

**UDP-Glucose:Glycoprotein Glucosyltransferase
(UGGT1) Promotes Substrate Solubility in the
Endoplasmic Reticulum**

by

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Dedication

For my Mom, Dad, Ann, and Ashley.

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excellence.

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Abstract

Protein folding in the endoplasmic reticulum (ER) is error-prone, and ER quality control processes exist (ERQC) to ensure only correctly folded proteins are exported from the ER en route to the Golgi compartment. Glycoproteins with amino acid mutations can be retained in the ER by ERQC, and this retention contributes to multiple human diseases, termed ER storage diseases. UDP-glucose:glycoprotein glucosyltransferase (UGGT1, GT1) is proposed to be a central component of ER glycoprotein quality control. The mechanism of UGGT1-mediated monoglucosylation of deglucosylated N-glycans of incompletely folded glycoproteins, and the subsequent reassociation of these glycoproteins with lectin-like chaperones (calreticulin and calnexin) in the ER has been extensively described. However, the extent to which UGGT1 influences the folding of ER substrate proteins has only been investigated for a few selected substrates. Using mouse embryonic fibroblasts lacking UGGT1, or those with UGGT1 complementation, we investigated the effect of monoglucosylation on the soluble/insoluble distribution of two misfolded alpha-1 antitrypsin (AAT) variants responsible for AAT deficiency disease, null Hong Kong (NHK) and Z allele (ATZ). Our results indicate that, whereas substrate solubility increases directly with the number of N-linked glycosylation sites, additional solubility is conferred by UGGT1 enzymatic activity at the expense of the pool of insoluble protein complexes in the ER. Monoglucosylation-dependent solubility decreases both BiP association with NHK and unfolded protein response (UPR) activation, and the solubility increase is blocked in cells deficient for calreticulin. These results suggest that UGGT1-dependent monoglucosylation of N-linked glycoproteins promotes substrate solubility in the ER.

Chapter 1

Introduction

1.1 Introduction

Over the past thirty years, multiple human genetic diseases have been identified where pathogenesis is directly linked to endoplasmic reticulum (ER) retention of misfolded glycoproteins, sometimes with mutation as modest as a single amino acid substitution[1]. These diseases have been termed ER storage diseases[2]. Interestingly, many of these mutant glycoproteins still possess the biological activity of their wild-type counterparts, as demonstrated by experiments in which a functional product is exported under conditions of altered temperature, or after treatment with chemical chaperones[3, 4]. The process whereby imperfectly-folded proteins are retained in the ER, and proteins with a native fold are permitted export from the ER, has been termed ER quality control (ERQC)[5]. In some instances, the pathogenicity associated with the expression of mutant glycoproteins might be reduced if ERQC were to be altered. Accordingly, much research effort has been directed toward understanding the mechanism of ERQC, with the goal of finding ways to usher problematic glycoproteins past the ERQC barrier without disrupting generalized ER function[6].

Glycosylation has been described as the most common post-translational modification of proteins[7], with a majority of glycoproteins bearing covalent attachment of oligosaccharide to asparagine side-chains (N-linked glycosylation). These oligosaccharides, termed N-linked glycans or simply N-glycans, are added in the ER where they serve as an entry pass to an intricate glycoprotein-specific portion of the ERQC system that ultimately enhances the export efficiency of high

quality glycoprotein products[8]. In overview, glycoprotein-specific ERQC functions through a multitude of proteins: sugar-modifying enzymes, sugar-binding lectins, and protein-binding chaperones. The N-glycans are trimmed during glycoprotein folding, and the various trimmed glycans facilitate recognition of substrate glycoproteins by various QC factors. This chapter highlights the mechanisms and principles of glycoprotein-specific ERQC, beginning with an introduction to general ERQC and implications for cell stress responses. Then, to focus on glycoprotein ERQC, I address the addition of N-linked oligosaccharides to nascent polypeptides, then shift to the cyclical deglycosylation and reglycosylation of N-glycans, and finally end with consideration of ER exit, retention, degradation, or accumulation of insoluble glycoprotein secretory products.

1.2 Protein folding, ER stress and the unfolded protein response (UPR)

The discovery of molecular chaperones in distinct cellular compartments challenged the concept that proteins fold spontaneously according to their amino acid sequence and length. Molecular chaperones are required for correct folding and assembly of proteins, and form the basis for the 'assisted folding hypothesis' [9, 10]. Experiments showed chaperones could also promote correct protein folding by reducing the propensity of proteins to aggregate both in vitro and in cells[9]. Mechanistically, interactions with chaperones may: 1) improve kinetics of appearance of functional conformation, or 2) prevent formation of incorrectly folded structures[9]. Proteins tend to aggregate because transiently exposed hydrophobic side-chains that normally form weak intramolecular bonds form weak intermolecular bonds with an adjacent molecule. This disrupts the native state of the attacked molecule, exposing hydrophobic residues that normally would not be exposed, and the chain reaction continues[10], unless molecular chaperones bind the exposed hydrophobic residues.

Generally, proteins recognized as unfolded by molecular chaperones are retained in the ER[11]. This can produce a situation where the amount of unfolded protein in the ER exceeds the folding capacity of the ER. This situation is known as ER stress[12], which triggers an ER stress response also known as the unfolded protein response (UPR)[13]. The acute UPR is an adaptive response to resolve and limit protein misfolding. Very briefly, the UPR utilizes three transmembrane signal transducers to reduce the amount of misfolded protein in the ER by two main mechanisms. First, mRNA translation is decreased globally—limiting entry of newly-synthesized proteins into an incapacitated ER protein folding environment—through phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2alpha) by the ER-localized transmembrane UPR signal transducer PERK. Second, UPR upregulates transcription of genes encoding protein translocation, folding, trafficking and degradative functions, to reduce misfolded protein accumulation through: 1) trafficking and cleavage-activation of ATF6, 2) IRE1-initiated unconventional splicing of XBP1 mRNA, and 3) phospho-eIF2alpha stimulated translation of ATF4[13]. Importantly, expression of many proteins involved in ERQC is increased by the UPR, highlighting the connection between ER stress and ERQC[13, 14].

BiP (immunoglobulin binding protein) is an HSP70 family member and molecular chaperone that is highly expressed in the ER[15]. Binding assays on random peptide libraries demonstrated that short hydrophobic peptides preferentially bound to BiP[15]. These and other studies led to the conclusion that BiP binds unfolded proteins in the ER[12]. BiP binding to unfolded proteins also involves an ADP-ATP cycle, where the ADP-bound BiP form has a high affinity for bound protein, and an exchange of ATP for ADP releases the substrate from BiP[12]. Since BiP binds unfolded but not correctly folded proteins, it acts as a quality control factor in the ER, retaining unfolded protein forms that have not yet reached a folding form that allows for ER exit[12].

Along with the BiP-based chaperone system, there is also a glycoprotein specific chaperone system termed the 'calnexin cycle', which involves the lectin-like chaperones calnexin (CNX) and calreticulin (CRT), and the glycan-modifying enzymes glucosidase II (GSII) and UDP-glucose:glycoprotein glucosyltransferase (UGGT1). These two chaperone systems can act on the same substrate, and can act as complementary ERQC systems if substrate interaction with BiP or CRT/CNX is impeded[16-18]. Before describing in depth the glycoprotein-specific calnexin cycle ERQC system, I will first describe the initial formation and processing of glycoproteins in the ER.

1.3 N-linked glycoprotein processing in the ER

1.3.1 Oligosaccharide formation and transfer to nascent polypeptides

N-linked oligosaccharide addition and processing begins with assembly of a tetradecaoligosaccharide core. Construction of the glucose₃mannose₉N-acetylglucosamine₂ (Glc₃Man₉GlcNAc₂) oligosaccharide begins and ends on a dolichyl pyrophosphate molecule anchored in the ER membrane. Multiple glycosyltransferases and sugar donors coordinate the step-wise assembly of the oligosaccharide, which initiates on the cytoplasmic face of the ER to produce Man₅GlcNAc₂, and is completed on the luminal side of the ER membrane[19]. The Glc₃Man₉GlcNAc₂ is a triantennary structure having three mannose chains (A, B, and C, SEE FIGURE 1.1) with three glucose residues attached to the A chain[20]. The end result of this process is a fully-formed dolichyl pyrophosphate-linked Glc₃Man₉GlcNAc₂ oligosaccharide on the luminal side of the ER membrane, ready to be used as a substrate for N-linked protein glycosylation[21].

Glc₃Man₉GlcNAc₂ oligosaccharides are transferred to nascent polypeptides by oligosaccharyltransferase (OST). The mammalian oligosaccharyltransferase (OST) exists as multiple isoforms, with multiple distinct

subunits. One subunit, STT3, plays a critical catalytic role in OST function[21, 22]. OST isoforms containing either of two STT3 subunits (STT3-A or STT3-B) have been identified, and have differences in both tissue distribution and enzymatic activity. OST isoforms containing STT3-B are much more active and less discriminating of donor sugar forms, transferring non-canonical $\text{Man}_9\text{GlcNAc}_2$ oligosaccharides at a higher rate than STT3-A containing isoforms[23]. Other mammalian OST subunits include ribophorin I, ribophorin II, OST48, DAD1, and keratinocyte-associated protein 2 (KCP2), and depletion of these subunits can have deleterious effects on OST stability and function[24, 25].

$\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ oligosaccharides are transferred cotranslationally to newly-synthesized glycoproteins as they are translocated into the ER lumen[26, 27]. The oligosaccharide is transferred to an acceptor Asn (Figure 1.1), in the context of the N-linked glycosylation sequon, Asn-X-Ser/Thr (where X can be any amino acid except proline)[28]. Transfer generally occurs when the acceptor Asn residue is 12-14 residues away from the ER membrane[29]. Recently, the structure for a bacterial OST was solved, and this structure illuminates the mechanism of N-linked glycosylation at the molecular level[30]. The peptide and lipid-linked oligosaccharide binding sites are on opposite sides of the OST, and there is a tunnel through which the acceptor Asn can contact the lipid-linked oligosaccharide to form the N-glycosidic bond[30]. Interestingly, post-translocational N-glycosylation of proteins retained in the ER has been reported, so N-glycosylation may not be strictly a cotranslocational event, although the majority of N-glycosylation does occur cotranslocationally[31, 32].

Not every potential N-glycosylation site receives an oligosaccharide, and regulation of N-glycosylation occupancy could serve as an early step affecting glycoprotein ERQC. In a recent study of the mouse N-glycoproteome, brain tissue was found to have a high rate of N-glycosylation site occupancy, with less than 2% of potentially glycosylated peptides detected in unglycosylated states[33]. This same study also showed that about half of the identified glycoproteins only had one N-glycan, and that mouse brain glycoproteins had the

highest amount of N-glycosylation per glycoprotein, when compared to kidney, liver and heart[33].

The cellular consequences of blockade of N-glycan addition have been studied using tunicamycin (TM), a nucleoside antibiotic which blocks the first step in N-linked oligosaccharide assembly, transfer of GlcNAc-1-P from UDP-GlcNAc to dolichyl-P by GlcNAc-1-P transferase[34]. When invertase from *Saccharomyces cerevisiae* was expressed heterologously in *Xenopus laevis* oocytes, complete blockade of N-glycosylation (by TM) treatment increased ER retention of invertase, significantly reducing the amount secreted – but the non-glycosylated secreted invertase still retained activity[8]. These studies indicate that inhibiting initial glycan addition to N-linked glycoproteins can significantly affect glycoprotein export from the ER, but does not necessarily preclude formation of biologically active products. However, in most instances, TM treatment does in fact trigger massive misfolding and accumulation of unglycosylated versions of glycoproteins in the ER, leading to ER stress[13, 35-37]. In many cases these misfolded proteins are bound to the protein chaperone BiP[12].

Our understanding of the biological roles of N-glycans in protein function, including roles in protein targeting, cell signaling and trafficking, and immune response, has been steadily increasing. Mutations in many of the steps of N-linked glycan biosynthesis and processing lead to devastating (often fatal) ‘congenital disorders of glycosylation’ (CDGs), highlighting the widespread importance of this common post-translational modification[19]. Among the most important molecular effects are the biophysical consequences of N-glycan addition to substrate glycoproteins in the ER. On the one hand, studies comparing thermal stability of purified glycoproteins to their unglycosylated counterparts produced in vitro through enzymatic deglycosylation probably do not accurately reflect these consequences, because N-glycans are rapidly processed to diverse forms through the action of ER and Golgi glycosidases and glycosyltransferases[38]. On the other hand, Schulke et al. compared the in vitro

thermal stability of glycosylated and non-glycosylated yeast invertase whose N-glycan forms remain of a high-mannose type throughout protein maturation; although the effect of glycosylation on thermal stability was somewhat minor[39], the major biophysical effect was that the non-glycosylated form was more susceptible to protein aggregation[40]. Although those studies did not specifically consider the contributions of glycoprotein ERQC, additional cellular studies revealed that blockade of N-glycan addition not only blocked invertase secretion[41] but also resulted in the protein acquiring an abnormally trypsin-resistant state[42] typically seen for insoluble protein complexes[43, 44]. Thus, existing data suggest that a common biophysical consequence of N-glycosylation of substrate glycoproteins in the ER is enhanced folding/solubility.

1.3.2 Processing of $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ to $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$

It has been known for more than thirty years that after $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ is added *en bloc* to proteins in the ER, the outermost glucose molecule is cleaved almost immediately by glucosidase I (GS1), forming $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$ [45]. GS1 is an integral membrane glycoprotein that cleaves the (α 1,2)-glycosidic linkage between the terminal and middle glucose on $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ N-glycans. GS1 is notable as being the enzyme deficient in congenital disorder of glycosylation type IIb (CDG-IIb)[46, 47]. The index patient with CDG-IIb was a compound heterozygote for two different GS1 missense mutations with enzymatic analysis (of liver tissue and skin fibroblasts) demonstrating an almost complete lack of GS1 activity. That patient survived embryonic development but did not live beyond 2.5 months of age, with multiple organ system failure including severe neurological defects (hypotonia, hypoventilation and seizures), dysmorphic features, and progressive hepatomegaly[47]. Thus, GS1 appears essential for postnatal human life.

GS1-mediated glucose trimming prevents re-binding of the processed N-glycan by OST[48] and also promotes recognition by a recently discovered

membrane-anchored ER protein called malectin[49]. The physiologic role of malectin is still not clear, but initial studies suggest that malectin plays a role in glycoprotein ERQC. In studies using the misfolded glycoprotein alpha-1 antitrypsin null Hong Kong (NHK) variant as a model substrate, malectin overexpression increased ER retention and decreased secretion of NHK. The secreted NHK displayed immature ER-type N-glycans, suggesting malectin overexpression may impair further glycan processing, disturbing ERQC[50]. It was also suggested that malectin may be involved in ER associated degradation (ERAD), as malectin overexpression increased NHK association with OS-9, a component of the ERAD machinery for glycoproteins[51].

After the terminal glucose residue is cleaved by GS1, the next glucose residue is cleaved from $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$ by glucosidase II (GS2) to form $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ [52-54] (GS2 also cleaves the innermost (alpha1,3)-linked glucose, the significance of which will be described further in the next section). GS2 is composed of two subunits: $\text{GS2}\alpha$, the catalytically active subunit, and $\text{GS2}\beta$, the subunit containing an HDEL ER retention signal, responsible for ER localization of the heterodimer[55]. The physiologic importance of GS2 and formation of $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ N-glycan forms is highlighted by mutations in the $\text{GS2}\beta$ subunit causing polycystic liver disease[56, 57].

Both glucosidases GS1 and GS2 can be inhibited in cells with either 1-deoxynojirimycin (DNJ; glucose analog) or castanospermine (CAS; indolizine alkaloid). Initial studies showed that pharmacological inhibition of ER glucosidases impairs trafficking of different glycoproteins to different degrees, but in general inhibits glycoprotein export with increased ER retention and increased ERAD[58, 59]. Interestingly, additional studies have shown that interfering with glucosidase activity can result in faster kinetics of glycoprotein folding yet with lesser efficiency (i.e. lower amounts of native forms produced), again, with more substrate glycoprotein degradation[60, 61]. Glucosidase inhibition can also activate the UPR[62]. As described below, these phenotypes are likely due to

absence of formation of Glc₁Man₉GlcNAc₂ N-glycan forms that serve a critical role in glycoprotein ERQC[48].

1.3.3 Processing Glc₁Man₉GlcNAc₂ to Man₉GlcNAc₂ – the calnexin cycle

Early studies demonstrated that monoglucosylated glycans accumulate when proteins are retained in the ER[50]. Glycoproteins with Glc₁Man₉GlcNAc₂ N-glycans are high-affinity substrates for the ER lectin-like chaperones calnexin (CNX) and calreticulin (CRT). Glycoprotein association with CNX or CRT marks the beginning of what has been termed the ‘calnexin cycle’[63]. In the calnexin cycle, CNX and CRT are thought to play equivalent roles, as they have very similar structures and mechanisms of action, although CNX is a type 1 integral ER membrane protein while CRT resides in the ER lumen[48]. The calnexin cycle is based on repeated association and disassociation of incompletely-folded glycoproteins with pro-folding chaperones CNX and CRT (Figure 1.2). The N-glycans attached to substrate glycoproteins cycle between monoglucosylated (Glc₁Man₉GlcNAc₂) and unglucosylated forms (Man₉GlcNAc₂) when bound or unbound to CNX/CRT, respectively. GS2 plays two critical functions in the cycle: 1) initial formation of Glc₁Man₉GlcNAc₂ N-glycans from Glc₂Man₉GlcNAc₂ precursors, and 2) removal of the terminal glucose residue from Glc₁Man₉GlcNAc₂ forms. Deglucosylation to the Man₉GlcNAc₂ form mediates glycoprotein release from CNX/CRT, as CNX and CRT have very low affinity for unglucosylated glycans. To complete the cycle, protein-bound Man₉GlcNAc₂ N-glycans are reglucosylated to the Glc₁Man₉GlcNAc₂ form by uridine diphosphate (UDP)-glucose:glycoprotein glucosyltransferase (UGGT1), which allows these glycoproteins to reassociate with CNX/CRT. As described further, below, UGGT1 can also reglucosylate Man₈GlcNAc₂ and Man₇GlcNAc₂ N-glycans formed through demannosylation in the ER, although at a lower efficiency.

UGGT1 plays a unique and critical role in the calnexin cycle because it is the only component that can recognize the folding status of glycoprotein

substrates. Indeed, UGGT1 selectively reglucosylates only incompletely folded substrates (Figure 1.2); correctly folded substrates are not reglucosylated by UGGT1, rather, they proceed along the ER-to-Golgi anterograde trafficking pathway. This function of UGGT1 to monitor folding of glycoprotein substrates and discriminate between correctly and incompletely folded forms matches the definition of glycoprotein ERQC, but the mechanism by which UGGT1 recognizes its substrates, and the downstream consequences of reglucosylation for specific substrates remain areas of active investigation.

1.3.4 Calnexin cycle components – CRT, CNX, ERp57, UGGT1

Calreticulin was first identified as a calcium-binding protein in skeletal muscle and named high-affinity calcium binding protein[64]. After being assigned many different names (e.g., calregulin, CRP55, CaBP3), a consensus for the term calreticulin (CRT) was finally achieved[65]. CRT was appreciated as having chaperone properties when it was demonstrated that it (and other ER chaperone proteins: BiP, GRP94, PDI) could be affinity purified by chromatography on a column of denatured (glyco)protein[66].

Calnexin (CNX) was discovered as a calcium-binding, integral membrane phosphoprotein with high sequence identity to CRT[67]. An ERQC function for CNX was hypothesized based on data that unassembled T-cell receptor (TCR) subunits and major histocompatibility complex class I proteins (MHC I) exhibited prolonged interaction with CNX while being retained in the ER[68, 69]. Additionally, CNX in HepG2 cells was found to associate with six major secretory glycoproteins including alpha-1 antitrypsin, and it was shown (by interfering with protein folding using the proline analog azetidine-2-carboxylic acid) that binding of glycoproteins to CNX was enhanced when they were misfolded[70].

ERp57 is a thiol oxidoreductase (with sequence similarity to protein disulfide isomerase) that binds to the P-domain of CNX and CRT[71]. Many (glyco)proteins require disulfide bond formation for correct folding, and ERp57

forms mixed disulfide bonds with glycoprotein folding intermediates[72]. ERp57 has also been shown to accelerate the folding of ribonuclease B[73]. Importantly the ERp57-mediated folding required a monoglucosylated glycan and CNX[73].

UGGT1 was first appreciated in *Trypanosoma cruzi* because this organism initially transfers non-glucosylated Man₉GlcNAc₂ oligosaccharides to ER proteins[74], yet glucosylated Glc₁Man₉GlcNAc₂, Glc₁Man₈GlcNAc₂, and Glc₁Man₇GlcNAc₂ oligosaccharides could be detected[75]. Monoglucosylation was found to require transport of a UDP-glucose donor into the ER lumen[76]. Using an in vitro assay (composed of rat liver microsomes, UDP-[¹⁴C]Glc as a glucose donor, and purified thyroglobulin) it was shown that formation of radiolabeled Glc₁Man₇₋₉GlcNAc₂ oligosaccharides occurred preferentially on denatured rather than native glycoprotein substrate[77], indicating that reglucosylation reflects the action of a ‘folding sensor’ in the cell. Folding sensor behavior was directly demonstrated by showing that reglucosylation activity is dependent on protein-based characteristics and not just accessibility of the oligosaccharides. Indeed, in vitro protease digestion of substrates abrogated reglucosylation activity even as the oligosaccharides were still recognized by lectin probes like concanavalin A[78]. By studying *Schizosaccharomyces pombe* bioengineered so that the UGGT1 enzyme was absent and only Man₉GlcNAc₂ N-glycans were transferred to glycoproteins—the formation of the Glc₁Man₉GlcNAc₂ glycan structure was found to be essential for cell viability under stress conditions[79], consistent with the idea that UGGT1 enzyme activity linked to the folding sensor may enhance glycoprotein folding quality and limit cytotoxicity.

