infectivity.[555]

FBS2 is a paralogue of FBS1 and has a wider tissue distribution. Recent evidence suggests that FBS2 attenuates cadmium toxicity by suppressing the ER stress response.[574,575] In addition, FBS2 ubiquitinates ERO1L, a thiol oxidase in the ER, and inhibits ER stress-induced apoptosis.[553] The expression of FBS2 also promotes the growth and proliferation of both normal gastric cells and gastric cancer cells, while it inhibits the apoptosis and invasion of the cancer cells.[576] FBS2 confers drug sensitization to cisplatin through decreasing the expression/phosphorylation of Chk1 in non-small cell lung cancer cells.[577] FBS2 also regulates antiviral immunity through the degradation of interferon-regulatory factor 3 (IRF3), a key transcription factor that is involved in the response to viral infections.[578] Of note, IRF3 degradation has been reported to be SCF complex-independent.

While the lectin property of FBS3 has been characterized, the function of FBS3 has been difficult to ascertain. Recently, and opposed to FBS1/FBS2,[538,540] FBS3 was found to localize in membranes upon *N*-myristoylation.[540] The SCF^{Fbs3} complex catalyzes the ubiquitination of glycoproteins in damaged lysosomes facilitating the recruitment of autophagic machinery to induce a process referred to as lysophagy.[540] Therefore, FBS3 is involved in the clearance of damaged lysosomes, and this role appears to be a unique feature of FBS3 among FBS proteins.[540]

Some plants contain F-box proteins with lectin domains.[579] The lectin Nictaba was originally found in tobacco leaves,[580] and upon a search for orthologous proteins in other plant genomes, the presence of putative F-box proteins comprised of a C-terminal domain homologous to Nictaba has been found in variety of plant species.[581] Gene expression of the *A. thaliana* F-box-Nictaba protein, At2g02360, can be induced with salicylic acid, heat stress and by a virulent *Pseudomonas syringae* infection.[582] At2g02360 binds to *N*- and *O*-glycans that contain *N*-acetyllactosamine, poly-*N*-acetyllactosamine structures as well as Lewis structures (Lewis A/X/Y).[582] Whether or not these F-box-lectin proteins indeed form SCF complexes in plants remains to be clarified.

4.3 Cytosolic peptide:*N*-glycanases (NGLY1)

For a polypeptide substrate to gain efficient entry into the interior of the cylinder-shaped 20S proteasome, where the active sites of proteases are located, the bulky modifications such as *N*-glycans and/or polyubiquitinated molecules may need to be removed. The peptide:*N*-glycanase (PNGase; Png1 in yeast[583] and NGLY1 in mammals[584]) are responsible for the removal of *N*-glycans from glycoproteins in the cytosol (Fig. 3).[585–588] Cytoplasmic PNGase activity is ubiquitous from yeast to mammals,[583,589–602] and has been shown to be involved in the ERAD process.[308,592,603–617] However, it should be noted that the occurrence of "non-glycosylated" proteins upon treatment with a proteasome inhibitor does not necessarily result in cytosolic deglycosylation by PNGase.[618] PNGase is required for the efficient degradation of at least a subset of misfolded *N*-glycoproteins,[614,616,619] while many ERAD substrates can be degraded quite efficiently even when PNGase activity is inhibited.[612,613,620] The critical role of NGLY1 in MHC class I antigen presentation has been well characterized.[621–625]

Considering the biochemical activity of deglycosylating enzymes, they also represent an *N*-glycoprotein-specific component for ERAD. In fact, it is known that Png1/NGLY1 participates in various protein-protein interactions; in yeast, Rad23 was identified as a Png1-binding protein through yeast two-hybrid screening[626] and its interaction was confirmed *in vitro*.[627–629] Rad23 binds to proteasomes[630] and is involved in proteasomal degradation.[614,631] The interaction of PNGase with RAD23 (HR23B in mammals) appears to be conserved between yeast and mammals, although the complex interphase is quite distinct between yeast and mammalian complex.[632,633]