Studies of purified UGGT1 have indicated that the molecular mechanism by which UGGT1 recognizes its glycoprotein substrates is bipartite[80, 81]. At minimum, the substrate must have the innermost N-acetylglucosamine residue of the N-glycan, and must also have exposed hydrophobic residues[80]. Using artificially constructed RNase heterodimers with mixed folded and unfolded halves, UGGT1 was found to reglucosylate N-glycans only on the misfolded

half[82], suggesting that for UGGT1-mediated reglucosylation, the N-glycan must be near the misfolded region of the substrate. Interestingly, for a different glycoprotein substrate UGGT1 was reported to reglucosylate N-glycans more distant (40 angstroms) from local hydrophobic regions[83]. Despite these substrate-specific differences, it appears from all studies that UGGT1 can survey glycoprotein substrates for misfolded regions and reglucosylate attached N-glycans when appropriate (Figure 1.2).

Based on the availability of chemical glucosidase inhibitors (e.g., DNJ), it was shown that when GS2 was inhibited, the effect of UGGT1-mediated reglucosylation was to promote glycoprotein reassociation with, and impaired release from, CRT/CNX — and this resulted in delayed glycoprotein export from the ER[84]. This finding indicated that CRT/CNX binding is normally dynamic, with monoglucosylation enhancing the CRT/CNX-associated state and GS2 activity enhancing the CRT/CNX-dissociated state — with each state having downstream consequences for substrate glycoproteins. Overall, the implication of the above findings for mammalian cells is that the absence or insufficiency of any of the three key N-glycan-modifying enzymes (GS1, GS2 and UGGT1), or other components of the CRT/CNX cycle (Figure 1.2), can perturb the cycle and thereby result in deleterious consequences for glycoprotein quality control.

1.3.5 Role of N-glycans and demannosylation in ER export and ERAD

Trimming of mannose residues from N-linked glycans is linked to productive transport of one subset of glycoproteins through the Golgi complex, and is also linked to the processing of another subset of glycoproteins entrapped in the ER — most typically misfolded glycoproteins targeted for ERAD[85].

For the subset of glycoproteins undergoing anterograde transport, demannosylation of N-glycans is a prerequisite for the formation of complex Golgi-modified glycans found on mature glycoproteins, which typically only have three or five core mannoses remaining from the original nine[86]. For this subset

of glycoproteins, trimming five mannoses from $\text{Man}_8\text{GlcNAc}_2$ to $\text{Man}_3\text{GlcNAc}_2$ forms occurs through the action of Golgi-localized mannosidases {Golgi ManI (A,B,C), Golgi ManII, and Golgi ManIII[86]}. These Golgi mannosidases are also able to remove the same mannoses from $\text{Glc}_{1-3}\text{Man}_{8-9}\text{GlcNAc}_2$ N-glycans, allowing further processing of aberrant N-glycans arriving at the Golgi due to abnormal or failed ER quality control[86, 87] — often, such misfolded glycoproteins are recognized by quality control systems that operate in the Golgi complex, resulting in delivery to lysosomes for degradation[88]. Class I mannosidases specifically hydrolyze only (alpha1,2)-mannose linkages and are found in the ER and Golgi, while class II mannosidases will hydrolyze (alpha1,2), (alpha1,3), and (alpha1,6)-mannose linkages and are found in the Golgi and lysosomal compartments[89].

For forward transport of secretory proteins out of the ER and into the Golgi, two non-mutually exclusive mechanisms have been proposed: 1) bulk-flow, and 2) receptor-mediated transport[90]. N-linked glycans are thought to play an important role in the second mechanism. For example, ERGIC-53 is one protein (along with ERGL and VIP36) that is thought to act as a cargo receptor, and transfer correctly folded substrates to COP-II vesicles for transport out of the ER, into the Golgi[85, 91-93]. Similar to UGGT1, ERGIC-53 may have a bipartite recognition signal, and be able to sense folding status of its substrates[92]. As an example, ERGIC-53 recognizes both the high-mannose type N-glycan and a beta-hairpin loop structure present only in correctly folded cathepsin Z, an ERGIC-53 binding substrate[92]. Further, a pathologic role for ERGIC-53 deficiency has been identified as a cause of a combined secretion defect for two serum glycoproteins and clotting factors, factor V (FV) and factor VIII (FVIII)[94].

For substrates entrapped in the ER, such as under conditions in which ER-to-Golgi transport is blocked pharmacologically (e.g., using carbonyl cyanide m-chlorophenylhydrazone, a calcium ionophore), glycoproteins accumulate with a specific attached high-mannose N-glycan form, $\text{Man}_8\text{GlcNAc}_2$, lacking the B-chain mannose (M8B)[95]. This $\text{Man}_8\text{GlcNAc}_2$ N-glycan form was also observed

using a temperature-sensitive model protein (ts045 VSV G protein) that is retained in the ER[96] — indicative of ER mannosidase I (ERmanI) activity with high specificity for the terminal B-chain mannose, allowing reglucosylation to continue to occur on the A-chain[71]. ERmanI is implicated in ERAD of glycoproteins (Figure 1.3), as inhibition of ERmanI stabilizes glycoproteins that are normally degraded[97, 98]. The mechanism of degradation involves recognition of the misfolded M8B-glycoprotein by the alpha-mannosidase I-like protein EDEMI[99-101]. EDEMI possesses little or no mannosidase activity, and seems to act as a 'holdase' that mediates transfer to other ERAD components, such as OS-9[99, 102]. There are two other EDEM homologs, EDEM2 and EDEM3. EDEM3 has some mannosidase activity, and the C-branch mannose of the M8B N-glycan is cleaved by EDEM3 (or possibly Golgi mannosidase I) allowing OS-9 to specifically recognize this N-glycan lacking the C-branch mannose[103], with subsequent retrotranslocation to the cytoplasm for ubiquitination and ERAD via proteasomal proteolysis[99].

Recent research is beginning to reveal the individual components of the mammalian ERAD/retrotranslocation complex, which has been termed the HRD1/SEL1L ERAD complex[99]. HRD1 is a transmembrane ubiquitin ligase which, together with SEL1L (that binds OS-9), forms a ubiquitin ligase complex[99]. OS-9 contains a mannose-receptor homology (MRH) domain, and it was shown that rather than binding to substrate N-glycans, OS-9 binds to N-linked glycans of SEL1L with this domain, suggesting a mechanism for OS-9 interaction with SEL1L, but complicating the true function of the OS-9 MRH domain[102]. Interestingly, ER-to-Golgi trafficking may also be necessary for ERAD of soluble glycoproteins[104]. Perhaps this transient exposure to the Golgi compartment allows for cleavage of the C-chain mannose, and subsequent targeting to the HRD1/SEL1L ERAD complex, possibly through direct interaction with OS-9[103].

One key question regarding ERQC is how the QC system distinguishes between terminally-misfolded glycoprotein forms and intermediate folding forms

that might have similar characteristics, targeting the former for degradation and the latter for reglucosylation and more refolding attempts[105]. In particular, if misfolded glycoprotein forms are in the calnexin cycle, but their foldedness does not improve during cycling, they could become trapped in the calnexin cycle. Based on data from cell-free assays, there is decreased efficiency of UGGT1-mediated reglucosylation of lower mannose N-glycan forms ($\text{Man}_8\text{GlcNAc}_2$, $\text{Man}_7\text{GlcNAc}_2$)[78]. Consequently, it was thought that N-glycan demannosylation—either of the B- and C- chains to decrease affinity of UGGT1, or of the A-chain to completely prevent UGGT1-mediated reglucosylation—might favor release of misfolded glycoproteins from the CNX cycle, leading to degradative fates[105].

In contrast to the finding above, it was recently shown in *S. pombe* that UGGT1-mediated reglucosylation efficiency does not decrease on mannose removal, while it is GS2 whose efficiency of deglucosylation decreases precipitously as mannose residues are removed from $\text{Man}_9\text{GlcNAc}_2$ N-glycans[106]. The MRH domain of OS-9 does interact specifically with N-glycans lacking terminal C- chain mannoses, including monoglucosylated forms ($\text{Glc}_1\text{M8C}$, $\text{Glc}_1\text{M7B,C}$)[103, 107]. This opens the possibility of an alternative means of glycoprotein disposal to that proposed in the previous paragraph, where in this instance removal of the terminal C- chain mannose by EDEM3 and subsequent recognition by OS-9 may lead to ERAD of monoglucosylated forms directly from the calnexin cycle[103, 106]. Interestingly, ERAD of the misfolded alpha-1 antitrypsin variant null Hong Kong (NHK) is accelerated when the glucosidase inhibitor CAS is added during chase after metabolic labeling, implying deglucosylation may not be necessary to target misfolded glycoproteins to ERAD[108].

Initial studies strongly implicated $\text{Man}_8\text{GlcNAc}_2$ N-glycan forms as sufficient for targeting to ERAD, but more recently it has been proposed that $\text{Man}_{5-7}\text{GlcNAc}_2$ N-glycans may serve as even stronger signals for ERAD (Figure 1.3). Using [^3H]-mannose pulse-labeling, Lederkremer's group showed that

asialoglycoprotein receptor H2a N-glycans accumulated in $\text{Man}_5\text{GlcNAc}_2$ and $\text{Man}_6\text{GlcNAc}_2$ forms when proteasomal proteolysis was inhibited with MG-132. When the mannosidase inhibitor deoxymannojirimycin (DMJ) was added during the chase, the N-glycan forms shifted to those with more mannose residues, namely $\text{Man}_{6-9}\text{GlcNAc}_2$, and degradation of H2a was inhibited. These effects were not seen with swainsinone, an inhibitor of class II mannosidases (such as ER and Golgi mannosidase II); thus, the authors postulated that endogenous ERmanI activity involves cleavage beyond the $\text{Man}_8\text{GlcNAc}_2$ form, all the way to $\text{Man}_5\text{GlcNAc}_2$, and that these further mannose cleavage events are required for efficient ERAD[109].

Based on in vitro studies[110, 111], it was not thought that ERmanI at physiological concentrations could cleave beyond the terminal B-chain mannose[48, 112, 113], but it was shown that ERmanI could cleave to the $\text{Man}_5\text{GlcNAc}_2$ form at high concentrations[114]. Indeed, it has been proposed that ERmanI is concentrated in a pericentriolar compartment called the ER quality control (ERQC) compartment, where it may reach a high enough concentration in vivo to cleave additional mannoses beyond the $\text{Man}_8\text{GlcNAc}_2$ form[109]. Characterization of the ERQC is in the early stages, but results do indicate its involvement in degradation of misfolded ER proteins by both ERAD and autophagy. First, when proteasomal proteolysis is inhibited by lactacystin treatment, the ERAD substrate H2a accumulates in the ERQC[109]. Second, when lysosomal degradation—and therefore autophagy—is inhibited by treatment with leupeptin, the lysosomal marker Lamp1 accumulates in the ERQC[109].

Intriguingly, with lactacystin treatment, CNX and CRT become concentrated in the ERQC compartment, while UGGT1 does not[115]. The significance of this finding is also unclear, but may indicate that once glycoproteins enter the ERQC compartment, UGGT1-mediated reglucosylation no longer has a major role in their eventual fate.

1.3.6 Insoluble protein aggregates accumulate in cytoplasmic compartments

If ER glycoproteins are not delivered to the Golgi or degraded through ERAD, they become especially susceptible to aggregation. These aggregates are often insoluble in nonionic detergent lysis buffers, such as 1% Triton X-100[116, 117]. Detergent insolubility has been observed for multiple mutant glycoproteins, and indeed such protein aggregation is thought to play a role in the pathogenesis of numerous diseases[118-122] such as neurodegenerative diseases and the liver disease associated with alpha-1 antitrypsin deficiency[117]. Thus, the mechanism(s) by which cells compartmentalize, degrade, and may potentially resolubilize insoluble aggregates is a critical area of medical research. Recent findings indicate that cells manage cytoplasmic and ER aggregates in distinct ways, but there is also evidence that these two pathways may intermingle[117].

In the cytoplasm, a new paradigm for managing misfolded proteins and protein aggregates has recently emerged. Briefly, misfolded proteins are ubiquitinated and targeted to the juxtannuclear QC compartment (JUNQ), a dynamic compartment that rapidly exchanges its contents with the cytosol. Alternatively, misfolded proteins can also be transported to insoluble protein deposits (IPODs), which tend to store insoluble protein aggregates at the cell periphery [117], and may be a site for misfolded proteins that overflow when their accumulation exceeds capacity of the JUNQ compartment. Proteins localized to JUNQ or IPODs are sensitive or resistant to trypsin digestion, respectively — supporting the idea that IPODs are sites where aggregated proteins are delivered[123]. The main route of degradation from the JUNQ compartment is through the ubiquitin-proteasome system (UPS), whereas insoluble aggregates in IPODs are thought to be degraded by autophagy (Atg8, the yeast homolog of the mammalian autophagy marker LC3, localizes to IPODs)[117]. The JUNQ compartment shares many characteristics with another cytoplasmic structure called the ‘aggresome’, which also exhibits juxtannuclear localization, ubiquitinated

proteins, proteasomes, and microtubular transport of incoming proteins[116, 117, 123, 124]. It seems that JUNQ and aggresomes may be different structures, as aggresomes colocalize with spindle pole bodies, while JUNQ does not[117].

The ER quality control (ERQC) system may help transport soluble or insoluble ER glycoproteins to similar or related cytoplasmic compartments, and the mechanism may be different for luminal and transmembrane ER glycoproteins (Figure 1.4). For example, cystic fibrosis transmembrane conductance regulator (CFTR, which has difficulty in anterograde transport from the ER) can accumulate in aggresomes[124], presumably after dislocation from the ER membrane, but there are no reports of luminal ER proteins accumulating in aggresomes. Rather, insoluble aggregates of the ER luminal glycoprotein fibronectin may be transported out of the ER through a vesicular system, for eventual lysosomal degradation. These ER-derived vesicles are distinct from COPII coated vesicles used for ER-to-Golgi transport[125, 126]. They also contain high amounts of EDEM1 and may be related to vesicles termed 'EDEMosomes', involved in degradation of ERAD components (ERAD tuning)[127]. Many questions remain regarding the ERQC of misfolded soluble and insoluble glycoproteins, but it is generally thought that soluble and insoluble glycoproteins are processed differently (Figure 1.4). For example, mutant alpha-1 antitrypsin Z allele (ATZ) is thought accumulate in the ER in both soluble and insoluble forms that are degraded by either the UPS or autophagy, respectively[128]. Insoluble forms of ER entrapped proteins are often described to accumulate in the ER-associated compartment (ERAC)[129, 130]. As an example, eGFP-tagged CFTR accumulates in both soluble and insoluble pools, and both localize to the ERAC. Whereas the soluble pool is degraded through the UPS, the insoluble pool is degraded by autophagy[129]. ERACs also have a juxtannuclear localization; some substrates are delivered and localized exclusively to the ERAC and cannot be found in the rest of the ER[130]. ERACs are proposed to promote ER homeostasis by partitioning aggregated (presumably non-functional, possibly toxic) proteins away from the rest of the ER[129]. Similar

to cytoplasmic aggresomes, microtubules are necessary for trafficking of substrate proteins to the ERAC[131]. Experimentally, immunofluorescent staining for the translocon component Sec63 can be used to identify the ERAC compartment[130]. Presently, experimental results do not clearly define the ERAC and another hypothesized 'ERQC compartment' as distinct compartments, therefore these two compartments will be termed ERQC/ ERAC for the remainder of this review (Figure 1.4).

Sequestrations of misfolded proteins in subregions of the ER (ERQC/ ERAC compartment) have also been called Russel bodies, a form of inclusion body[129, 131, 132] that may be either electron-dense or electron-lucent, depending upon the misfolded substrate contained within them[133]. Again, it is unclear whether these unique names reflect unique structures, or if there is overlap between them. Classically, Russel bodies are filled with protein aggregates of misfolded immunoglobulin along with certain ER resident proteins such as CNX, whereas others (BiP, PDI, ERp72) are excluded, suggesting selective protein entry into, or exclusion from, this compartment[132]. As CNX is thought to interact with glycoprotein substrates, its inclusion in Russel bodies may signify an activity to limit, or even reverse, glycoprotein aggregation. Thus, in future work, it will be important to examine and understand the effects of N-linked glycoprotein processing on the aggregation of misfolded secretory glycoprotein substrates.

1.4 Conclusion

N-linked glycosylation of proteins in the ER initiates entry into a complex glycoprotein-specific ERQC system that maintains the fidelity of the secretory and transmembrane proteome. The effectiveness of the ERQC is quite sensitive to glycoproteins with even slight amino acid substitutions, targeting them for ERAD (despite that they may still have the potential to contribute a measure of bioactivity). The CNX cycle figures prominently into glycoprotein ERQC, with

UGGT1, the reglucosylating enzyme serving as a primary folding sensor to mediate downstream consequences for non-native glycoprotein folding forms. How the glycoprotein ERQC system functions to degrade terminally misfolded glycoproteins, and what happens when these degradation pathways are overwhelmed are key questions for future exploration. Although many of the components of glycoprotein ERQC have been identified, the recent discovery of new proteins (e.g., malectin) makes it likely that additional glycoprotein ERQC components remain to be identified. Our understanding of the mechanistic details of glycoprotein ERQC will help to expose therapeutic targets for the ever-increasing list of ER storage diseases[2].

1.5 Figures

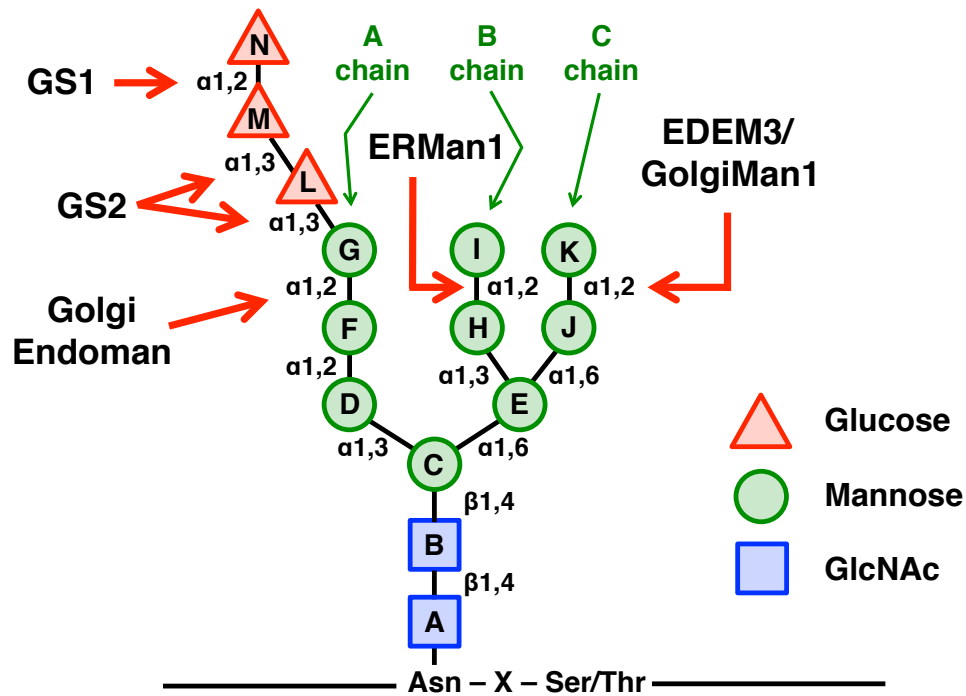


Figure 1.1 Structure of N-linked glycans

N-linked oligosaccharides are assembled on the ER membrane, and then transferred en bloc to Asn side chains of newly translocated proteins harboring consensus N-glycosylation sites (Asn-X(any amino acid except proline)-Ser/Thr). The 14-sugar form, starting from the Asn residue, contains two N-acetylglucosamines (GlcNAc, squares), nine mannoses (circles), and three glucoses that are all on the A-chain branch of the triantennary mannose structure (triangles). Glycosidic bond types are indicated, as are ER and Golgi localized glycosidases responsible for trimming the N-glycan to smaller forms.