In mammalian cells, various PNGase-binding proteins have been isolated using two hybrid screening using mice Ngly1 as the bait,[634] and the interactions have been confirmed *in vitro*.[633,635] Except for HR23B, all of the binding proteins binds to Ngly1 through the N-terminal region of Ngly1 containing a domain called PUB (PNGase and UBA or UBX-containing proteins)[636] or PUG (peptide:*N*-glycanases and other putative UBA or UBX domain-containing proteins),[637] which are known to mediate various protein-protein interactions.[635,638–640] Proteins that have been identified include ubiquitin, mouse S4 (19S proteasome subunit), UBX domain protein 1 (UBXN1) and gp78,[634] and many of those interactions were confirmed *in vitro* or *in cellulo*.[628,629,641,642] Moreover, the binding of Ngly1 to ERAD-related molecules such as p97/Cdc48[629,641] or Der1[643] has been reported. In this connection, it should be noted that FBS1/FBS2 also binds to the ER membrane through p97/VCP/Cdc48.[570] Since NGLY1 and FBS1/2 both recognize *N*-glycans, their reactions would be expected to be competitive. Indeed, FBS1 can protect misfolded glycoproteins from the action of NGLY1 *in vitro*.[644] How these two proteins—a deglycosylating enzyme and a glycan-recognizing ubiquitin ligase subunit—coordinate their respective functions will be an interesting future topic. In any case, these protein-protein interactions may be critical for the formation of an "*N*-glycoprotein degradation complex," which facilitates the sequential reactions during the ERAD process, i.e. ubiquitination, deglycosylation, deubiquitination, and proteasomal proteolysis, in a highly efficient manner.[645,646]

In addition to the N-terminal PUB domain, mammalian NGLY1 contain a C-terminal PAW (PNGase and other worm) domain, which can interact with high mannose-type glycans.[305,647] NGLY1 has a lectin-like activity[648] and free oligosaccharides, one of the reaction products of PNGase, inhibit the activity of this enzyme.[649] Interestingly, the *Dictyostelium discoideum* PNGase C-terminal PAW domain is replaced by an L-type lectin domain,[600] suggesting that this is a result of convergent evolution and one can assume that glycan binding is a beneficial property for the function of the cytosolic PNGase. NGLY1 can also bind to *N*,*N*′-diacetylchitobiose-($GlcNAc_2$)-containing glycans.[649–651] Haloacetamidyl derivatives of $GlcNAc_2$-containing glycans are therefore potent inhibitors/labeling reagents of the Png1/NGLY1 and other PNGase orthologues.[650,652–654] This probe can selectively bind to the catalytic Cys residue, even though the protein contains numerous other surface Cys residues.[650,652]

In yeast cells, the absence of Png1 results in a significant reduction in the level of cytosolic free oligosaccharides, indicating that the majority of the free oligosaccharides are generated by the deglycosylation of misfolded glycoproteins destined for degradation by ERAD.[498,655,656] These results have led to the proposal that the structures/amounts of free oligosaccharides can be a read-out for the overall processing of *N*-glycans during the ERAD process.[498,656] However, this is not the case with mammalian cells, since the knockdown[657] or knockout[658] of Png1 minimally changed the structure/amount of the free oligosaccharides. Therefore, the mechanism for formation of free oligosaccharides appears to be distinct between yeast and mammalian cells (reviewed in[659,660]). Mammalian cells produce two cytosolic glycosidases, an endo-β-N-acetylglucosaminidase[661] and a cytosolic α-mannosidase, MAN2C1.[662–664] They constitute a "non-lysosomal" degradation pathway for free oligosaccharides in mammalian cells.[646,660,665]