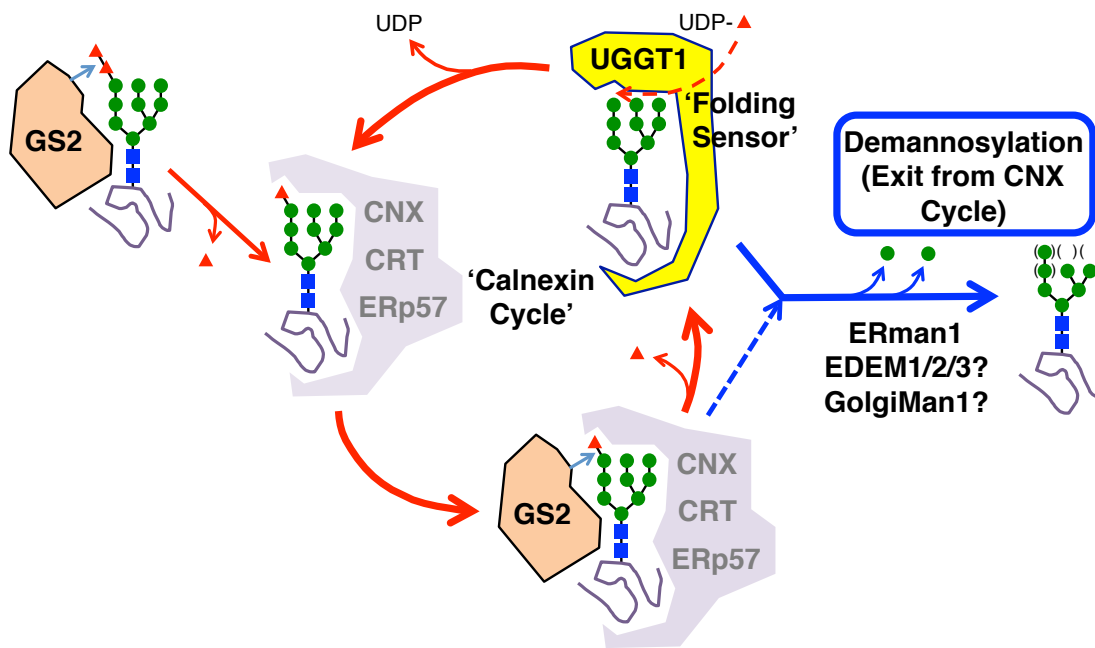


Figure 1.2 N-glycan processing in the calnexin cycle

Glycoproteins first enter the calnexin cycle when the penultimate glucose of the attached N-glycan is cleaved by glucosidase II (GS2). The resulting monoglucosylated N-glycan can serve as a high-affinity substrate for the lectin-like chaperones calnexin (CNX) and calreticulin (CRT) the associated oxidoreductase ERp57. The ability of the substrate to re-associate with the lectin-like chaperones is terminated when GS2 acts to remove the terminal glucose residue from the N-glycan. At this point, the glycoprotein substrate's folding status is surveyed by the 'folding sensor' component of the calnexin cycle, UDP-glucose:glycoprotein glucosyltransferase (UGGT1). UGGT1 reglucosylates nearly-native folding forms of glycoprotein substrates, converting them again into high-affinity substrate for CNX/CRT, and therefore allowing for another round (and potentially, multiple rounds) of CNX/CRT association. Substrates are eventually released from the calnexin cycle upon demannosylation of N-glycans. The mechanism for permanent exit from the cycle involves either termination of UGGT1 reglucosylation activity of demannosylated N-glycans, or active recognition of demannosylated forms by ER exit machinery or ERAD components.

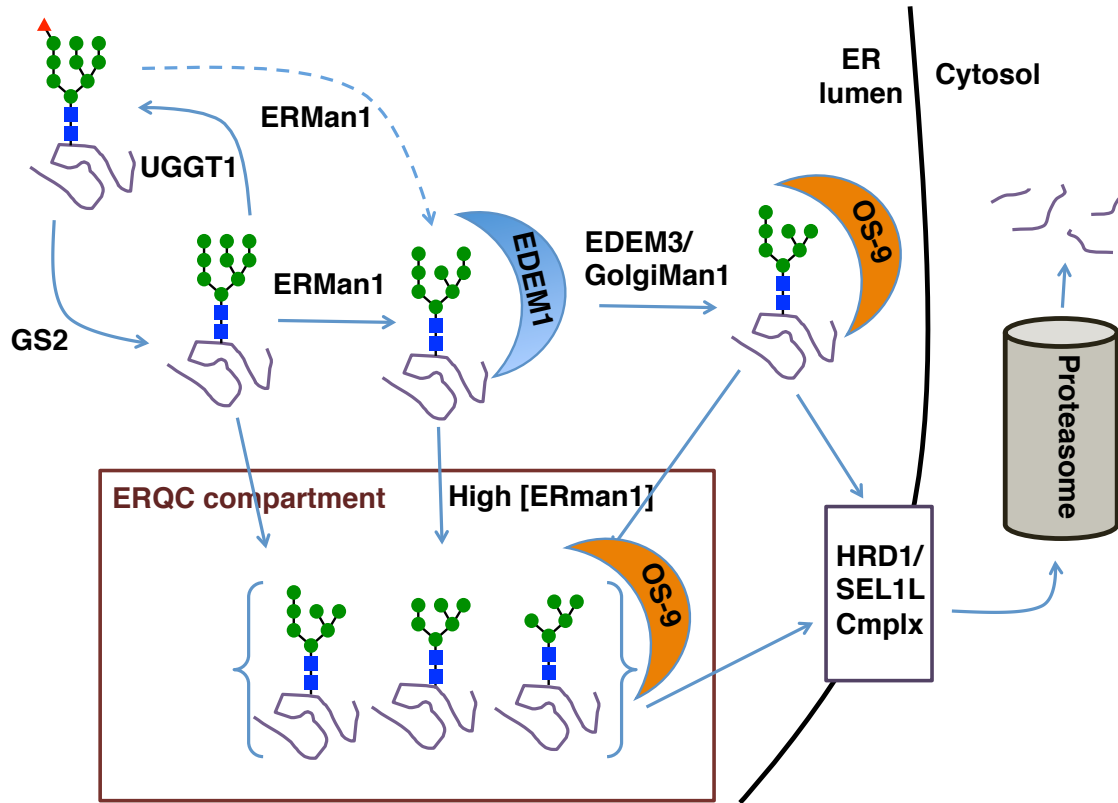


Figure 1.3 Glycoprotein ERAD

Degradation of terminally misfolded glycoproteins through ERAD is likely initiated by cleavage of the terminal B-chain mannose of Man₉GlcNAc₂ (and possibly Glc₁Man₉GlcNAc₂) N-glycan forms by ER mannosidase I (ERman1). This results in formation and recognition of this specific Man₈GlcNAc₂ (M8B) form by ER degradation enhancing alpha-mannosidase-like 1 (EDEM1). Then, removal of the terminal C-chain mannose — either: 1) directly by EDEM3 or possibly Golgi mannosidase I, or 2) by highly concentrated ERman1 in the ERQC compartment — exposes an alpha1-6 linked mannose that is recognized by OS-9. OS-9 facilitates transport of the misfolded substrate to the core ERAD HRD1/SEL1L complex, and subsequent retrotranslocation to the cytoplasm for degradation by the 26S proteasome.

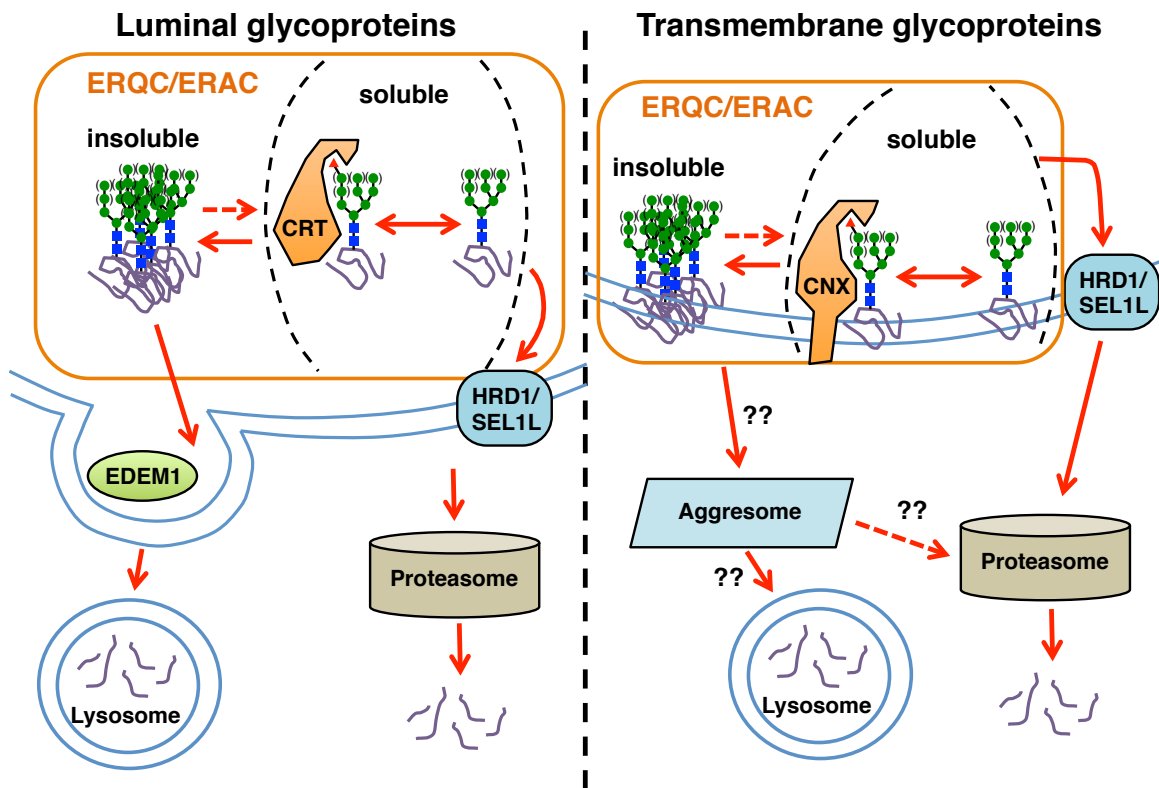


Figure 1.4 ERQC of insoluble glycoproteins

Some misfolded glycoproteins are known to form insoluble aggregates or ordered polymers in the ER, and UGGT1-mediated modification of glycosylation status (monoglucosylated or unglucosylated) may play a role to limit insolubility. The soluble-to-insoluble transition may be related to compartmentalization in the ERQC/ERAC compartment, and insoluble substrates may also be resolubilized. Soluble forms of both luminal and transmembrane glycoproteins tend to be degraded through ERAD, while insoluble forms tend to be degraded via autophagy, although the mechanism by which insoluble ER proteins get to the lysosome is not entirely clear. Luminal insoluble glycoproteins may be packaged into EDEM1-containing vesicles (EDEMosomes) and transported to the lysosome. Transmembrane insoluble glycoproteins could accumulate in the aggresome and then be degraded by the proteasome, or targeted by an unknown mechanism to the lysosome for degradation.

1.6 References

1. Welch, W.J., *Role of quality control pathways in human diseases involving protein misfolding*. Semin Cell Dev Biol, 2004. **15**(1): p. 31-8.
2. Kim, P.S. and P. Arvan, *Endocrinopathies in the family of endoplasmic reticulum (ER) storage diseases: disorders of protein trafficking and the role of ER molecular chaperones*. Endocr Rev, 1998. **19**(2): p. 173-202.
3. Bhargava, A., et al., *Osteopetrosis mutation R444L causes endoplasmic reticulum retention and misprocessing of vacuolar H⁺-ATPase α 3 subunit*. J Biol Chem, 2012. **287**(32): p. 26829-39.
4. Drumm, M.L., et al., *Chloride conductance expressed by delta F508 and other mutant CFTRs in Xenopus oocytes*. Science, 1991. **254**(5039): p. 1797-9.
5. de Silva, A.M., W.E. Balch, and A. Helenius, *Quality control in the endoplasmic reticulum: folding and misfolding of vesicular stomatitis virus G protein in cells and in vitro*. J Cell Biol, 1990. **111**(3): p. 857-66.
6. Balch, W.E., et al., *Adapting proteostasis for disease intervention*. Science, 2008. **319**(5865): p. 916-9.
7. Apweiler, R., H. Hermjakob, and N. Sharon, *On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database*. Biochim Biophys Acta, 1999. **1473**(1): p. 4-8.
8. Roitsch, T. and L. Lehle, *Expression of yeast invertase in oocytes from Xenopus laevis. Secretion of active enzyme differing in glycosylation*. Eur J Biochem, 1989. **181**(3): p. 733-9.
9. Ellis, R.J. and S.M. Hemmingsen, *Molecular chaperones: proteins essential for the biogenesis of some macromolecular structures*. Trends Biochem Sci, 1989. **14**(8): p. 339-42.
10. Ellis, R.J., *Molecular chaperones: assisting assembly in addition to folding*. Trends Biochem Sci, 2006. **31**(7): p. 395-401.
11. Schroder, M. and R.J. Kaufman, *Divergent roles of IRE1 α and PERK in the unfolded protein response*. Curr Mol Med, 2006. **6**(1): p. 5-36.
12. Schroder, M. and R.J. Kaufman, *The mammalian unfolded protein response*. Annu Rev Biochem, 2005. **74**: p. 739-89.
13. Malhotra, J.D. and R.J. Kaufman, *The endoplasmic reticulum and the unfolded protein response*. Semin Cell Dev Biol, 2007. **18**(6): p. 716-31.
14. Olivari, S. and M. Molinari, *Glycoprotein folding and the role of EDEM1, EDEM2 and EDEM3 in degradation of folding-defective glycoproteins*. FEBS Lett, 2007. **581**(19): p. 3658-64.
15. Gething, M.J., *Role and regulation of the ER chaperone BiP*. Semin Cell Dev Biol, 1999. **10**(5): p. 465-72.
16. Kim, P.S. and P. Arvan, *Calnexin and BiP act as sequential molecular chaperones during thyroglobulin folding in the endoplasmic reticulum*. J Cell Biol, 1995. **128**(1-2): p. 29-38.

17. Molinari, M., et al., *Sequential assistance of molecular chaperones and transient formation of covalent complexes during protein degradation from the ER*. J Cell Biol, 2002. **158**(2): p. 247-57.
18. Molinari, M. and A. Helenius, *Chaperone selection during glycoprotein translocation into the endoplasmic reticulum*. Science, 2000. **288**(5464): p. 331-3.
19. Larkin, A. and B. Imperiali, *The expanding horizons of asparagine-linked glycosylation*. Biochemistry, 2011. **50**(21): p. 4411-26.
20. Parodi, A.J., G.Z. Lederkremer, and D.H. Mendelzon, *Protein glycosylation in Trypanosoma cruzi. The mechanism of glycosylation and structure of protein-bound oligosaccharides*. J Biol Chem, 1983. **258**(9): p. 5589-95.
21. Burda, P. and M. Aebi, *The dolichol pathway of N-linked glycosylation*. Biochim Biophys Acta, 1999. **1426**(2): p. 239-57.
22. Wacker, M., et al., *N-linked glycosylation in Campylobacter jejuni and its functional transfer into E. coli*. Science, 2002. **298**(5599): p. 1790-3.
23. Kelleher, D.J., et al., *Oligosaccharyltransferase isoforms that contain different catalytic STT3 subunits have distinct enzymatic properties*. Mol Cell, 2003. **12**(1): p. 101-11.
24. Roboti, P. and S. High, *The oligosaccharyltransferase subunits OST48, DAD1 and KCP2 function as ubiquitous and selective modulators of mammalian N-glycosylation*. J Cell Sci, 2012. **125**(Pt 14): p. 3474-84.
25. Wilson, C.M. and S. High, *Ribophorin I acts as a substrate-specific facilitator of N-glycosylation*. J Cell Sci, 2007. **120**(Pt 4): p. 648-57.
26. Rothman, J.E. and H.F. Lodish, *Synchronised transmembrane insertion and glycosylation of a nascent membrane protein*. Nature, 1977. **269**(5631): p. 775-80.
27. Bergman, L.W. and W.M. Kuehl, *Co-translational modification of nascent immunoglobulin heavy and light chains*. J Supramol Struct, 1979. **11**(1): p. 9-24.
28. Aebi, M., et al., *N-glycan structures: recognition and processing in the ER*. Trends Biochem Sci, 2010. **35**(2): p. 74-82.
29. Nilsson, I.M. and G. von Heijne, *Determination of the distance between the oligosaccharyltransferase active site and the endoplasmic reticulum membrane*. J Biol Chem, 1993. **268**(8): p. 5798-801.
30. Lizak, C., et al., *X-ray structure of a bacterial oligosaccharyltransferase*. Nature, 2011. **474**(7351): p. 350-5.
31. Bolt, G., C. Kristensen, and T.D. Steenstrup, *Posttranslational N-glycosylation takes place during the normal processing of human coagulation factor VII*. Glycobiology, 2005. **15**(5): p. 541-7.
32. Geiger, R., et al., *Folding, quality control, and secretion of pancreatic ribonuclease in live cells*. J Biol Chem, 2011. **286**(7): p. 5813-22.
33. Zielinska, D.F., et al., *Precision mapping of an in vivo N-glycoproteome reveals rigid topological and sequence constraints*. Cell, 2010. **141**(5): p. 897-907.

34. Varki, A., *Essentials of glycobiology*, 1999, Cold Spring Harbor Laboratory Press, [Bethesda National Center for Biotechnology Information]: Cold Spring Harbor, NY.
35. Watowich, S.S. and R.I. Morimoto, *Complex regulation of heat shock- and glucose-responsive genes in human cells*. *Mol Cell Biol*, 1988. **8**(1): p. 393-405.
36. Dorner, A.J., et al., *The stress response in Chinese hamster ovary cells. Regulation of ERp72 and protein disulfide isomerase expression and secretion*. *J Biol Chem*, 1990. **265**(35): p. 22029-34.
37. Dorner, A.J., D.G. Bole, and R.J. Kaufman, *The relationship of N-linked glycosylation and heavy chain-binding protein association with the secretion of glycoproteins*. *J Cell Biol*, 1987. **105**(6 Pt 1): p. 2665-74.
38. Trombetta, E.S., *The contribution of N-glycans and their processing in the endoplasmic reticulum to glycoprotein biosynthesis*. *Glycobiology*, 2003. **13**(9): p. 77R-91R.
39. Wang, C., et al., *Influence of the carbohydrate moiety on the stability of glycoproteins*. *Biochemistry*, 1996. **35**(23): p. 7299-307.
40. Schulke, N. and F.X. Schmid, *The stability of yeast invertase is not significantly influenced by glycosylation*. *J Biol Chem*, 1988. **263**(18): p. 8827-31.
41. Bergh, M.L., et al., *Expression of the Saccharomyces cerevisiae glycoprotein invertase in mouse fibroblasts: glycosylation, secretion, and enzymatic activity*. *Proc Natl Acad Sci U S A*, 1987. **84**(11): p. 3570-4.
42. Feldman, R.I., M. Bernstein, and R. Schekman, *Product of SEC53 is required for folding and glycosylation of secretory proteins in the lumen of the yeast endoplasmic reticulum*. *J Biol Chem*, 1987. **262**(19): p. 9332-9.
43. An, S.J., et al., *Analysis of the association of syncollin with the membrane of the pancreatic zymogen granule*. *J Biol Chem*, 2000. **275**(15): p. 11306-11.
44. Kopper, R.A., et al., *Peanut protein allergens: the effect of roasting on solubility and allergenicity*. *Int Arch Allergy Immunol*, 2005. **136**(1): p. 16-22.
45. Hubbard, S.C. and P.W. Robbins, *Synthesis and processing of protein-linked oligosaccharides in vivo*. *J Biol Chem*, 1979. **254**(11): p. 4568-76.
46. Volker, C., et al., *Processing of N-linked carbohydrate chains in a patient with glucosidase I deficiency (CDG type IIb)*. *Glycobiology*, 2002. **12**(8): p. 473-83.
47. De Praeter, C.M., et al., *A novel disorder caused by defective biosynthesis of N-linked oligosaccharides due to glucosidase I deficiency*. *Am J Hum Genet*, 2000. **66**(6): p. 1744-56.
48. Helenius, A. and M. Aebi, *Roles of N-linked glycans in the endoplasmic reticulum*. *Annu Rev Biochem*, 2004. **73**: p. 1019-49.

49. Schallus, T., et al., *Malectin: a novel carbohydrate-binding protein of the endoplasmic reticulum and a candidate player in the early steps of protein N-glycosylation*. Mol Biol Cell, 2008. **19**(8): p. 3404-14.
50. Galli, C., et al., *Malectin participates in a backup glycoprotein quality control pathway in the mammalian ER*. PLoS One, 2011. **6**(1): p. e16304.
51. Chen, Y., et al., *Role of malectin in Glc(2)Man(9)GlcNAc(2)-dependent quality control of alpha1-antitrypsin*. Mol Biol Cell, 2011. **22**(19): p. 3559-70.
52. Reitman, M.L., I.S. Trowbridge, and S. Kornfeld, *A lectin-resistant mouse lymphoma cell line is deficient in glucosidase II, a glycoprotein-processing enzyme*. J Biol Chem, 1982. **257**(17): p. 10357-63.
53. Grinna, L.S. and P.W. Robbins, *Glycoprotein biosynthesis. Rat liver microsomal glucosidases which process oligosaccharides*. J Biol Chem, 1979. **254**(18): p. 8814-8.
54. Kornfeld, S., E. Li, and I. Tabas, *The synthesis of complex-type oligosaccharides. II. Characterization of the processing intermediates in the synthesis of the complex oligosaccharide units of the vesicular stomatitis virus G protein*. J Biol Chem, 1978. **253**(21): p. 7771-8.
55. Trombetta, E.S., J.F. Simons, and A. Helenius, *Endoplasmic reticulum glucosidase II is composed of a catalytic subunit, conserved from yeast to mammals, and a tightly bound noncatalytic HDEL-containing subunit*. J Biol Chem, 1996. **271**(44): p. 27509-16.
56. Drenth, J.P., et al., *Germline mutations in PRKCSH are associated with autosomal dominant polycystic liver disease*. Nat Genet, 2003. **33**(3): p. 345-7.
57. Li, A., et al., *Mutations in PRKCSH cause isolated autosomal dominant polycystic liver disease*. Am J Hum Genet, 2003. **72**(3): p. 691-703.
58. Peyrieras, N., et al., *Effects of the glucosidase inhibitors nojirimycin and deoxynojirimycin on the biosynthesis of membrane and secretory glycoproteins*. EMBO J, 1983. **2**(6): p. 823-32.
59. Lodish, H.F. and N. Kong, *Glucose removal from N-linked oligosaccharides is required for efficient maturation of certain secretory glycoproteins from the rough endoplasmic reticulum to the Golgi complex*. J Cell Biol, 1984. **98**(5): p. 1720-9.
60. Hebert, D.N., B. Foellmer, and A. Helenius, *Calnexin and calreticulin promote folding, delay oligomerization and suppress degradation of influenza hemagglutinin in microsomes*. EMBO J, 1996. **15**(12): p. 2961-8.
61. D'Alessio, C., et al., *Genetic evidence for the heterodimeric structure of glucosidase II. The effect of disrupting the subunit-encoding genes on glycoprotein folding*. J Biol Chem, 1999. **274**(36): p. 25899-905.
62. Lin, Z.P., et al., *Prevention of brefeldin A-induced resistance to teniposide by the proteasome inhibitor MG-132: involvement of NF-kappaB activation in drug resistance*. Cancer Res, 1998. **58**(14): p. 3059-65.