Significant progress has been made concerning the functional roles of cytosolic PNGase in various organisms. In *Neurospora crassa*, the PNG1 mutant shows temperature-sensitive growth and strong hyphae polarity defects[654,666,667] Strikingly, the PNG1 in this fungus contains mutations in which all of the three essential amino acids in the catalytic triad, i.e. Cys, His and Asp are modified,[668,669] and therefore is enzymatically inactive.[654] It is predicted that some fungal PNGase orthologues have an essential deglycosylation-independent function.[670] In *Dictyostelium discoidium*, a mutant shows slow growth and developmental defects in

cell aggregation during multicellular development.[600,671] In *C. elegans*, the PNG-1 mutant exhibited abnormal axon branching of VC4/VC5 egg-laying neurons, and hence egg-laying behavior defects.[672] The mutant also showed a very strong sensitivity towards proteasome inhibitors.[673–675] In *D. melanogaster*, severe growth delay,[653] sensitivity towards proteasome inhibitors[675,676] with dysregulated NRF1 function,[677] and a compromised BMP signaling pathway in a context-dependent fashion was observed for mutants of *pngl*, a fly orthologue of *NGLY1*.[602]

Functional studies of NGLY1 have accelerated since it was revealed that a human recessive genetic disorder referred to as an NGLY1 deficiency[678] or a congenital disorder of deglycosylation (CDDG)[679] is caused by mutations in the *NGLY1* gene.[680] Patient-derived cells had compromised NGLY1 activity,[679] as evidenced by deglycosylation-dependent fluorescence assays.[681] Typical clinical features of NGLY1 deficiency patients include developmental delay, hypoalacrima, seizure, motor deficits, bone malmorphology and movement disorders.[678,682–686] It has been suggested that the phenotypic consequences of the *NGLY1* defect may be greatly affected by the genetic background of the individual as well as environmental factors such as food.[677,687,688] There is no established treatment for this disease.

NGLY1 has long been believed to be mainly involved in quality control machinery for newly synthesized proteins. Abundant recent evidence indicates that this enzyme-mediated deglycosylation is also involved in the activation of a transcription factor, NRF1/NFE2L1 (Fig. 4A).[673,674,689] NRF1 (nuclear factor erythroid 2-related factor 1) is known to be involved in the response to various types of cellular stress such as oxidative response, and also is involved in the proteasome "bounce-back" recovery pathway when proteasome activity is inhibited.[690] Consequently, NGLY1-KO animals/cells are very sensitive towards proteasome inhibitors, due to compromised NRF1 functions[673–676,689] (Fig. 4A), and the genetic interaction between NGLY1 and NRF1 has been further confirmed through unbiased transcriptome or genome-wide screening analyses.[677,691] In this connection, an NGLY1 inhibitor potentiated the cytotoxicity of proteasome inhibitors that were developed as anti-cancer agents,[692] and therefore inhibiting NGLY1 may represent a new therapeutic approach for treating cancer.[689] Indeed, the knockdown/knockout of NGLY1 inhibits the growth of certain cancer cells.[693,694]