63. Hammond, C., I. Braakman, and A. Helenius, *Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control*. Proc Natl Acad Sci U S A, 1994. **91**(3): p. 913-7.
64. Ostwald, T.J. and D.H. MacLennan, *Isolation of a high affinity calcium-binding protein from sarcoplasmic reticulum*. J Biol Chem, 1974. **249**(3): p. 974-9.
65. Michalak, M., et al., *Calreticulin*. Biochem J, 1992. **285 (Pt 3)**: p. 681-92.
66. Nigam, S.K., et al., *A set of endoplasmic reticulum proteins possessing properties of molecular chaperones includes Ca(2+)-binding proteins and members of the thioredoxin superfamily*. J Biol Chem, 1994. **269**(3): p. 1744-9.
67. Wada, I., et al., *SSR alpha and associated calnexin are major calcium binding proteins of the endoplasmic reticulum membrane*. J Biol Chem, 1991. **266**(29): p. 19599-610.
68. David, V., et al., *Interaction with newly synthesized and retained proteins in the endoplasmic reticulum suggests a chaperone function for human integral membrane protein IP90 (calnexin)*. J Biol Chem, 1993. **268**(13): p. 9585-92.
69. Galvin, K., et al., *The major histocompatibility complex class I antigen-binding protein p88 is the product of the calnexin gene*. Proc Natl Acad Sci U S A, 1992. **89**(18): p. 8452-6.
70. Ou, W.J., et al., *Association of folding intermediates of glycoproteins with calnexin during protein maturation*. Nature, 1993. **364**(6440): p. 771-6.
71. Trombetta, E.S. and A.J. Parodi, *Quality control and protein folding in the secretory pathway*. Annu Rev Cell Dev Biol, 2003. **19**: p. 649-76.
72. Molinari, M. and A. Helenius, *Glycoproteins form mixed disulphides with oxidoreductases during folding in living cells*. Nature, 1999. **402**(6757): p. 90-3.
73. Zapun, A., et al., *Enhanced catalysis of ribonuclease B folding by the interaction of calnexin or calreticulin with ERp57*. J Biol Chem, 1998. **273**(11): p. 6009-12.
74. Parodi, A.J. and L.A. Quesada-Allue, *Protein glycosylation in Trypanosoma cruzi. I. Characterization of dolichol-bound monosaccharides and oligosaccharides synthesized "in vivo"*. J Biol Chem, 1982. **257**(13): p. 7637-40.
75. Parodi, A.J. and J.J. Cazzulo, *Protein glycosylation in Trypanosoma cruzi. II. Partial characterization of protein-bound oligosaccharides labeled "in vivo"*. J Biol Chem, 1982. **257**(13): p. 7641-5.
76. Perez, M. and C.B. Hirschberg, *Topography of glycosylation reactions in the rough endoplasmic reticulum membrane*. J Biol Chem, 1986. **261**(15): p. 6822-30.

77. Trombetta, S.E., M. Bosch, and A.J. Parodi, *Glucosylation of glycoproteins by mammalian, plant, fungal, and trypanosomatid protozoa microsomal membranes*. *Biochemistry*, 1989. **28**(20): p. 8108-16.
78. Sousa, M.C., M.A. Ferrero-Garcia, and A.J. Parodi, *Recognition of the oligosaccharide and protein moieties of glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase*. *Biochemistry*, 1992. **31**(1): p. 97-105.
79. Fanchiotti, S., et al., *The UDP-Glc:Glycoprotein glucosyltransferase is essential for Schizosaccharomyces pombe viability under conditions of extreme endoplasmic reticulum stress*. *J Cell Biol*, 1998. **143**(3): p. 625-35.
80. Sousa, M. and A.J. Parodi, *The molecular basis for the recognition of misfolded glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase*. *EMBO J*, 1995. **14**(17): p. 4196-203.
81. Trombetta, S.E. and A.J. Parodi, *Purification to apparent homogeneity and partial characterization of rat liver UDP-glucose:glycoprotein glucosyltransferase*. *J Biol Chem*, 1992. **267**(13): p. 9236-40.
82. Ritter, C. and A. Helenius, *Recognition of local glycoprotein misfolding by the ER folding sensor UDP-glucose:glycoprotein glucosyltransferase*. *Nat Struct Biol*, 2000. **7**(4): p. 278-80.
83. Taylor, S.C., et al., *The ER protein folding sensor UDP-glucose glycoprotein-glucosyltransferase modifies substrates distant to local changes in glycoprotein conformation*. *Nat Struct Mol Biol*, 2004. **11**(2): p. 128-34.
84. Labriola, C., J.J. Cazzulo, and A.J. Parodi, *Retention of glucose units added by the UDP-GLC:glycoprotein glucosyltransferase delays exit of glycoproteins from the endoplasmic reticulum*. *J Cell Biol*, 1995. **130**(4): p. 771-9.
85. Ruddock, L.W. and M. Molinari, *N-glycan processing in ER quality control*. *J Cell Sci*, 2006. **119**(Pt 21): p. 4373-80.
86. Moremen, K.W., *Golgi alpha-mannosidase II deficiency in vertebrate systems: implications for asparagine-linked oligosaccharide processing in mammals*. *Biochim Biophys Acta*, 2002. **1573**(3): p. 225-35.
87. Gabel, C.A. and J.E. Bergmann, *Processing of the asparagine-linked oligosaccharides of secreted and intracellular forms of the vesicular stomatitis virus G protein: in vivo evidence of Golgi apparatus compartmentalization*. *J Cell Biol*, 1985. **101**(2): p. 460-9.
88. Arvan, P., et al., *Secretory pathway quality control operating in Golgi, plasmalemmal, and endosomal systems*. *Traffic*, 2002. **3**(11): p. 771-80.
89. Daniel, P.F., B. Winchester, and C.D. Warren, *Mammalian alpha-mannosidases--multiple forms but a common purpose?* *Glycobiology*, 1994. **4**(5): p. 551-66.
90. Warren, G. and I. Mellman, *Bulk flow redux?* *Cell*, 1999. **98**(2): p. 125-7.

91. Appenzeller, C., et al., *The lectin ERGIC-53 is a cargo transport receptor for glycoproteins*. Nat Cell Biol, 1999. **1**(6): p. 330-4.
92. Appenzeller-Herzog, C., et al., *Carbohydrate- and conformation-dependent cargo capture for ER-exit*. Mol Biol Cell, 2005. **16**(3): p. 1258-67.
93. Kamiya, Y., et al., *Molecular basis of sugar recognition by the human L-type lectins ERGIC-53, VIPL, and VIP36*. J Biol Chem, 2008. **283**(4): p. 1857-61.
94. Zhang, B., et al., *Bleeding due to disruption of a cargo-specific ER-to-Golgi transport complex*. Nat Genet, 2003. **34**(2): p. 220-5.
95. Kabcenell, A.K. and P.H. Atkinson, *Processing of the rough endoplasmic reticulum membrane glycoproteins of rotavirus SA11*. J Cell Biol, 1985. **101**(4): p. 1270-80.
96. Suh, K., J.E. Bergmann, and C.A. Gabel, *Selective retention of monoglucosylated high mannose oligosaccharides by a class of mutant vesicular stomatitis virus G proteins*. J Cell Biol, 1989. **108**(3): p. 811-9.
97. Jakob, C.A., et al., *Degradation of misfolded endoplasmic reticulum glycoproteins in Saccharomyces cerevisiae is determined by a specific oligosaccharide structure*. J Cell Biol, 1998. **142**(5): p. 1223-33.
98. Tokunaga, F., et al., *Endoplasmic reticulum (ER)-associated degradation of misfolded N-linked glycoproteins is suppressed upon inhibition of ER mannosidase I*. J Biol Chem, 2000. **275**(52): p. 40757-64.
99. Hoseki, J., R. Ushioda, and K. Nagata, *Mechanism and components of endoplasmic reticulum-associated degradation*. J Biochem, 2010. **147**(1): p. 19-25.
100. Molinari, M., et al., *Role of EDEM in the release of misfolded glycoproteins from the calnexin cycle*. Science, 2003. **299**(5611): p. 1397-400.
101. Oda, Y., et al., *EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin*. Science, 2003. **299**(5611): p. 1394-7.
102. Christianson, J.C., et al., *OS-9 and GRP94 deliver mutant alpha1-antitrypsin to the Hrd1-SEL1L ubiquitin ligase complex for ERAD*. Nat Cell Biol, 2008. **10**(3): p. 272-82.
103. Hosokawa, N., et al., *Human OS-9, a lectin required for glycoprotein endoplasmic reticulum-associated degradation, recognizes mannose-trimmed N-glycans*. J Biol Chem, 2009. **284**(25): p. 17061-8.
104. Caldwell, S.R., K.J. Hill, and A.A. Cooper, *Degradation of endoplasmic reticulum (ER) quality control substrates requires transport between the ER and Golgi*. J Biol Chem, 2001. **276**(26): p. 23296-303.
105. Fagioli, C. and R. Sitia, *Glycoprotein quality control in the endoplasmic reticulum. Mannose trimming by endoplasmic reticulum mannosidase I times the proteasomal degradation of unassembled immunoglobulin subunits*. J Biol Chem, 2001. **276**(16): p. 12885-92.

106. Stigliano, I.D., et al., *Glucosidase II and N-glycan mannose content regulate the half-lives of monoglucosylated species in vivo*. Mol Biol Cell, 2011. **22**(11): p. 1810-23.
107. Satoh, T., et al., *Structural basis for oligosaccharide recognition of misfolded glycoproteins by OS-9 in ER-associated degradation*. Mol Cell, 2010. **40**(6): p. 905-16.
108. Liu, Y., et al., *Oligosaccharide modification in the early secretory pathway directs the selection of a misfolded glycoprotein for degradation by the proteasome*. J Biol Chem, 1999. **274**(9): p. 5861-7.
109. Avezov, E., et al., *Endoplasmic reticulum (ER) mannosidase I is compartmentalized and required for N-glycan trimming to Man5-6GlcNAc2 in glycoprotein ER-associated degradation*. Mol Biol Cell, 2008. **19**(1): p. 216-25.
110. Tremblay, L.O. and A. Herscovics, *Cloning and expression of a specific human alpha 1,2-mannosidase that trims Man9GlcNAc2 to Man8GlcNAc2 isomer B during N-glycan biosynthesis*. Glycobiology, 1999. **9**(10): p. 1073-8.
111. Herscovics, A., *Structure and function of Class I alpha 1,2-mannosidases involved in glycoprotein synthesis and endoplasmic reticulum quality control*. Biochimie, 2001. **83**(8): p. 757-62.
112. Cabral, C.M., Y. Liu, and R.N. Sifers, *Dissecting glycoprotein quality control in the secretory pathway*. Trends Biochem Sci, 2001. **26**(10): p. 619-24.
113. Vallee, F., et al., *Structural basis for catalysis and inhibition of N-glycan processing class I alpha 1,2-mannosidases*. J Biol Chem, 2000. **275**(52): p. 41287-98.
114. Herscovics, A., P.A. Romero, and L.O. Tremblay, *The specificity of the yeast and human class I ER alpha 1,2-mannosidases involved in ER quality control is not as strict previously reported*. Glycobiology, 2002. **12**(4): p. 14G-15G.
115. Kamhi-Nesher, S., et al., *A novel quality control compartment derived from the endoplasmic reticulum*. Mol Biol Cell, 2001. **12**(6): p. 1711-23.
116. Buchberger, A., B. Bukau, and T. Sommer, *Protein quality control in the cytosol and the endoplasmic reticulum: brothers in arms*. Mol Cell, 2010. **40**(2): p. 238-52.
117. Kaganovich, D., R. Kopito, and J. Frydman, *Misfolded proteins partition between two distinct quality control compartments*. Nature, 2008. **454**(7208): p. 1088-95.
118. Perlmutter, D.H., *Autophagic disposal of the aggregation-prone protein that causes liver inflammation and carcinogenesis in alpha-1-antitrypsin deficiency*. Cell Death Differ, 2009. **16**(1): p. 39-45.
119. Kroeger, H., et al., *Endoplasmic reticulum-associated degradation (ERAD) and autophagy cooperate to degrade polymerogenic mutant serpins*. J Biol Chem, 2009. **284**(34): p. 22793-802.

120. Rutishauser, J. and M. Spiess, *Endoplasmic reticulum storage diseases*. Swiss Med Wkly, 2002. **132**(17-18): p. 211-22.
121. Kruse, K.B., et al., *Mutant fibrinogen cleared from the endoplasmic reticulum via endoplasmic reticulum-associated protein degradation and autophagy: an explanation for liver disease*. Am J Pathol, 2006. **168**(4): p. 1299-308; quiz 1404-5.
122. Fujita, E., et al., *Two endoplasmic reticulum-associated degradation (ERAD) systems for the novel variant of the mutant dysferlin: ubiquitin/proteasome ERAD(I) and autophagy/lysosome ERAD(II)*. Hum Mol Genet, 2007. **16**(6): p. 618-29.
123. Ben-Gedalya, T., et al., *Cyclosporin-A-induced prion protein aggresomes are dynamic quality-control cellular compartments*. J Cell Sci, 2011. **124**(Pt 11): p. 1891-902.
124. Kopito, R.R., *Aggresomes, inclusion bodies and protein aggregation*. Trends Cell Biol, 2000. **10**(12): p. 524-30.
125. Zuber, C., et al., *EDEM1 reveals a quality control vesicular transport pathway out of the endoplasmic reticulum not involving the COPII exit sites*. Proc Natl Acad Sci U S A, 2007. **104**(11): p. 4407-12.
126. Le Fourn, V., et al., *Large protein complexes retained in the ER are dislocated by non-COPII vesicles and degraded by selective autophagy*. Cell Mol Life Sci, 2013.
127. Bernasconi, R. and M. Molinari, *ERAD and ERAD tuning: disposal of cargo and of ERAD regulators from the mammalian ER*. Curr Opin Cell Biol, 2011. **23**(2): p. 176-83.
128. Kruse, K.B., J.L. Brodsky, and A.A. McCracken, *Characterization of an ERAD gene as VPS30/ATG6 reveals two alternative and functionally distinct protein quality control pathways: one for soluble Z variant of human alpha-1 proteinase inhibitor (A1PiZ) and another for aggregates of A1PiZ*. Mol Biol Cell, 2006. **17**(1): p. 203-12.
129. Fu, L. and E. Sztul, *ER-associated complexes (ERACs) containing aggregated cystic fibrosis transmembrane conductance regulator (CFTR) are degraded by autophagy*. Eur J Cell Biol, 2009. **88**(4): p. 215-26.
130. Huyer, G., et al., *A striking quality control subcompartment in Saccharomyces cerevisiae: the endoplasmic reticulum-associated compartment*. Mol Biol Cell, 2004. **15**(2): p. 908-21.
131. Leitman, J., et al., *Compartmentalization of Endoplasmic Reticulum Quality Control and ER-Associated Degradation Factors*. DNA Cell Biol, 2013. **32**(1): p. 2-7.
132. Kopito, R.R. and R. Sitia, *Aggresomes and Russell bodies. Symptoms of cellular indigestion?* EMBO Rep, 2000. **1**(3): p. 225-31.
133. Ordonez, A., et al., *Endoplasmic reticulum polymers impair luminal protein mobility and sensitise to cellular stress in alpha (1) -antitrypsin deficiency*. Hepatology, 2012.

Chapter 2

UDP-glucose:glycoprotein glucosyltransferase (UGGT1) promotes substrate solubility in the endoplasmic reticulum

2.1 Introduction

Although the amino acid sequence of a protein has all the information required to fold into its native functional conformation[1], folding pathways and efficiency are dramatically influenced by the environment[2]. Newly-made secretory and transmembrane proteins in the endoplasmic reticulum (ER) require the assistance of chaperones and enzymes to fold to their native conformation[2]. Recognition, retention, refolding, and degradation of misfolded protein substrates in the ER are referred to, collectively, as ER quality control[2-6]. Protein folding failure in the ER can lead to insolubility, either by formation of protein aggregates, or by ordered polymerization. Once rendered insoluble in the ER, entrapped proteins are either degraded (through autophagy), or can persist as undegraded insoluble complexes possibly contributing to cell toxicity, or may be re-solubilized[7-9]. The mechanisms that promote protein solubility in the ER are poorly understood.

The folding, trafficking and degradation of glycoproteins is coupled with the modification of N-linked oligosaccharides[10, 11]. Upon translocation into the ER, a preassembled oligosaccharide core (Glc₃Man₉GlcNAc₂) is transferred to asparagine residues within the acceptor sequence Asn-Xxx-Ser/Thr. Glucosidases I and II sequentially trim this core to a monoglucosylated glycan that can mediate binding to lectin-like ER chaperones calreticulin (CRT) and

calnexin (CNX). Interaction with CRT and CNX serves to retain improperly folded substrates in the ER as a key step in glycoprotein quality control, as well as to promote folding through interactions with ERp57[12]. Upon release from CRT/CNX, glucosidase II can remove the remaining glucose to produce a product that if folded properly traffics to the Golgi, or if folded improperly is directed to ER-associated degradation (ERAD) or is subject to reglucosylation for interaction with CRT/CNX. Uridine diphosphate (UDP)-glucose:glycoprotein glucosyltransferase (UGGT1) is proposed to be a central gatekeeper for ER quality control for glycoproteins, as it recognizes the partially folded status as a substrate to reglucosylate the deglucosylated N-glycan to direct another round of interaction with CRT/CNX[10, 13].

There are two UGGT genes in the mouse, *Uggt1* and *Uggt2*[14]. The product of *Uggt1* has reglucosylation activity in vitro and its deletion destroys reglucosylation activity in cells and is embryonic lethal at day E13 in mice[15]. In contrast, the product of *Uggt2* has not been demonstrated to have reglucosylation activity and its function remains unknown. Interestingly, exchange of the UGGT1 80% amino terminal substrate recognition domain into UGGT2 partially restores reglucosylation activity in vitro, demonstrating that the carboxy terminal 20% of UGGT2 can function as a glucosyltransferase in the proper context[16].

While the purported mechanism for UGGT1-mediated quality control—reiterative monoglucosylation of deglucosylated N-glycans on incompletely folded glycoproteins, and subsequent rounds of association of these glycoproteins with CRT/CNX—has been extensively described[17-21], it remains unclear the extent to which UGGT1 influences the susceptibility of ER substrate proteins to form insoluble protein aggregates or polymers. In this study, we used two different alpha-1 antitrypsin (AAT) variants prone to misfolding, to investigate the impact of UGGT1 on the solubility of these glycoprotein substrates. One variant is the well-studied ERAD substrate known as null Hong Kong variant (NHK); the other is the more common mutant Z allele (ATZ). Both alleles cause AAT deficiency

(the most common genetic liver disease in children), which is a representative of the class of ‘conformational diseases’ caused by retention of misfolded glycoproteins in the ER[22-25]. In this report, using *Uggt1*^{-/-} mouse embryonic fibroblasts (MEFs) expressing NHK or ATZ, and complemented with plasmid-encoded UGGT1, we demonstrate that UGGT1 enzymatic activity—in conjunction with lectin-like chaperones—helps to limit aggregation and polymer formation of misfolded glycoprotein substrates, decreasing their BiP association, and decreasing ER stress.

2.2 Results

UGGT1 increases solubility of alpha-1 antitrypsin (AAT) mutants: NHK and ATZ. *Uggt1*^{-/-} MEFs have undetectable levels of UGGT1 protein and enzymatic monoglucosylation activity[15]. UGGT1 in the ER is associated with misfolded NHK and ATZ variants of AAT[26, 27]. ATZ is predisposed to form insoluble ordered polymers[28] whereas ER-entrapped NHK can be recovered in a detergent-soluble pool in UGGT1-expressing cells[29].

To directly investigate the effect of UGGT1 on NHK, ATZ, or wild-type AAT (wt-AAT) solubility, *Uggt1*^{-/-} MEFs (or those complemented with plasmid-encoded UGGT1) expressing these substrates were metabolically labeled with [³⁵S]-methionine/cysteine to approach steady state. By quantitative immunoprecipitation (Figure 2.S1A), we determined that over the course of the metabolic labeling period (24 h), a fraction of mutant NHK molecules was secreted; a second portion was recovered in the intracellular detergent-soluble supernatant fraction; and a third was recovered as aggregates that failed to be solubilized from *Uggt1*^{-/-} MEFs lysed in nonionic detergents (Figure 2.1A). This ‘detergent-insoluble fraction’ was indeed solubilized in SDS-containing detergent extracts.

Whereas endoglycosidase H(EndoH)-resistant NHK molecules were found in the secreted fraction, both the soluble and detergent-insoluble intracellular

fractions were EndoH-sensitive, indicating that neither the soluble nor insoluble intracellular pool had arrived in a Golgi compartment from which they could be further modified by Golgi N-glycan processing enzymes (Figure 2.2D). We confirmed that a fraction of mutant NHK molecules forms detergent-insoluble protein aggregates by several independent approaches. First, pulse-chase experiments demonstrated that newly-synthesized NHK was not initially recovered in the detergent-insoluble fraction but became so as a function of chase, indicating time-dependent specificity (Figure 2.2C). Second, we compared solubility of NHK using three standard detergent lysis methods [(1) CHAPS (2%w/v); (2) Nonidet P-40 (1%v/v); (3) Triton X-100 (0.5%w/v) and sodium deoxycholate (0.5%w/v)] that have been used for analysis of AAT and its variants[27, 30, 31]: in each case, a detergent-insoluble fraction of NHK was recovered (Figure 2.S1B). Finally, intracellular wt-AAT was always undetectable in the insoluble fraction (Figure 2.1C, 2.2A), indicating that recovery in the detergent-insoluble pool was not caused by failure to disrupt cellular membranes from *Ugg1*^{-/-} MEFs. These findings plus recent reports[32] provide strong evidence that NHK is predisposed to aberrant protein aggregation.

Complementation of *Ugg1*^{-/-} MEFs with UGGT1 greatly expanded the steady-state intracellular pool of NHK molecules (Figure 2.1A). Whereas only half as many NHK molecules were secreted, there was a great expansion of the pool of soluble intracellular NHK molecules (red bar, Figure 2.1A, middle), and a decrease in the pool of detergent-insoluble NHK (blue bar, Figure 2.1A, middle). Additionally, the total soluble (extracellular+soluble)/insoluble NHK ratio increased with UGGT1 cotransfection (Figure 2.1A, right). Thus, the net effect of coexpressing UGGT1 was to increase the solubility of NHK.

The same experimental analysis was used to investigate ATZ trafficking in the ER. Whereas NHK cannot undergo loop-sheet polymerization, recovery of detergent insoluble ATZ is thought to reflect the formation of ordered polymers[28]. Here as well, UGGT1 expression increased the total soluble/insoluble ratio of ATZ almost 2-fold, (with little effect on the secreted

fraction, Figure 2.1B). Also, similarly to NHK, in pulse-chase experiments, ATZ was not detected in the detergent-insoluble fraction initially, but accumulated in the insoluble fraction over time (Figure 2.2B). Furthermore, to directly detect ATZ polymers in transfected cells, we utilized a recently developed monoclonal antibody (2C1) that specifically recognizes ATZ polymers[33].

Immunofluorescent co-staining with 2C1 and polyclonal anti-AAT antibody—and subsequent quantitation of signal intensity in the entire cell volume through Z-stack analysis—revealed a decrease in the ratio of 2C1 to AAT signal in cells cotransfected with UGGT1 (Figures 2.S2A-B).

Thus, for mutant glycoproteins that are prone to misfolding in two distinctly different ways [formation of ordered polymers (ATZ) or formation of disordered aggregates (NHK)], UGGT1 increased substrate solubility. By contrast, UGGT1 had no significant effect on the behavior of wt-AAT, consistent with the observation that wt-AAT molecules were soluble in *Uggt1*^{-/-} MEFs even before complementation with UGGT1 (Figure 2.1C).

UGGT1 promotes NHK solubility through monoglucosylation activity. To investigate whether increased NHK solubility was due to UGGT1 chaperone function or enzymatic reglucosylation activity[20], we eliminated the enzymatic contribution of UGGT1 either by glucosidase inhibition (with castanospermine (CAS), which prevents formation of unglucosylated N-glycans that serve as substrates for UGGT1-mediated monoglucosylation) or by complementation with UGGT1 variants lacking monoglucosylation activity. When CAS was included throughout the labeling period, UGGT1 did not increase solubility of NHK (Figure 2.3A). Furthermore, a catalytically-inactive UGGT1 (lacking six amino acids within the catalytic site, called Δ UGGT1[14]) was similarly unable to augment the solubility of NHK (Figure 2.3A). However, a UGGT1/UGGT2 chimera (in which the C-terminal 20% of UGGT2 replaced the C-terminal 20% of UGGT1) bearing ~half of the enzymatic activity of native UGGT1[16] increased NHK solubility, and this effect was similarly eliminated by CAS treatment (Figure 2.3A). In parallel to

these NHK results, we found that enhanced solubility of ATZ also correlated with the presence of enzymatic UGGT1 monoglucosylation activity (Figure 2.S3A).

Since lectin-like chaperones CRT and CNX recognize monoglucosylated N-glycans, as an indirect measure of monoglucosylation we examined NHK association with CNX at the end of the 24 h metabolic labeling of *Uggt1*^{-/-} MEFs ± UGGT1 complementation. As in previous experiments, 2% CHAPS was included in the cell lysis buffer to maintain CNX–substrate interactions[34]. However, only when *Uggt1*^{-/-} MEFs were complemented with enzymatically active glucosyltransferase was the CNX-NHK association detected (Figure 2.3B).

To directly establish that increased NHK solubility in UGGT1-expressing cells is caused by enzymatic monoglucosylation activity, we used jack-bean alpha mannosidase [JBM, an exo-mannosidase that cleaves eight mannose residues from unglucosylated ER-type N-glycans, but only five mannose residues from monoglucosylated ER-type N-glycans (Figure 2.3C)] to create diagnostic digests to assess the glycosylation status of steady-state soluble and detergent-insoluble pools. Upon JBM digestion, intracellular soluble NHK from UGGT1-expressing MEFs produced a predominant higher molecular mass digestion product distinct from the primary form recovered in *Uggt1*^{-/-} MEFs (Figure 2.3D, left panel). By contrast, in spite of UGGT1 expression, intracellular detergent-insoluble NHK, upon JBM digestion, produced a predominant lower molecular mass digestion product that co-migrated with that from UGGT1-deficient MEFs (Figure 2.3D, right panel). Strikingly, upon UGGT1-complementation, the majority of detergent-insoluble NHK lacks monoglucosylated N-glycans whereas the majority of soluble NHK from the same cells bear monoglucosylated N-glycans. The findings strongly suggest that increased solubility of the substrate is a consequence of its N-glycan monoglucosylation.

UGGT1 maintains solubility of NHK over time. As noted above, all newly synthesized NHK, ATZ and wt-AAT molecules begin in the soluble fraction; thus the accumulation of NHK and ATZ molecules takes time. While steady-state

labeling provides useful conditions for determining the sizes of soluble and insoluble intracellular pools, it does not provide insight into the entry or exit of molecules in these fractions. Therefore, after steady-state labeling, we followed pools of NHK for up to 6 h in the absence of further metabolic labeling. As previously observed (Figure 2.1A), intracellular NHK solubility was enhanced in UGGT1-expressing cells (Figure 2.4A-B). At 3 h and 6 h after the steady-state labeling period, the pool of soluble NHK decreased in *Uggt1*^{-/-} MEFs both without and with UGGT1 complementation. However, without UGGT1 complementation, some of the molecules formerly in the soluble pool entered and expanded the detergent-insoluble pool (Figure 2.4A-B), whereas in the presence of UGGT1, expansion of the detergent-insoluble pool was prevented. Indeed, despite a decrease in soluble NHK as a function of chase time, the relative increase in NHK solubility as a consequence of UGGT1 activity was maintained at all times (Figure 2.4C). Thus, the results suggest that UGGT1 activity limits misfolded glycoprotein entry into detergent-insoluble complexes.

ERAD inhibition further expands NHK solubility induced by UGGT1. To investigate whether NHK is degraded by ER-associated degradation (ERAD) during the course of our experiments, NHK transfected cells were treated with kifunensine (KIF, a mannosidase inhibitor) for the entire 24 h metabolic labeling period. KIF potently inhibits glycoprotein ERAD[35], including the degradation of NHK[36, 37]. By measuring the amount of steady-state NHK recovered in all fractions and normalizing this total to its synthesis in the same cells, the amount of NHK degraded under each experimental condition could be deduced (Figure 2.5A-B). When KIF was present, the amount of NHK recovered in the insoluble fraction increased, both with and without UGGT1 co-transfection (Figure 2.5A-B). However, in the presence of UGGT1, KIF treatment also promoted an increase in total soluble NHK (Figure 2.5A-C). Likewise, using a pulse-chase technique, when UGGT1 was present, KIF treatment stabilized soluble NHK in cells (Figure 2.5D). Interestingly, this KIF-induced increase in soluble NHK was not seen in

Uggt1^{-/-} MEFs cotransfected with empty vector (Figure 2.5B). As ERAD inhibition expands NHK solubility caused by UGGT1 activity, the effect of UGGT1 to promote solubility of NHK in the absence of ERAD inhibition is likely to be larger than the magnitude shown in Figures 2.1-2.3.

Relative contribution of N-glycan addition versus monoglucosylation on the solubility of misfolded mutant AAT. NHK, ATZ and wt-AAT each have three N-linked glycosylation sites at positions N46, N83, and N247. To investigate the contribution of each of these N-glycans to NHK solubility, we eliminated N-glycosylation at these sites by mutagenizing each Asn acceptor to Gln, and prepared all possible combinations of one-site or two-site N-glycosylation mutants. We then measured the total soluble/insoluble ratio for each of the seven constructs, with or without UGGT1 complementation (Figure 2.6A-B). The majority of unglycosylated NHK (NHK-N46Q_N83Q_N247Q or NHKQQQ) was insoluble, and UGGT1 cotransfection had no effect on the solubility of this construct (Figure 2.6B, bars 1-2). For the NHK constructs with only one N-glycosylation site (NHK-N83Q_N247Q, NHK-N46Q_N247Q, and NHK-N46Q_N83Q), in the absence of UGGT1, solubility did not increase, but solubility was enhanced by expression of UGGT1 (Figure 2.6B, bars 3-8). The largest UGGT1-mediated solubility increase occurred with the NHK-N46Q_N247Q mutant, perhaps indicating particular importance of the N83 N-glycan (Figure 2.6B, bars 5-6). For the NHK constructs with two N-glycosylation sites (NHK-N247Q, NHK-N83Q, NHK-N46Q), solubility increased both with and without UGGT1, but the increase was greater when UGGT1 was present (Figure 2.6B, bars 9-14), and each of the double-glycan mutants behaved comparably. Similar to Figure 2.1A, the fully glycosylated protein was the most soluble NHK construct, but UGGT1 expression conferred additional solubility (Figure 2.6B, bars 15-16). The results indicate that each N-linked glycan contributes to the solubility of NHK, but maximal solubility is achieved only in UGGT1-expressing cells.

UGGT1 cotransfection reduces NHK association with BiP, and BiP-Luciferase response. The binding of unfolded and misfolded proteins in the ER to BiP activates the Unfolded Protein Response (UPR)[38-40]. To examine BiP association with NHK, DSP-crosslinked cell lysates were analyzed by immunoprecipitation with anti-AAT, and the immunoprecipitates subjected to reducing SDS-PAGE and immunoblotting with anti-BiP and anti-AAT. Consistent with the metabolic labeling results of Figure 2.1A, there was increased NHK in the soluble fraction of *Uggt1*^{-/-} MEFs complemented with UGGT1 (Figure 2.7A, lower panel). However, despite that there was more NHK present in the soluble pool, the actual amount of BiP associated with NHK in that pool decreased (Figure 2.7A, quantified in Figure 2.7B). To investigate UPR activation in cells expressing NHK, ATZ, or wt-AAT, we co-expressed BiP-Luciferase and normalized the signal from the ER stress response reporter directly to the abundance of the misfolded protein substrate. Interestingly, neither wt-AAT nor mutant ATZ expression (data not shown) increased BiP-Luciferase activity, regardless of the presence or absence of UGGT1 expression (Figure 2.7C, bars 1-2), consistent with previous reports[41-43]. By contrast, NHK expression induced an appreciable BiP-Luciferase response that was attenuated in cells complemented with UGGT1 (Figure 2.7C, bars 3-4). Notably, UGGT1 complementation did not diminish (and actually augmented) UPR activation to tunicamycin treatment, presumably because of the increased protein load in the ER and the fact that reglucosylation activity is irrelevant in cells in which N-linked glycosylation has been blocked by tunicamycin (Figure 2.7C, bars 5-6). Taken together, the results of Figure 7 indicate that BiP-association with a misfolded glycoprotein substrate decreases as a consequence of substrate monoglucosylation, which reduces UPR activation in these cells.

The ER lectin calreticulin is linked to increased solubility of monoglucosylated glycoprotein substrate. To explore whether UGGT1-mediated solubility of NHK is due to increased interaction with lectin-like chaperones, we performed CRT

siRNA-mediated knockdown in *Uggt1*^{-/-} MEFs (CRT, the most abundant soluble ER lectin, is likely to interact with a high fraction of soluble glycoprotein substrates). In both DSP crosslinked and non-crosslinked cell lysates, interaction between NHK and CRT was readily demonstrated (Figure 2.8A). CRT siRNA knockdown (KD) was more than 50% efficient in *Uggt1*^{-/-} MEFs at 48 h post-siRNA treatment, and this itself did not upregulate other ER chaperones, as well as CNX (Figure 2.8B). However, upon NHK transfection in these cells, CRT KD largely blocked the increase of NHK solubility caused by UGGT1 complementation (Figure 2.8C-E). Also, CRT KD increased the proportion of insoluble NHK, and blocked the effect of UGGT1 to decrease the proportion of insoluble NHK (Figure 2.8D). The data indicate that abundance of CRT is a key factor in promoting solubility of monoglucosylated N-linked glycoprotein substrates in the ER.

2.3 Discussion

Protein solubility, or more importantly, the formation of insoluble protein aggregates or polymers, is thought to play a fundamental role in the pathology of a wide array of human diseases[44]. Mutations in secreted proteins can disrupt their normal ER folding program, resulting in aggregation and cell stress/death[45-47]. Regulation of aggregate/polymer formation and the role of chaperone interaction in this process are incompletely understood. In this report we describe how UGGT1 enzymatic activity correlates with increased solubility of two model glycoproteins susceptible to aggregation and polymerization.

A large body of work has delineated the key role of UGGT1 in reglucosylation of nonnative glycoprotein folding intermediates, and subsequent reassociation with the lectin-like chaperones CRT and CNX[17-21, 48, 49]. Here, we show that the ATZ allele of AAT, which is prone to misfolding, formed more insoluble polymers in UGGT1-deficient cells (Figure 2.1B). The null Hong Kong (NHK) variant of AAT also accumulated in the insoluble fraction, and the

proportion detected in the insoluble pool after 24 h labeling decreased in the presence of UGGT1 activity (Figure 2.1A). Interestingly, UGGT1 maintained the solubility of both nonnative ER forms of these AAT variants, but the consequences of this increased solubility were different for NHK and ATZ. For NHK, soluble nonnative forms were retained in the ER, and the secretion rate was lower upon UGGT1 complementation; thus, UGGT1 increases quality control for secretion of nonnative NHK. For ATZ, UGGT1 retained soluble nonnative forms in the ER and decreased the rate of entry into the insoluble fraction, without altering secretion. In parallel with these biochemical studies, the amount of ATZ recognized by a polymer-specific antibody also decreased upon UGGT1 complementation (Figure 2.S2A-B). The reason for solubility affecting trafficking of NHK and ATZ in different ways was not elucidated by this study, but likely involves different maturation pathways for the two proteins, possibly dependent on either retentive ER chaperone interactions and/or interactions with ER exit complexes.

Importantly, our results demonstrate from multiple independent approaches that UGGT1 requires its reglucosylation activity to promote NHK solubility. First, castanospermine treatment—which blocks glucose removal from N-glycans and prevents the formation of unglucosylated N-glycan substrates for UGGT1—decreased UGGT1-mediated solubility. Second, overexpression of a catalytically-inactive UGGT1 enzyme did not increase NHK solubility. In contrast, NHK solubility was increased by expression of a chimeric UGGT1/UGGT2 protein that contains the 20% carboxy terminal catalytic domain of UGGT2[16]. Interestingly, although UGGT2 does not have reglucosylation activity *in vitro*, the chimeric protein harboring the catalytic domain of UGGT2 can function to promote substrate solubility (Figure 2.3A). Lastly, UGGT1 had no effect on the solubility of NHK with all three N-glycosylation sites disrupted, which prevents formation of the UGGT1 substrate (Figure 2.6A-B).

To characterize the eventual fate of NHK molecules in the soluble and insoluble fractions, we performed 6 h chase after 24 h labeling experiments.

Interestingly, insoluble NHK was quite stable over a 6 h chase, and actually increased in amount with time in the absence of UGGT1, with soluble molecules entering the insoluble fraction (Figure 2.4A-B). Theoretically, the amount of NHK in the insoluble fraction after 24 h is determined by three processes: 1) entry from the soluble fraction, 2) degradation of insoluble forms, or 3) solubilization of insoluble forms to enter the soluble fraction. Interestingly, monoglucosylated NHK was not detected in the insoluble fraction (Figure 2.3D), indicating that UGGT1 cannot reglucosylate molecules that have entered the insoluble fraction, and therefore UGGT1 may not promote actual re-solubilization. However, we cannot rule out the possibility that insoluble molecules that are reglucosylated may immediately re-enter the soluble fraction, and thus are not detected in the insoluble fraction based on jack-bean alpha-mannosidase digestion. Another possibility for the absence of monoglucosylated NHK in the insoluble fraction—based on a previous finding that NHK is found in ER subcompartments called inclusion bodies[50]—is that NHK may be physically sequestered away from UGGT1. Lastly, perhaps the insoluble NHK forms a tight aggregate such that the recognition site for UGGT1 is not exposed.

Since ERAD is thought to require soluble substrates for degradation[51], the finding that UGGT1 promotes glycoprotein solubility could have implications for the degradation of glycoproteins in the ER. Indeed, upon ERAD inhibition with kifunensine, total soluble NHK increased, and this effect was most prominent when UGGT1 was present (Figure 2.5A-C). Insoluble NHK also seemed to increase upon ERAD inhibition, presumably due to the greater amount of soluble NHK available for entry to the insoluble fraction (although we cannot rule out that kifunensine may directly inhibit degradation of insoluble NHK).

Another finding from this study involves the contribution of the three N-glycans of NHK to its solubility. First, we found that although solubility of NHK did increase directly by adding hydrophilic N-glycans to the polypeptide, a major fraction of this solubilization was UGGT1-dependent (Figure 2.6A-B). This indicates that, at least for NHK, UGGT1-mediated reglucosylation contributes to

substrate solubility, in addition to solubility afforded by adding hydrophilic N-glycans to the hydrophobic polypeptide. CRT knockdown greatly decreased NHK solubilization in response to UGGT1 enzymatic activity (Figure 2.8C-E). From this, we infer that this UGGT1-dependent solubilization effect is due to reassociation with CRT/CNX, and is not a direct contribution from the glucose added to the N-glycan A-chain.

The CNX cycle hypothesis[49] proposes that UGGT1 reglucosylates nonnative glycoprotein folding intermediates and promotes their reassociation with CRT/CNX for further folding attempts. We directly tested how BiP interaction is involved in UGGT1-enhanced NHK solubility. Indeed, we show that while the amount of soluble NHK increased with UGGT1 overexpression, the amount of BiP bound to soluble NHK clearly decreased (Figure 2.7A-B). This appears to be an example of BiP acting as a second level ER quality controller for glycoproteins when interaction with their primary set of chaperones is disturbed[15, 52].

The role of UGGT1 in glycoprotein trafficking is intimately linked with the lectin-like chaperones CNX and CRT[21]. Previous studies have implied that NHK does not interact with calreticulin[53, 54]; however, we found that NHK extensively associates with CRT in *Uggt1*^{-/-} MEFs cotransfected with NHK and UGGT1 (Figure 2.8A). siRNA-mediated CRT knockdown strikingly reduced the ability of UGGT1 to increase NHK solubility (Figure 2.8C-E). The fact that >99% of intracellular CRT resides in the soluble fraction (data not shown) presumably explains its ability to promote the solubility of monoglucosylated NHK.

The exact mechanisms governing protein solubility and quality control in the ER are incompletely understood, but this study highlights how the ER enzyme UGGT1 can improve glycoprotein quality control by promoting solubility of glycoprotein substrates that are prone to misfolding (Figure 2.9). Our results contribute to the CNX cycle theory by demonstrating that UGGT1-dependent lectin interaction can increase glycoprotein substrate solubility in the ER. Further studies on the effect of glycan modifications and chaperone interactions should

provide additional clues to the mechanism(s) of maintaining solubility of glycoproteins during ER folding.

2.4 Materials and methods

Reagents and Antibodies

Castanospermine was from Tocris Bioscience. Kifunensine was from Cayman Chemical. Protein A-Sepharose, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), *N*-ethylmaleimide (NEM), phenylmethanesulfonyl fluoride (PMSF), leupeptin, antipain, pepstatin A, dithiobis succinimidyl propionate (DSP), and jack-bean alpha-mannosidase (JBM) were from Sigma. Chymostatin was from VWR. Endoglycosidase H (EndoH) and PNGaseF were from New England Biolabs. Rabbit anti-AAT antibody was from DAKO. Mouse mAb anti-KDEL and rabbit anti-CNX (used for immunoblotting) were from Enzo Life Sciences. Rabbit anti-CRT was from Thermo Scientific. Rabbit mAb anti-GAPDH was from Cell Signaling. Rabbit anti-BiP was from P. Arvan. Rabbit anti-CNX (used for IP) antibody was a kind gift from A. Helenius. Rabbit polyclonal anti-UGGT1 antibody was a kind gift from A. Parodi. Mouse monoclonal anti-antitrypsin polymer antibody (2C1) was a kind gift from E. Miranda and D. Lomas.

CDNAs and Constructs

NHK and ATZ mutant constructs were made by QuikChange mutagenesis (Stratagene) from wt-AAT in pcDNA3.1 using standard methods. wt-AAT, NHK, and ATZ constructs were modified by S. H. Back to express AAT and EGFP as two separate proteins from a single mRNA by CAP-dependent and IRES-dependent translation. UGGT1 and variants were previously published[16]. NHK N-glycosylation site mutants were constructed using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Stratagene).

Cell Culture

Uggt1^{-/-} MEFs[15] were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS and glutamax (Gibco). Cells were cultured at 37°C in a 5% CO₂ incubator.