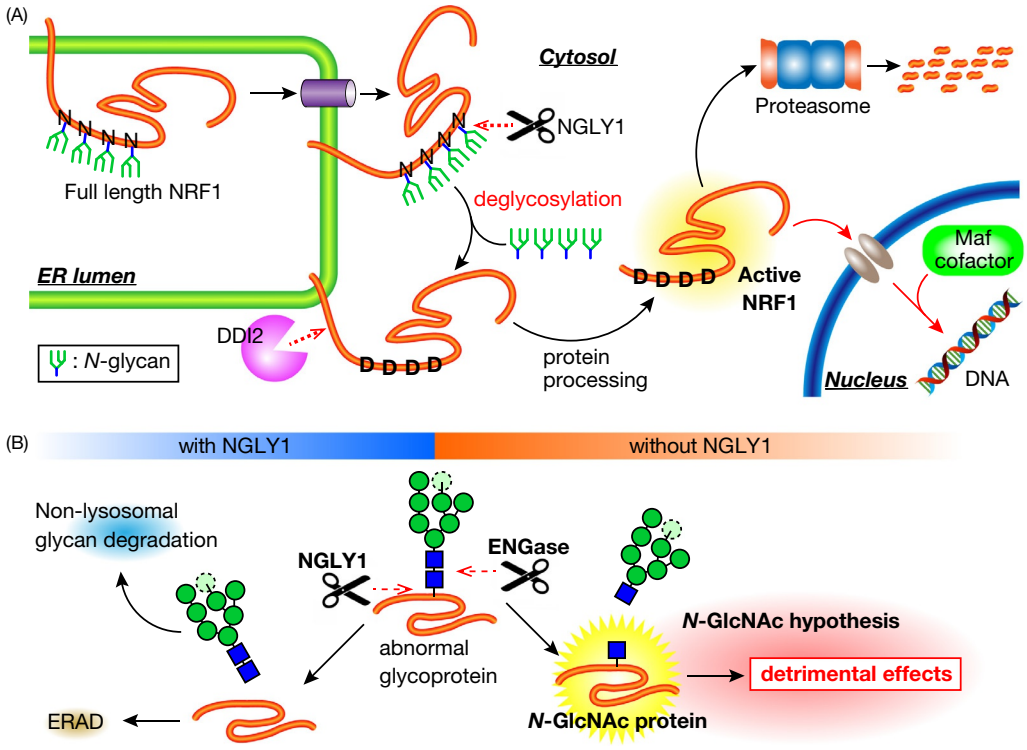

Fig. 4 (A) NGLY1-dependent activation of NRF1, a transcription factor (B) Schematic representation of the "*N*-GlcNAc hypothesis" (A) NRF1 is a transcription factor that is normally synthesized as an *N*-glycoprotein in the ER. During the activation process, which can be induced by various types of stress, NRF1 is translocated into the cytosol, where NGLY1 deglycosylates the molecule. Since NGLY1 is an amidase, *N*-glycosylated Asn residues are converted into Asp residues, and this conversion is critical for the full activation of this molecule. Deglycosylated NRF1 is further processed by the aspartyl protease DDI2 to become the fully activated NRF1. (B) *N*-GlcNAc hypothesis. Under normal conditions, misfolded glycoproteins that are released into the cytosol are deglycosylated by the action of NGLY1, and the deglycosylated proteins are then degraded by proteasomes. However, when NGLY1 activity is compromised, ENGase, another cytosolic deglycosylating enzyme, stochastically acts on misfolded glycoproteins, forming "*N*-GlcNAc proteins". The formation of excess amounts of *N*-GlcNAc proteins appear to be somehow detrimental to cells/animals. (A) Lehrbach, N. J.; Ruvkun, G. *Elife* **2016**, *5*, e17721; Lehrbach, N. J., Breen, P. C.; Ruvkun, G. *Cell* **2019**, *177*, 737–750; Tomlin, F. M.; Gerling-Driessen, U. I. M.; Liu Y. C.; Flynn, R. A.; Vangala, J. R., et al. *ACS Cent. Sci.* **2017**, *3*, 1143–1155; Koizumi, S.; Irie, T.; Hirayama, S.; Sakurai, Y.; Yashiroda, H.; et al. *Elife* **2016**, *5*, e18357. (B) Huang, C.; Harada, Y.; Hosomi, A.; Masahara-Negishi, Y.; Seino, J.; et al. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 1398–1403; Fujihira, H.; Masahara-Negishi, Y.; Tamura, M.; Huang, C.; Harada, Y.; et al. *PLoS Genet.* **2017**, *13*, e1006696.

NGLY1-KO cells or patient-derived cells exhibit abnormal mitochondria functions/morphology.[695,696] It was suggested that a defect of mitophagy, a process that involves clearing damaged mitochondria, leads to the leakage of mitochondrial nucleic acids into the cytosol, thus activating innate immune nucleic acid sensors and type I interferon response.[696] How this immunological phenomenon relates to the symptoms of patients remains unclarified. In this connection, NGLY1 has recently been identified as a host factor required for the replication of the enterovirus 71.[697] It is tempting to speculate that the chronic upregulation of the innate immune response may somehow be related to impaired virus replication.