Steady-State Metabolic Labeling/Pulse-Labeling Analysis

Uggt1^{-/-} MEFs were transiently transfected using Lipofectamine 2000 transfection reagent (Life Technologies) with AAT constructs and either UGGT1 (1:3 molar ratio AAT:UGGT1) or an equal microgram amount of empty vector (pEDΔC). The next day, cells were trypsinized and plated in separate 6-well plates, for pulse-labeling or steady-state analysis. Complete media supplemented with 0.1 mCi/ml [³⁵S]-methionine and [³⁵S]-cysteine (Tran³⁵S-label, MP Biomedicals), was added to steady state wells 24 h post-transfection, and media and cells were collected 48 h post-transfection (steady-state labeling). Cells in the remaining wells were starved for 20 min in media lacking met/cys, pulsed for 20 min with starvation media supplemented with 0.1 mCi/ml [³⁵S]-met/cys, and collected in the same manner as for steady-state labeling (next paragraph).

For each well, media was collected on ice, centrifuged at 10,000 X g for 10 min at 4°C, and this cleared supernatant was called the extracellular fraction. Cells were lysed in 550 μl 2%CHAPS/HBS (50 mM HEPES, 200 nM NaCl) pH 6.8 plus 200 mM PMSF, 20 mM NEM, and protease inhibitors (10 μg/ml final conc. chymostatin, leupeptin, antipain, and pepstatin A) for 20 min on ice. Cells were scraped from the wells with a rubber policeman and lysate was transferred to a centrifuge tube. Then, 100 μl lysis buffer was used to wash the well (to collect as much cellular material as possible), and this wash was added to the same centrifuge tube. Lysates were centrifuged at 10,000 X g for 10 min at 4°C, and the supernatant was transferred to a new tube and called the soluble fraction. Pellets from this centrifugation step were washed with 150 μl lysis buffer, centrifuged at 10,000 X g for 10 min at 4°C, and then the wash was removed. Pellets were then solubilized by addition of 100 μl 1% SDS, sonicated,

and boiled at 95°C for 5 min. 1 ml 1% Triton X-100 was added to the pellet/SDS mixture, followed by centrifugation at 10,000 x g for 10 min at RT. Unless indicated otherwise, equal proportions of each fraction were used for immunoprecipitation.

Pulse-Chase Analysis

Uggt1^{-/-} MEFs were transfected as for steady-state labeling, but the starvation, pulse, and chase were performed 24 h after transfection. Preparation of soluble and insoluble fractions, and double anti-AAT immunoprecipitation were similar to steady-state labeling analysis, but samples were not subjected to PNGaseF digestion.

Immunoprecipitation/Gel Electrophoresis

Trichloroacetic acid (TCA)-normalized volumes of extracellular, soluble, and insoluble fractions were incubated at 4°C o/n with anti-AAT antibody plus Protein A-sepharose. Beads were washed 3x with ice-cold 0.5% CHAPS/HBS pH 7.4, and then the wash was removed completely by careful aspiration with a small-bore pipette tip. To reduce background, antigen-antibody complexes were disrupted using the previously described 1% SDS/1% Triton X-100 resuspension method, and 1 ml of this supernatant was then incubated o/n at 4°C with anti-AAT antibody plus Protein A-sepharose, then washed and dried by aspiration (double IP). In most cases, beads were then subjected to PNGaseF digestion for 1 h, then 3 µl 5x sample buffer plus dithiothreitol (DTT) was added, and this bead suspension was boiled at 95°C for 5 min. Bead supernatants were analyzed by SDS-PAGE on 10% Tris-HCl Criterion gels or Mini-PROTEAN gels (Bio-Rad) at 200 V for 40 min. Gels were then Coomassie stained, destained, and dried in a gel-vacuum dryer for 2 h at 80°C. Finally, dried gels were exposed to a phosphor screen o/n and imaged on a Typhoon imager (GE Healthcare) and bands quantified with ImageQuant (GE Healthcare).

Dithiobis Succinimidyl Propionate (DSP) Crosslinking

At 48 h post-transfection, NHK transfected cells were crosslinked in 2 mM DSP in 4% DMSO/96% PBS for 30 min at RT. Soluble and insoluble fractions were prepared and TCA-normalized volumes were subjected to anti-AAT double IP, and analyzed by reducing SDS-PAGE (breaks DSP crosslink).

BiP-Luciferase Assay

ER stress response was measured 48 h post-transfection using BiP-Luciferase expression plasmid[55], and a Dual-Luciferase Reporter Assay System (Promega), and a parallel set of wells were steady-state labeled to determine total intracellular AAT in each transfection group.

Immunofluorescence

Uggt1^{-/-} MEFs were transfected with ATZ +/- UGGT1 cotransfection, plated on chamber slides, and fixed with 4% paraformaldehyde solution 48 h post-transfection. Cells were permeabilized and blocked in blocking buffer (1% BSA, 0.1% Triton X-100, 0.01% NaN₃ in PBS) for 1 h, and incubated o/n with 2C1 culture media supernatant (CMS) (1:10) and anti-AAT antibody (1:1000) in blocking buffer. Secondary antibody (Alexa 350, 555 (1:1000); Invitrogen) incubation was at RT for 1 h. Slides were imaged on an Olympus FV500 confocal microscope with Fluoview software (Olympus). Z-stack analysis was performed using Metamorph software (Molecular Devices).

siRNA Knockdown Experiment

Uggt1^{-/-} MEFs were transfected with Lipofectamine RNAiMAX transfection reagent (Life Technologies) at 0 h with either an equal mixture of two non-overlapping CRT siRNAs (Silencer Select, Life Technologies), or non-targeting Negative Control No. 1 siRNA, at a 10 nM final concentration in transfection media. Each transfection group was then transfected with Lipofectamine 2000 at 48 h with DNA expression plasmids plus the same siRNAs as the 0 h

transfection. Cells were trypsinized and split into two groups. One group was steady state labeled from 72-96 h, and the other group was collected for immunoblot analysis at 96 h.

Statistical Analysis

Using Prism software (Graph Pad Software), Student's t-test was used throughout.

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2.6 Figures

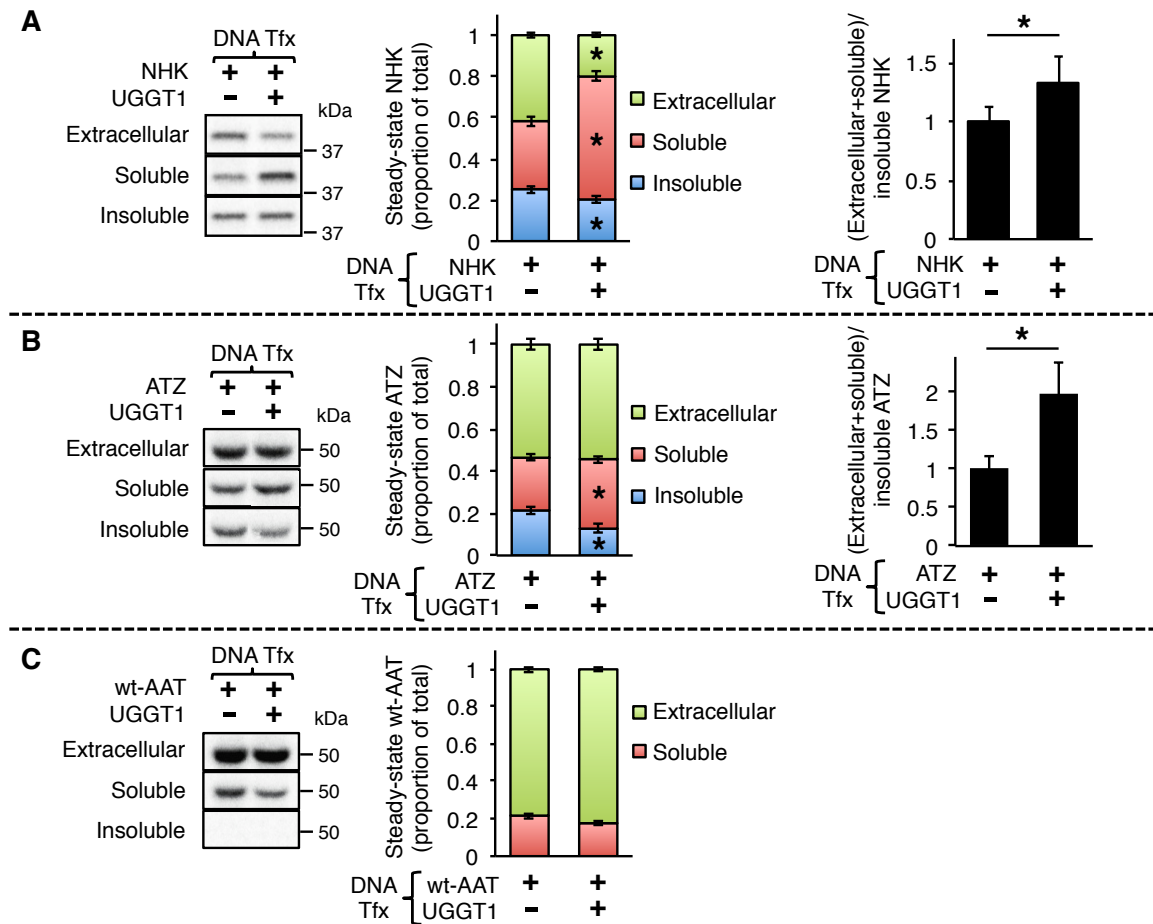


Figure 2.1 UGGT1 increases the solubility of alpha-1 antitrypsin mutants: NHK and ATZ

A, B, C 24 h metabolic labeling of *Uggt1*^{-/-} MEFs transfected with **(A)** NHK, **(B)** ATZ or **(C)** wt-AAT +/- UGGT1 cotransfection. *Uggt1*^{-/-} MEFs were transfected with expression vectors encoding the misfolded alpha-1 antitrypsin variants null Hong Kong (NHK), the Z allele (ATZ), or wild-type AAT (wt-AAT), and cotransfected with either empty vector or UGGT1 expression vector. Cells were radiolabeled from 24 to 48 h post-transfection in complete media plus 10 μ Ci/ml [³⁵S]-methionine/cysteine. Extracellular, soluble and insoluble fractions were produced (Experimental Procedures) and TCA-precipitation normalized volumes of each fraction were subjected to double anti-AAT quantitative immunoprecipitation and PNGaseF digestion, and analyzed by reducing SDS-PAGE and autoradiography. Substrate solubility was calculated by dividing total soluble (extracellular+intracellular soluble) substrate amount by insoluble substrate amount. Student's t-test was used for statistical testing of the center panels, and paired Student's t-test was used for statistical testing of the right-most panels (* = p \leq 0.05). Error bars represent standard error of the mean (SEM). For ATZ: All UGGT1+ bands darkened with imaging software to make synthesis (data not shown) +/- UGGT1 appear equal.

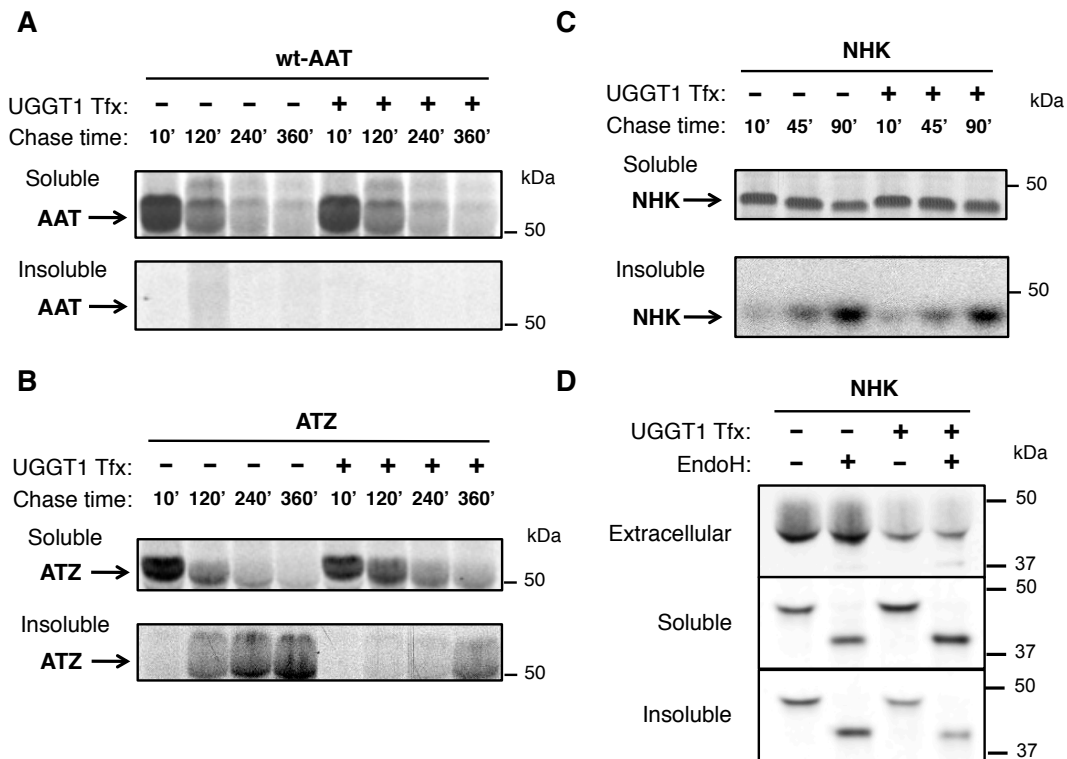


Figure 2.2 NHK and ATZ, but not wt-AAT, become insoluble over time

A) Transient transfection of *Uggt1*^{-/-} MEFs with wt-AAT +/- UGGT1 and analysis by pulse-chase at the indicated time points (Experimental Methods). No wt-AAT was detected in the insoluble fraction at any time point. **B)** same as **A** for ATZ. Here, ATZ progressively accumulates in the insoluble fraction over time. **C)** same as **A** for NHK. Here, NHK progressively accumulates in the insoluble fraction over time. Soluble NHK was run on a non-reducing gel because under reducing conditions, the reduced IP antibody physically pushes all NHK bands down to the same molecular weight. For **A**, **B** and **C**, four times the TCA-normalized amount of insoluble fraction was used for IP, compared to soluble fraction (actual relative amount of insoluble ATZ and NHK is 25% of what is seen in the gel image). **D)** EndoH digest of extracellular, soluble and insoluble NHK, after steady-state labeling. The majority of extracellular NHK is EndoH-resistant, evidence of having Golgi-modified N-glycans typically found on secreted glycoproteins. NHK in the soluble and insoluble fractions is completely EndoH-sensitive, indicating that these forms are not modified by Golgi-localized N-glycan modifying enzymes.

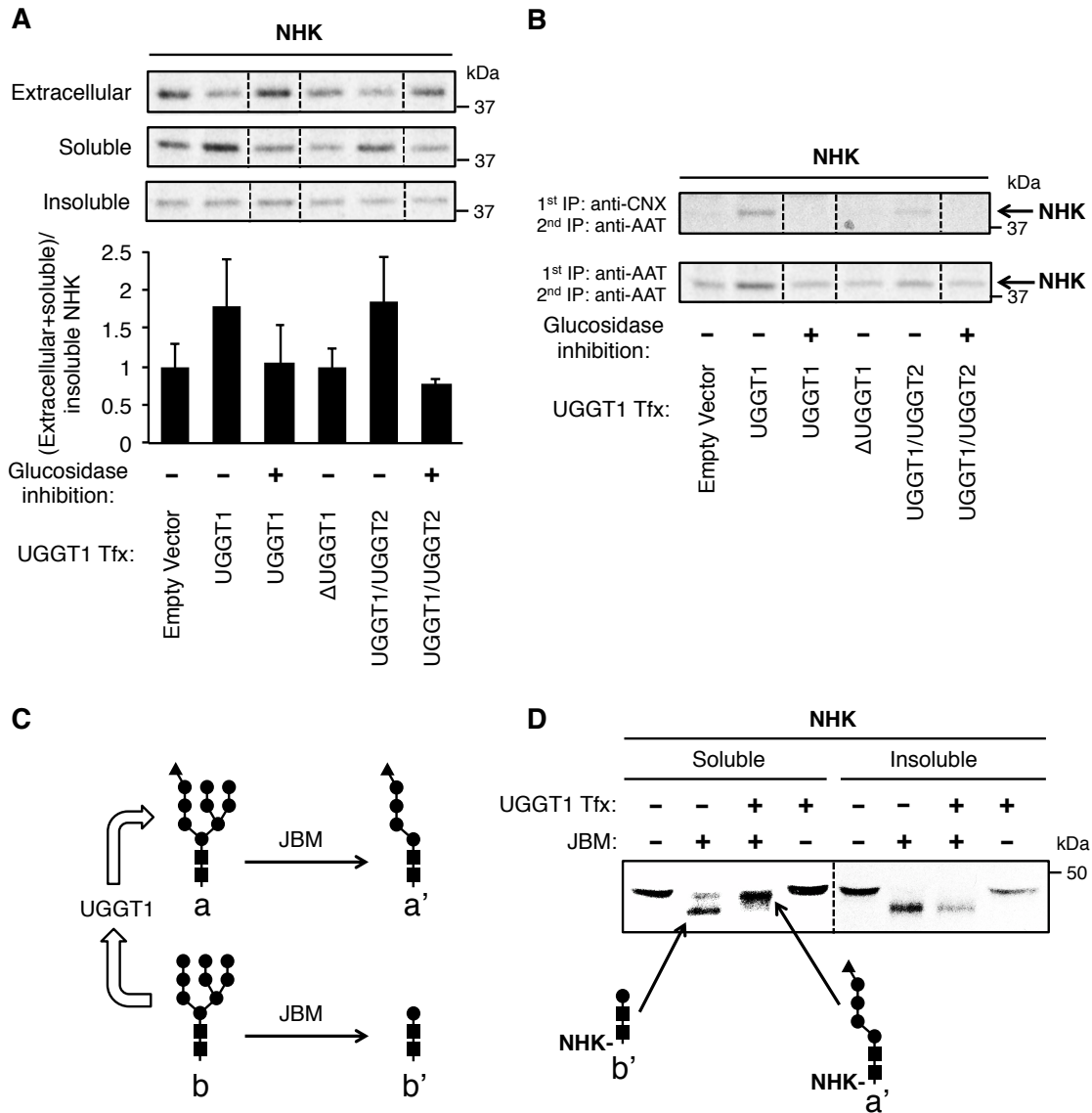


Figure 2.3 UGGT1 promotes solubility of NHK through monoglucosylation activity

A) Cotransfection with NHK and UGGT1 engineered variants and analysis of NHK. After 24 h metabolic labeling in the presence or absence of glucosidase inhibitor castanospermine, total soluble/insoluble ratios of NHK were determined. An increased total soluble/insoluble ratio was observed only when UGGT1 reglucosylation activity was expected, and this effect was blocked by glucosidase inhibition. Error bars represent SEM. **B)** Estimate of the proportion of soluble NHK associated with CNX in the various experimental conditions from **A**. Soluble material was subjected to anti-CNX/anti-AAT antibody tandem IP and anti-AAT antibody double IP. Appreciable NHK was only detected after anti-CNX/anti-AAT tandem IP when UGGT1 reglucosylation activity and monoglucosylated substrate was expected. **C)** Cartoon of Jack-bean alpha-

mannosidase (JBM) and UGGT1 activity. **D)** JBM digestion of N-glycans on NHK immunopurified from 24 h labeled soluble and insoluble fractions. Bands corresponding to monoglucosylated or unglucosylated N-glycans are as indicated. Soluble and insoluble fractions were run on the same gel, but insoluble bands were darkened with imaging software to appear similarly dense to soluble bands.

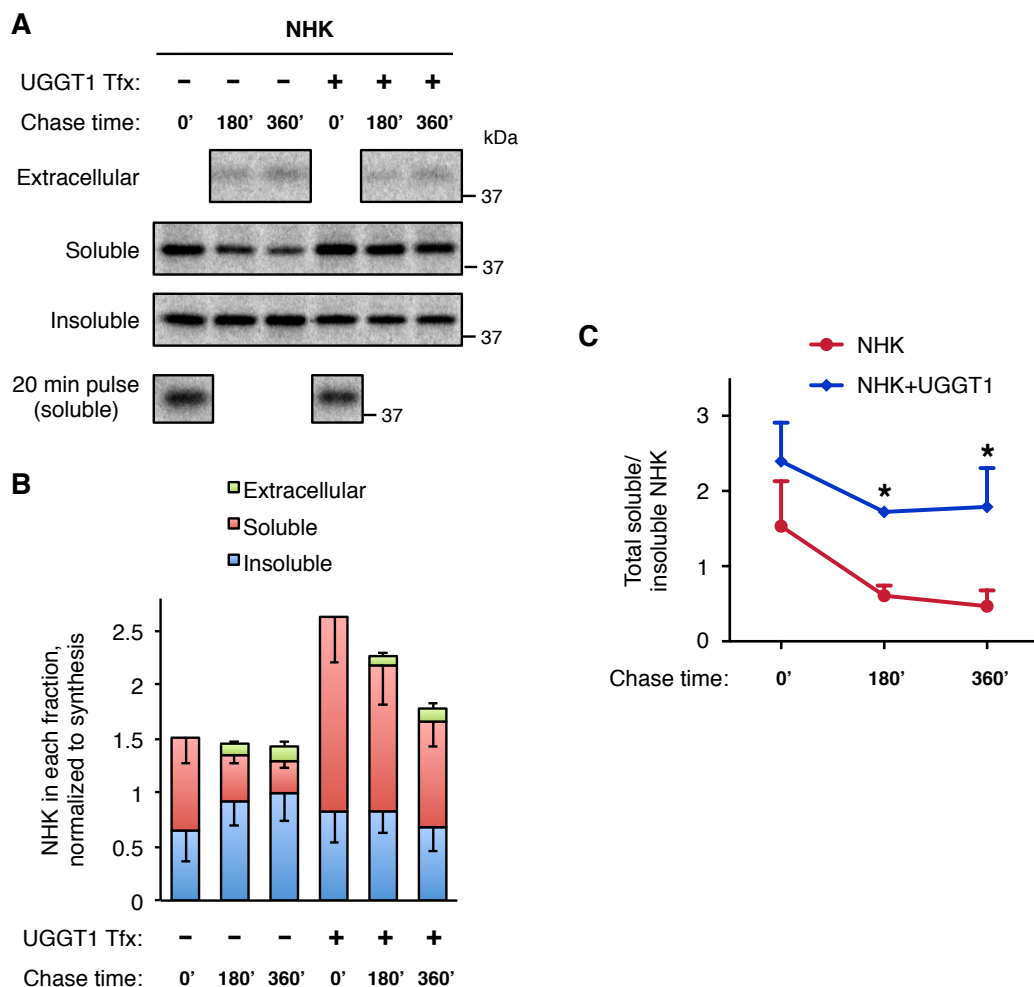


Figure 2.4 UGGT1 maintains increased total soluble/insoluble ratio for NHK during 6 h chase after 24 h labeling

A) 24 h labeling and chase analysis of NHK. After 24 h labeling, cells were chased with unlabeled media for 3 or 6 h, then extracellular, soluble, and insoluble fractions were subjected to anti-AAT double IP. The amount of newly-synthesized NHK recovered from a 20 min pulse-labeling was used to quantify NHK synthesis. **B)** Total amount of NHK remaining in each fraction after chase, normalized to synthesis. Error bars represent SEM. **C)** Plot of total soluble/insoluble NHK at 0, 3, and 6 h chase point. Student's t-test was used for statistical testing (* = $p \leq 0.05$). Error bars represent SEM.

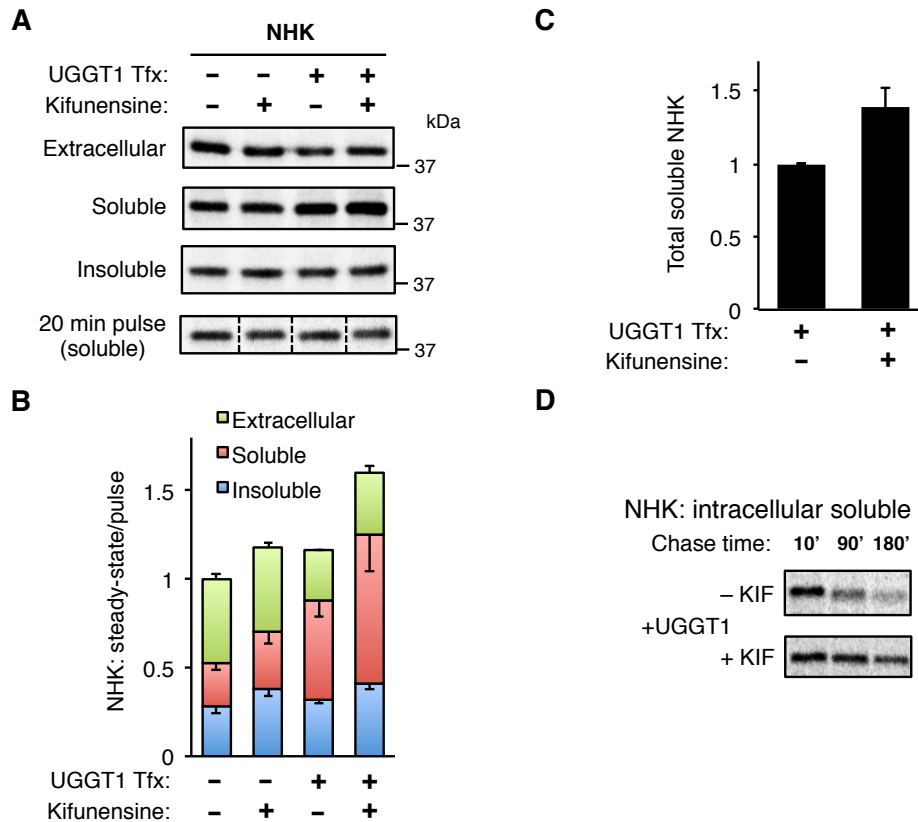


Figure 2.5 ERAD inhibition increases UGGT1-dependent NHK solubility

A) Effect of kifunensine on intracellular NHK. After cotransfection with NHK and UGGT1, 24 h metabolic labeling, and treatment with the mannosidase inhibitor kifunensine, amounts of NHK in extracellular, soluble and insoluble fractions were determined. Amount of newly-synthesized NHK recovered from a 20 min pulse-labeling was used to quantify NHK synthesis. **B)** Quantification of NHK in extracellular, soluble and insoluble fractions, normalized to synthesis. Error bars represent SEM. **C)** Total soluble NHK from **B**, with UGGT1 cotransfection, with and without kifunensine treatment. Error bars represent SEM. **D)** Pulse-chase experiment demonstrating effect of kifunensine to limit degradation of soluble NHK when UGGT1 is present.

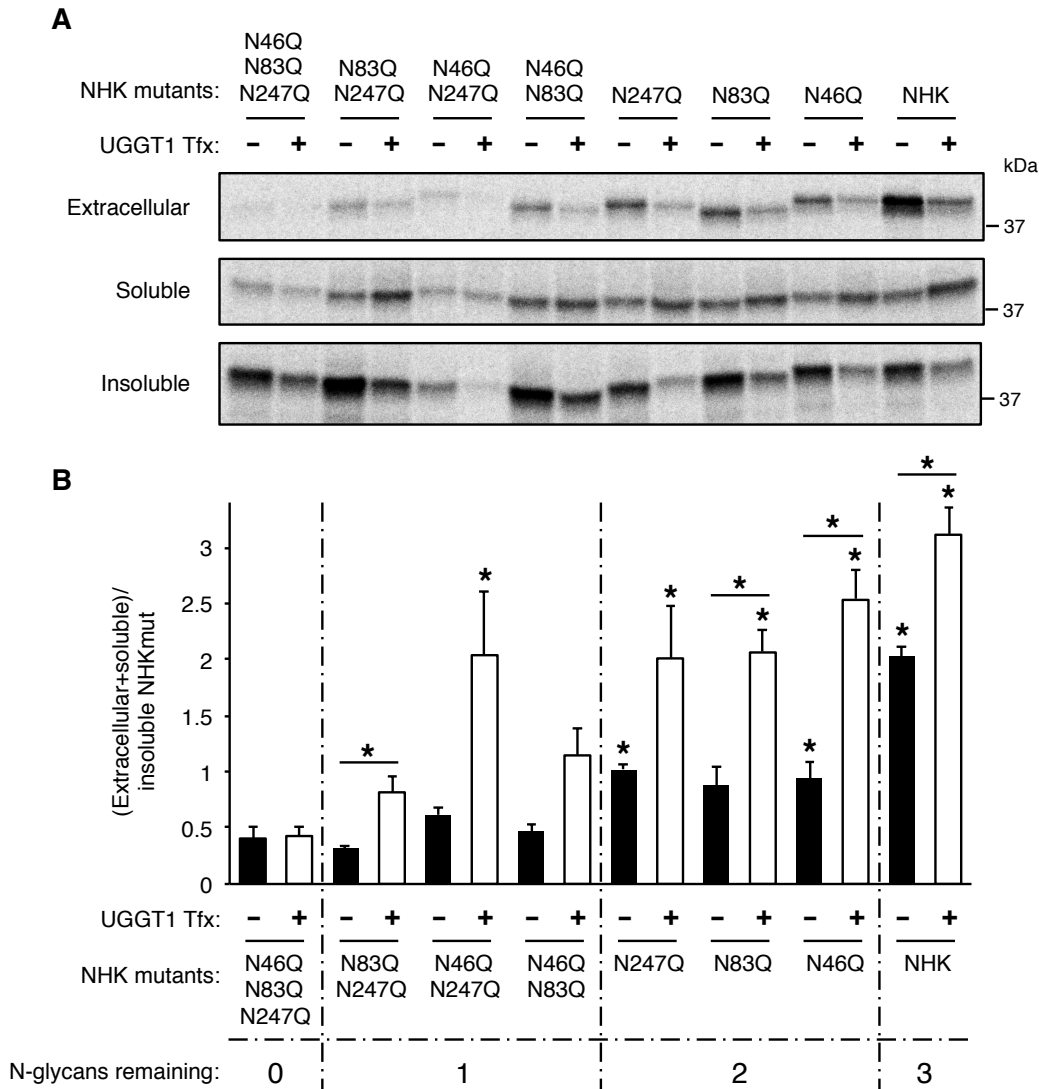


Figure 2.6 UGGT1-mediated solubility of NHK requires N-glycan on NHK
A) Analysis of NHK N-glycosylation mutants in transfected *Ugg1*^{-/-} MEFs. *Ugg1*^{-/-} MEFs were transfected with expression vectors encoding NHK N-glycosylation site mutants with all combinations of the three N-glycosylation sites mutated [(Asp(N) to Gln(Q); 1. NHK-N46,83,247QQQ, 2. NHK-N83,247QQ, 3. NHK-N46,247QQ, 4. NHK-N46,83QQ, 5. NHK-N247Q, 6. NHK-N83Q, 7. NHK-N46Q], cotransfected with either empty vector or UGGT1 expression vector, and subjected to steady-state labeling analysis. **B)** Quantification of three replicates of experiment in **A**. A significant portion of the increased solubility of NHK with increasing numbers of N-glycans added is due to UGGT1 reglycosylation activity. Student's t-test was used for statistical testing (* = p≤0.05). Asterisks directly above bars indicate a significant difference in total soluble/insoluble ratio between that bar and non-glycosylated NHK (NHKQQQ), either with or without UGGT1 cotransfection. Error bars represent SEM.

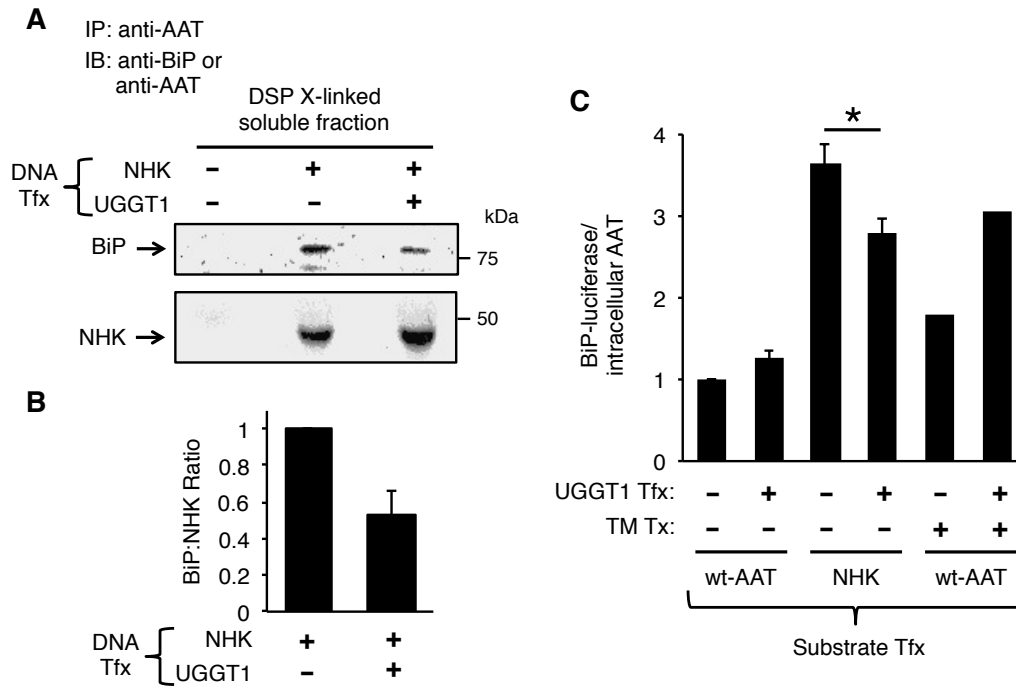


Figure 2.7 UGGT1 cotransfection reduces NHK association with BiP and reduces expression from the BiP promoter

A) Amount of BiP associated with NHK determined by immunoblotting of soluble material from DSP-crosslinked *Uggt1*^{-/-} MEFs—with or without UGGT1 cotransfection—with anti-BiP or anti-AAT antibodies. **B)** Quantification of three replicates of experiment in **A**. UGGT1 cotransfection reduces the amount of BiP associated with NHK per molecule. Error bars represent SEM. **C)** BiP-Luciferase response to transient transfection of NHK or wt-AAT is reported normalized to total intracellular (soluble+insoluble) amount of AAT. Tunicamycin treatment (6 h, 5 µg/ml) was included in cells transfected with AAT as a control for UPR activation. Student's t-test was used for statistical testing (* = p≤0.05). Error bars represent SEM.

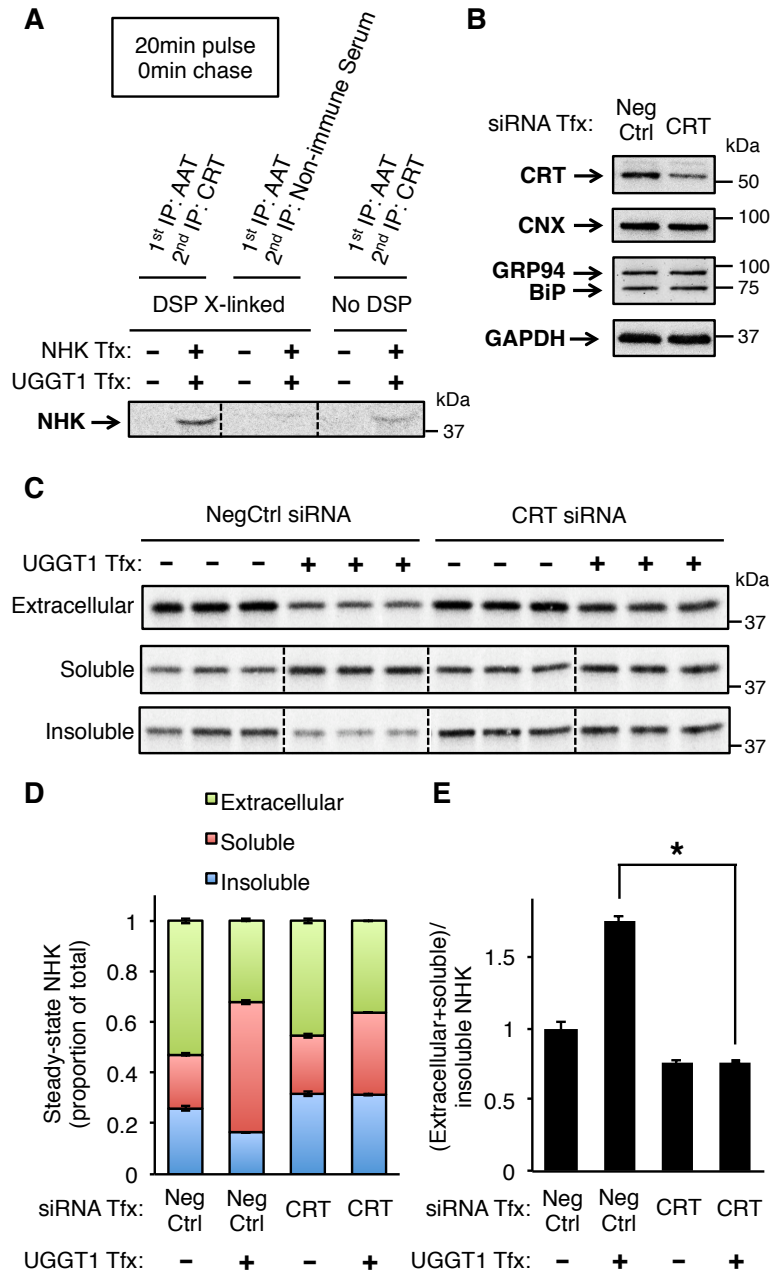


Figure 2.8 Calreticulin (CRT) siRNA knockdown reduces UGGT1-mediated increased solubility of NHK

A) Tandem anti-AAT/anti-CRT IP of soluble material from *Uggt1*^{-/-} MEFs cotransfected with NHK and UGGT1 and treated with or without DSP crosslinking. The results demonstrate association between NHK and CRT. **B)** Effect of CRT knockdown on chaperone expression. CRT siRNA transfection greatly reduces the amount of CRT protein without any compensatory increase in CNX, BiP or GRP94 protein. **C)** Steady-state labeling analysis of *Uggt1*^{-/-} MEFs transfected with CRT or NegCtrl siRNAs, and cotransfected with NHK and either

UGGT1 or empty vector (Experimental Methods). **D)** Quantification of **C**, each fraction represented as proportion of total. **E)** The effect of UGGT1 on the total soluble/insoluble ratio of NHK upon CRT knockdown. Student's t-test was used for statistical testing (* = $p \leq 0.05$). Error bars represent SEM.

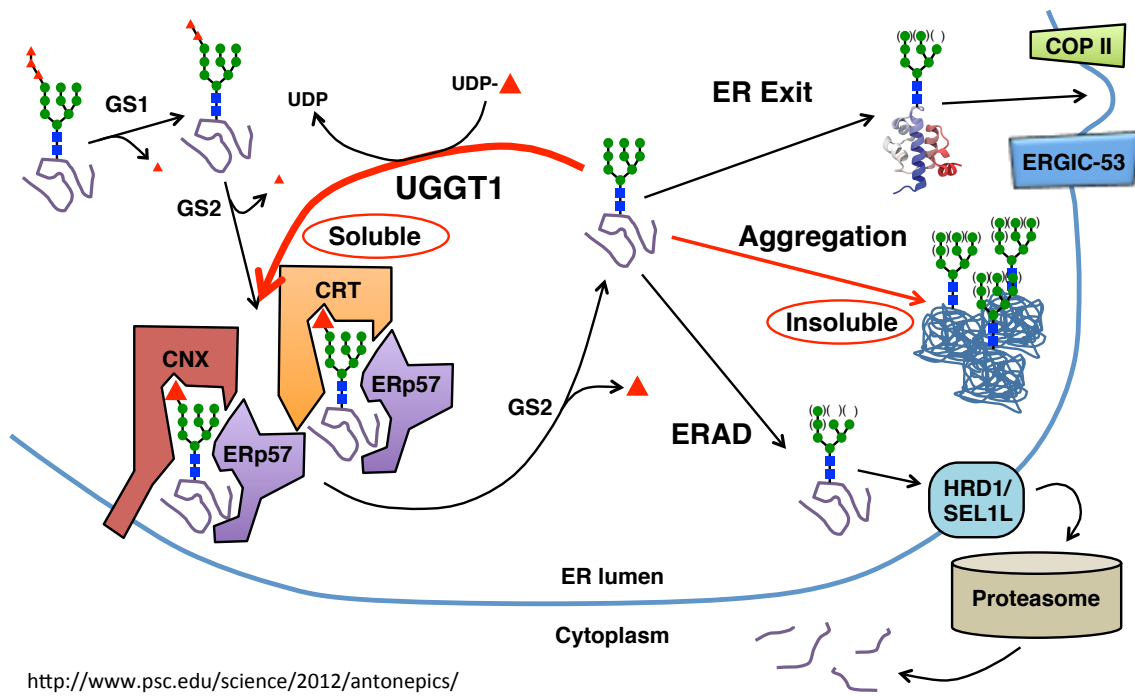


Figure 2.9 UGGT1 promotes solubility of glycoproteins through promoting interaction with CNX and CRT

Model depicts the role of UGGT1 in promoting solubility of glycoproteins through directing interaction with CNX and CRT. Monoglucosylated NHK is not observed in the insoluble fraction, but only in the soluble fraction in association with CRT and CNX.

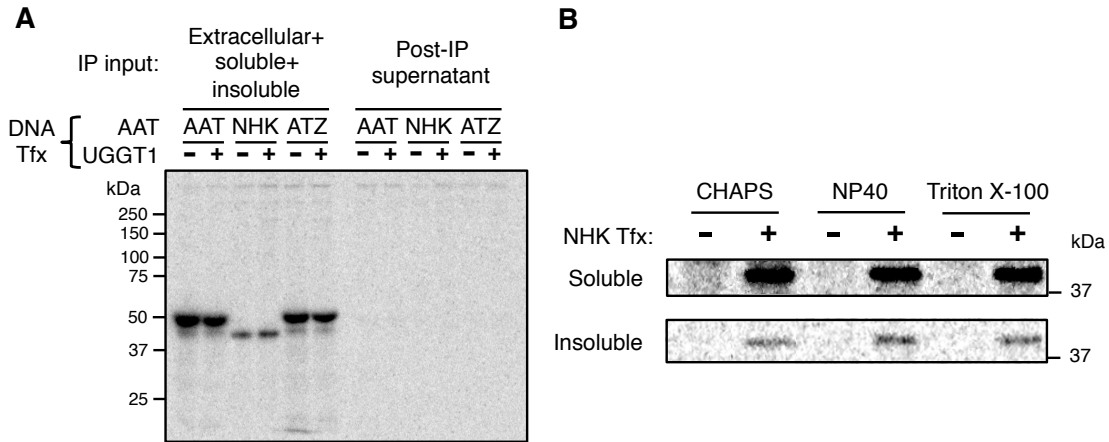


Figure 2.S1A The anti-alpha-1 antitrypsin (AAT) immunoprecipitation (IP) is almost 100% efficient

Equal fractions of cell media, soluble and insoluble fractions were pooled and subjected to one o/n immunoprecipitation (IP) with anti-AAT and Protein A-sepharose. Then, this IP supernatant was collected, remaining beads were washed 3X and resuspended with the 1% SDS/1% Triton X-100 method, and both the IP supernatant and SDS/Triton mixture were subjected to another round of anti-AAT IP. There was little to no AAT found in the IP supernatants.

Figure 2.S1B NHK is found in the insoluble pellet from three different non-ionic detergent lysis buffers

Equal fractions of soluble and insoluble fractions from cells lysed in CHAPS (2% w/v); Nonidet P-40 (1% v/v); or Triton X-100 (0.5% w/v) and sodium deoxycholate (0.5% w/v) were subjected to double IP with anti-AAT and Protein A-sepharose. Similar amounts of NHK were found in all three insoluble fractions.