Ngly1-KO in a C57BL/6 mouse genetic background is embryonically/perinatally lethal.[687] Moreover, liver-specific KO mice were reported to develop an abnormal nuclear morphology and, upon various types of food stress, developed hepatic steatosis.[688] Surprisingly, the additional KO of another cytosolic deglycosylating enzyme, ENGase, or a mixed background, resulted in the partial rescue of the lethality.[687] These results indicate that the phenotypes of an NGLY1-deficiency are largely dependent on the genetic background of the subjects. A recent, *in silico* screening of FDA-approved drugs identified a proton pump inhibitor as a potent ENGase inhibitor, suggesting that it has potential for use as a drug for the treatment of NGLY1-deficiency.[698] A fluorescence resonance energy transfer (FRET)-based assay probe for ENGase has been reported, which will facilitate the high throughput screening for ENGase inhibitors.[699] It is also noteworthy that sulforaphane, known as an activator of NRF2, a paralogue of NRF1 but with a function independent from NGLY1, can ameliorate mitochondrial defects and also suppress the innate immune response, suggesting that an NRF2 inducer also may correct some of the symptoms of an NGLY1-deficiency.[675,696] Both *Ngly1*-KO cells and *Engase Ngly1* double-KO cells showed a similar sensitivity towards proteasome inhibitors, strongly indicating that the NRF1-related pathway and the ENGase-related pathway are independent.[688] Therefore it is assumed that the pathogenesis of NGLY1-deficiency proceeds through multiple independent pathways.[585] Most recently, NGLY1-KO cells were shown to be resistant to hypotonicity-induced cell lysis, and NGLY1 appears to regulate the transcription of aquaporin1, which may contribute to the hypotonicity-resistance.[700] Interestingly, this effect is independent of enzyme activity.[700] This finding may explain why NGLY1-patients develop alacrima. Surprisingly, ENGase also regulates the transcription of aquaporins, although the mechanistic insights remain unclarified.

Regarding the mechanism responsible for the rescue of lethality by ENGase-KO, it was proposed that the accumulation of excess levels of *N*-GlcNAc proteins (ENGase product) may be the cause for the detrimental effects including lethality (*N*-GlcNAc hypothesis) (Fig. 4B).[619,687] A recent glycoproteomics analysis indeed indicated that the cytosolic *N*-GlcNAc proteins are generated by the action of cytosolic ENGase (Jason C. Maynard and Alma L. Burlingame, UCSF, unpublished observation).

Specific Asn-linked oligosaccharides have been identified in the urine of patients with an NGLY1-deficiency.[701] Moreover, Asn-GlcNAc has been identified as a serum marker for these patients.[702] These compounds therefore can serve as sensitive diagnostic markers for an NGLY1-deficiency in the future.

5 Perspectives

The progress of the research topics discussed in this chapter over the last decade is truly remarkable. These studies include the structural biology of OST/UGGT (although not covered in this chapter, the recent cryo EM structure of the Alg6 protein is of special note[703]), clarifications of the role of EDEMs, functional studies for FBS proteins or NGLY1, and the discovery of various human genetic disorders and information on the mutations of genes involved (e.g. OST subunits, NGLY1). It should be noted that ER homeostasis/quality control is tightly associated with a number of human diseases.[704–707] Many of these disorders have been shown, or are predicted, to occur due to defects in the ER quality control/homeostasis machinery. Given the fact that there are examples where proteins, although sensed and eliminated by the ERAD system, still retain their biochemical activity,[708,709] the clarification of this (glycan-based) recognition system is of great importance, since it may become a therapeutic target when we find approaches for controlling the recognition system.

In some cases, carbohydrate binding-independent functions of lectins[197,218,219,402,403] or enzyme-independent functions of enzymes[381,654,700,710] have been proposed. Efforts should be directed at accumulating comprehensive data on their roles and functions in order to understand the diverse roles of these lectins/enzymes in various cellular processes.[27]

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