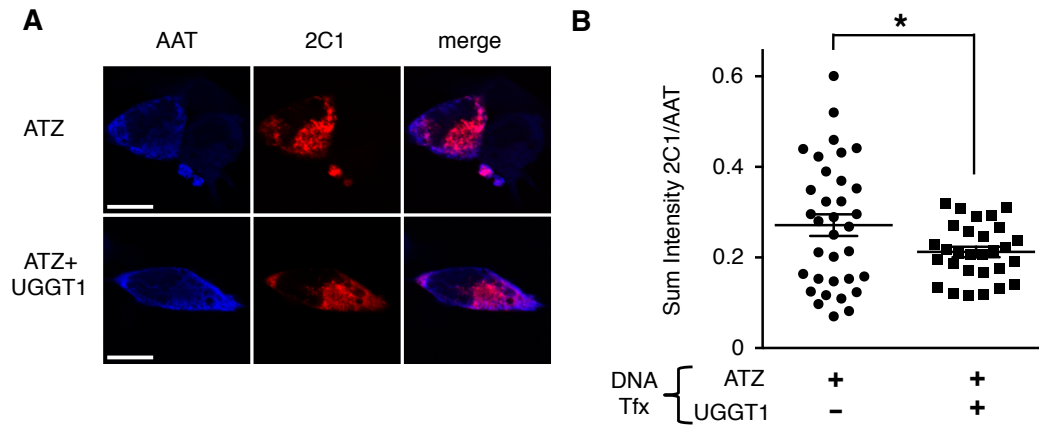


Figure 2.S2A The 2C1 mAb recognizes ATZ polymers

Uggt1^{-/-} MEFs were transfected with ATZ with or without UGGT1 complementation, fixed at 48 h post-transfection, and immunostained with anti-AAT and the polymer-specific mAb 2C1. Bar, 10 μ m.

Figure 2.S2B 2C1/ATZ ratio is reduced with UGGT1 cotransfection

The ratio of 2C1 mAb to polyclonal anti-AAT immunofluorescence in individual cells was quantified (Metamorph software), using Z-stack imaging to account for any difference in microscopic depth of the respective subcompartments in which ATZ accumulates. Cytoplasmic GFP co-expressed after an internal ribosomal entry sequence was used to define the full three-dimensional volume of each cell examined. Student's t-test was used for statistical testing (* = $p \leq 0.05$). Error bars represent SEM.

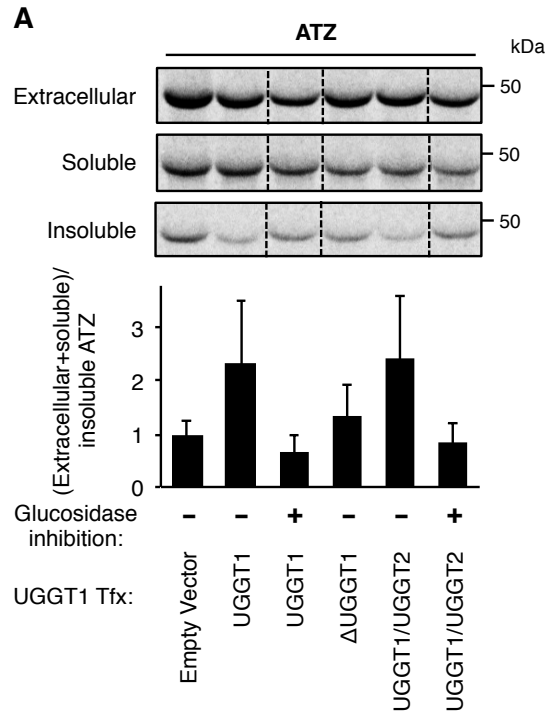


Figure 2.S3 Enhanced intracellular solubility of ATZ correlates with the presence of enzymatic UGGT1 monoglucosylation activity

After cotransfection with ATZ and UGGT1 engineered variants, 24 h metabolic labeling, and treatment with the glucosidase inhibitor castanospermine, total soluble/insoluble ratios of ATZ were determined. An increased total soluble/insoluble ratio was observed only when UGGT1 reglucosylation activity was expected, and this effect was blocked by glucosidase inhibition. Error bars represent range.

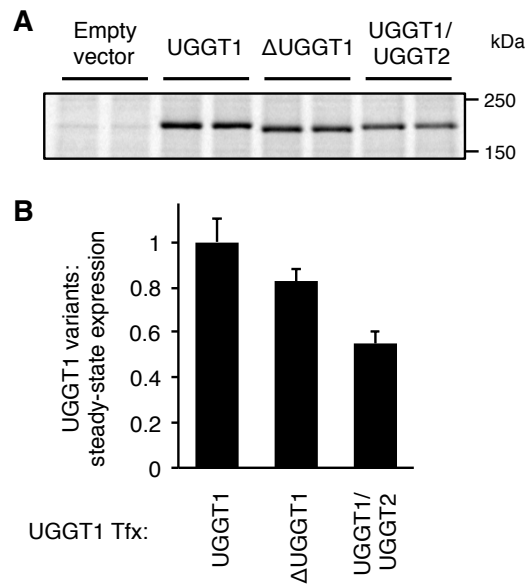


Figure 2.S4 UGGT1 engineered variants are expressed at comparable levels in *Uggt1*^{-/-} MEFs

Uggt1^{-/-} MEFs were transfected with expression vectors encoding UGGT1, Δ UGGT1, and UGGT1/UGGT2, and subjected to steady-state labeling analysis. Expression levels were determined by double anti-UGGT1 immunoprecipitation. Error bars represent SEM.

2.7 References

1. Anfinsen, C.B., *Principles that govern the folding of protein chains*. Science, 1973. **181**(4096): p. 223-30.
2. Braakman, I. and N.J. Buleid, *Protein folding and modification in the mammalian endoplasmic reticulum*. Annu Rev Biochem, 2011. **80**: p. 71-99.
3. Anelli, T. and R. Sitia, *Protein quality control in the early secretory pathway*. EMBO J, 2008. **27**(2): p. 315-27.
4. Hebert, D.N. and M. Molinari, *In and out of the ER: protein folding, quality control, degradation, and related human diseases*. Physiol Rev, 2007. **87**(4): p. 1377-408.
5. Trombetta, E.S. and A.J. Parodi, *Quality control and protein folding in the secretory pathway*. Annu Rev Cell Dev Biol, 2003. **19**: p. 649-76.
6. Ellgaard, L., M. Molinari, and A. Helenius, *Setting the standards: quality control in the secretory pathway*. Science, 1999. **286**(5446): p. 1882-8.
7. Hidvegi, T., et al., *An autophagy-enhancing drug promotes degradation of mutant alpha1-antitrypsin Z and reduces hepatic fibrosis*. Science, 2010. **329**(5988): p. 229-32.
8. Rampelt, H., et al., *Metazoan Hsp70 machines use Hsp110 to power protein disaggregation*. EMBO J, 2012. **31**(21): p. 4221-35.
9. Ishida, Y. and K. Nagata, *Autophagy eliminates a specific species of misfolded procollagen and plays a protective role in cell survival against ER stress*. Autophagy, 2009. **5**(8): p. 1217-9.
10. Hebert, D.N. and M. Molinari, *Flagging and docking: dual roles for N-glycans in protein quality control and cellular proteostasis*. Trends Biochem Sci, 2012. **37**(10): p. 404-10.
11. Helenius, A. and M. Aebl, *Roles of N-linked glycans in the endoplasmic reticulum*. Annu Rev Biochem, 2004. **73**: p. 1019-49.
12. Zapun, A., et al., *Enhanced catalysis of ribonuclease B folding by the interaction of calnexin or calreticulin with ERp57*. J Biol Chem, 1998. **273**(11): p. 6009-12.
13. Maattanen, P., et al., *Protein quality control in the ER: the recognition of misfolded proteins*. Semin Cell Dev Biol, 2010. **21**(5): p. 500-11.
14. Arnold, S.M., et al., *Two homologues encoding human UDP-glucose:glycoprotein glucosyltransferase differ in mRNA expression and enzymatic activity*. Biochemistry, 2000. **39**(9): p. 2149-63.
15. Molinari, M., et al., *Persistent glycoprotein misfolding activates the glucosidase II/UGT1-driven calnexin cycle to delay aggregation and loss of folding competence*. Mol Cell, 2005. **20**(4): p. 503-12.
16. Arnold, S.M. and R.J. Kaufman, *The noncatalytic portion of human UDP-glucose: glycoprotein glucosyltransferase I confers UDP-glucose binding*

- and transferase function to the catalytic domain.* J Biol Chem, 2003. **278**(44): p. 43320-8.
17. Sousa, M.C., M.A. Ferrero-Garcia, and A.J. Parodi, *Recognition of the oligosaccharide and protein moieties of glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase.* Biochemistry, 1992. **31**(1): p. 97-105.
 18. Sousa, M. and A.J. Parodi, *The molecular basis for the recognition of misfolded glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase.* EMBO J, 1995. **14**(17): p. 4196-203.
 19. Hebert, D.N., B. Foellmer, and A. Helenius, *Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum.* Cell, 1995. **81**(3): p. 425-33.
 20. Parodi, A.J., *Protein glucosylation and its role in protein folding.* Annu Rev Biochem, 2000. **69**: p. 69-93.
 21. D'Alessio, C., J.J. Caramelo, and A.J. Parodi, *UDP-Glc:glycoprotein glucosyltransferase-glucosidase II, the ying-yang of the ER quality control.* Semin Cell Dev Biol, 2010. **21**(5): p. 491-9.
 22. Perlmutter, D.H., *Pathogenesis of chronic liver injury and hepatocellular carcinoma in alpha-1-antitrypsin deficiency.* Pediatr Res, 2006. **60**(2): p. 233-8.
 23. Kim, P.S. and P. Arvan, *Endocrinopathies in the family of endoplasmic reticulum (ER) storage diseases: disorders of protein trafficking and the role of ER molecular chaperones.* Endocr Rev, 1998. **19**(2): p. 173-202.
 24. Rutishauser, J. and M. Spiess, *Endoplasmic reticulum storage diseases.* Swiss Med Wkly, 2002. **132**(17-18): p. 211-22.
 25. Schroder, M. and R.J. Kaufman, *ER stress and the unfolded protein response.* Mutat Res, 2005. **569**(1-2): p. 29-63.
 26. Choudhury, P., et al., *Intracellular association between UDP-glucose:glycoprotein glucosyltransferase and an incompletely folded variant of alpha1-antitrypsin.* J Biol Chem, 1997. **272**(20): p. 13446-51.
 27. Schmidt, B.Z. and D.H. Perlmutter, *Grp78, Grp94, and Grp170 interact with alpha1-antitrypsin mutants that are retained in the endoplasmic reticulum.* Am J Physiol Gastrointest Liver Physiol, 2005. **289**(3): p. G444-55.
 28. Gooptu, B., U.I. Ekeowa, and D.A. Lomas, *Mechanisms of emphysema in alpha1-antitrypsin deficiency: molecular and cellular insights.* Eur Respir J, 2009. **34**(2): p. 475-88.
 29. Liu, Y., et al., *Intracellular disposal of incompletely folded human alpha1-antitrypsin involves release from calnexin and post-translational trimming of asparagine-linked oligosaccharides.* J Biol Chem, 1997. **272**(12): p. 7946-51.
 30. Galli, C., et al., *Malectin participates in a backup glycoprotein quality control pathway in the mammalian ER.* PLoS One, 2011. **6**(1): p. e16304.

31. Kroeger, H., et al., *Endoplasmic reticulum-associated degradation (ERAD) and autophagy cooperate to degrade polymerogenic mutant serpins*. J Biol Chem, 2009. **284**(34): p. 22793-802.
32. Greenblatt, E.J., J.A. Olzmann, and R.R. Kopito, *Derlin-1 is a rhomboid pseudoprotease required for the dislocation of mutant alpha-1 antitrypsin from the endoplasmic reticulum*. Nat Struct Mol Biol, 2011. **18**(10): p. 1147-52.
33. Miranda, E., et al., *A novel monoclonal antibody to characterize pathogenic polymers in liver disease associated with alpha1-antitrypsin deficiency*. Hepatology, 2010. **52**(3): p. 1078-88.
34. Solda, T., et al., *Substrate-specific requirements for UGT1-dependent release from calnexin*. Mol Cell, 2007. **27**(2): p. 238-49.
35. Tokunaga, F., et al., *Endoplasmic reticulum (ER)-associated degradation of misfolded N-linked glycoproteins is suppressed upon inhibition of ER mannosidase I*. J Biol Chem, 2000. **275**(52): p. 40757-64.
36. Brodsky, J.L., *The protective and destructive roles played by molecular chaperones during ERAD (endoplasmic-reticulum-associated degradation)*. Biochem J, 2007. **404**(3): p. 353-63.
37. Hosokawa, N., et al., *Stimulation of ERAD of misfolded null Hong Kong alpha1-antitrypsin by Golgi alpha1,2-mannosidases*. Biochem Biophys Res Commun, 2007. **362**(3): p. 626-32.
38. Malhotra, J.D. and R.J. Kaufman, *The endoplasmic reticulum and the unfolded protein response*. Semin Cell Dev Biol, 2007. **18**(6): p. 716-31.
39. Dorner, A.J., D.G. Bole, and R.J. Kaufman, *The relationship of N-linked glycosylation and heavy chain-binding protein association with the secretion of glycoproteins*. J Cell Biol, 1987. **105**(6 Pt 1): p. 2665-74.
40. Dorner, A.J., L.C. Wasley, and R.J. Kaufman, *Increased synthesis of secreted proteins induces expression of glucose-regulated proteins in butyrate-treated Chinese hamster ovary cells*. J Biol Chem, 1989. **264**(34): p. 20602-7.
41. Graham, K.S., A. Le, and R.N. Sifers, *Accumulation of the insoluble PiZ variant of human alpha 1-antitrypsin within the hepatic endoplasmic reticulum does not elevate the steady-state level of grp78/BiP*. J Biol Chem, 1990. **265**(33): p. 20463-8.
42. Hidvegi, T., et al., *Regulator of G Signaling 16 is a marker for the distinct endoplasmic reticulum stress state associated with aggregated mutant alpha1-antitrypsin Z in the classical form of alpha1-antitrypsin deficiency*. J Biol Chem, 2007. **282**(38): p. 27769-80.
43. Hidvegi, T., et al., *Accumulation of mutant alpha1-antitrypsin Z in the endoplasmic reticulum activates caspases-4 and -12, NFkappaB, and BAP31 but not the unfolded protein response*. J Biol Chem, 2005. **280**(47): p. 39002-15.
44. Balch, W.E., et al., *Adapting proteostasis for disease intervention*. Science, 2008. **319**(5865): p. 916-9.

45. Wu, J. and R.J. Kaufman, *From acute ER stress to physiological roles of the Unfolded Protein Response*. Cell Death Differ, 2006. **13**(3): p. 374-84.
46. Menon, S., et al., *Oxidoreductase interactions include a role for ERp72 engagement with mutant thyroglobulin from the rdw/rdw rat dwarf*. J Biol Chem, 2007. **282**(9): p. 6183-91.
47. Wang, S. and R.J. Kaufman, *The impact of the unfolded protein response on human disease*. J Cell Biol, 2012. **197**(7): p. 857-67.
48. Cannon, K.S. and A. Helenius, *Trimming and readdition of glucose to N-linked oligosaccharides determines calnexin association of a substrate glycoprotein in living cells*. J Biol Chem, 1999. **274**(11): p. 7537-44.
49. Hammond, C., I. Braakman, and A. Helenius, *Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control*. Proc Natl Acad Sci U S A, 1994. **91**(3): p. 913-7.
50. Zuber, C., et al., *EDEM1 reveals a quality control vesicular transport pathway out of the endoplasmic reticulum not involving the COPII exit sites*. Proc Natl Acad Sci U S A, 2007. **104**(11): p. 4407-12.
51. Nakatsukasa, K. and J.L. Brodsky, *The recognition and retrotranslocation of misfolded proteins from the endoplasmic reticulum*. Traffic, 2008. **9**(6): p. 861-70.
52. Molinari, M., et al., *Sequential assistance of molecular chaperones and transient formation of covalent complexes during protein degradation from the ER*. J Cell Biol, 2002. **158**(2): p. 247-57.
53. Cameron, P.H., et al., *Calnexin phosphorylation attenuates the release of partially misfolded alpha1-antitrypsin to the secretory pathway*. J Biol Chem, 2009. **284**(50): p. 34570-9.
54. Oda, Y., et al., *EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin*. Science, 2003. **299**(5611): p. 1394-7.
55. Tirasophon, W., A.A. Welihinda, and R.J. Kaufman, *A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endoribonuclease (Ire1p) in mammalian cells*. Genes Dev, 1998. **12**(12): p. 1812-24.

Chapter 3

Conclusions

3.1 Introduction

Protein folding in the endoplasmic reticulum (ER) is an orchestrated process, where a variety of resident ER proteins assist with the correct folding of substrate proteins. Molecular chaperones and folding catalysts facilitate the advancement of substrate proteins towards a native folded state, without becoming engaged in the final structure of the substrate[1, 2]. The presence of chaperones and the extremely high protein concentration in the ER limits the ability of in vitro protein folding studies to accurately reflect protein folding within the ER environment[3]. Chaperone function results in protein folding assistance by limiting the formation of incorrectly folded 'off-pathway' structures, thereby improving kinetics of appearance of 'on-pathway' intermediates that lead to the native state[2]. Protein aggregates are examples of incorrectly folded 'off-pathway' structures, and experiments have shown that molecular chaperones can reduce the propensity of substrate proteins to aggregate, both in vitro and in cells[2].

Protein aggregates are medically relevant, as they are thought to play a pathogenic role in multiple human disease states, including neurodegenerative diseases and alpha-1 antitrypsin (AAT) deficiency. These aggregates can be experimentally detected as insoluble species in cell lysis buffers based on nonionic detergents. Such aggregates can easily reach both a large size and density, and can often be isolated merely by centrifugation, to separate crude cell lysates into soluble supernatant and detergent-insoluble pellet fractions. I have

utilized this simple, reproducible method to study the role of UGGT1 in maintaining solubility of glycoproteins in the ER, using wild-type AAT and two misfolded AAT variants as model glycoproteins. The Z allele (ATZ) variant has a single missense mutation (K342E) that results in formation of insoluble ordered polymers in hepatocytes, and this has been observed both in vivo and in cell culture[4]. The null Hong Kong (NHK) variant harbors a nonsense mutation that leads to truncation of the carboxy terminus of the protein[5]. NHK also becomes insoluble in cells, however, its truncation precludes ordered polymer formation. Instead, it is thought that insoluble NHK forms disordered protein aggregates[6]. In contrast, wt-AAT forms neither ordered polymers nor disordered aggregates, and cannot be detected as an insoluble species under normal conditions. One feature shared between wild-type AAT, ATZ, and NHK is that they each contain three N-linked glycans.

Processing of N-linked glycans on glycoproteins in the ER is an intricate process that is critical for maintaining homeostasis of the glycoproteome, including folding, trafficking, and disposal of distinct components. UDP-glucose:glycoprotein glucosyltransferase (UGGT1) is central to these processes. As a critical component of glycoprotein ER quality control (ERQC), UGGT1 acts as a 'folding sensor', specifically reglucosylating intermediates in glycoprotein folding. This activity is required for lectin-like reassociation with ER chaperones calreticulin (CRT) and calnexin (CNX), which for some glycoprotein substrates can assist in additional folding attempts, for other substrates can promote export from the ER, and for yet other substrates may either limit or promote ERAD[7]. A central idea conveyed by this dissertation is that promotion of glycoprotein interactions with lectin-like ER chaperones assists in keeping these substrates in a soluble state, which is necessary for delivery of these substrates to their respective biological fates. In contrast, defects in the reglucosylation of N-linked glycoprotein substrates, and impaired interaction with lectin-like ER chaperones, may enhance formation of insoluble glycoprotein aggregates. Almost certainly, this is a problem for the cell, leading to specialized coping mechanisms, including

sequestration, degradation, and possibly resolubilization, of such aggregates. Failure of such mechanisms may underlie proteotoxicity, and prevention of insolubility is likely to enhance cell survival. The effect of UGGT1 on the solubility, folding, and delivery of glycoprotein substrates may well explain why UGGT1 is essential for embryo survival, as there is reason to believe that failure of reglucosylation underlies severe placental vascular insufficiency, which may be responsible for the embryonic lethal phenotype of *Uggt1*^{-/-} mice at E13 (Figure 3.1). The other components of the calnexin cycle are also essential for mammalian development, as whole-body genetic deletion of CRT, CNX, and ERp57 in mice results in embryonic or perinatal lethality[8-10].

To study the effect of UGGT1 on solubility of NHK and ATZ, *Uggt1*^{-/-} MEFs transiently expressing these substrates, in the presence or absence of UGGT1 complementation, were labeled with [³⁵S]-methionine/cysteine to approach steady state. This long-term labeling allowed for recovery of chemical quantities of substrate in three different pools: 1) secreted (extracellular), 2) intracellular soluble, and 3) detergent-insoluble. Amounts of AAT substrates in each fraction were measured by quantitative anti-AAT immunoprecipitation (Chapter 2.4, Materials and methods). To exclude differences in substrate protein expression (synthesis) between individual samples, each transfected sample was analyzed in parallel by short-term pulse-labeling, so that the steady state amounts recovered could be normalized to substrate synthesis from the same transfection. The actual experimental results have been described in Chapter 2; in this chapter I will highlight key findings and propose future studies that emerge from these findings.

3.2 Similarities and differences in the effect of UGGT1 activity on ER trafficking of NHK and ATZ

For both NHK and ATZ, the ratio of total soluble (extracellular+intracellular soluble)/insoluble substrate increases when UGGT1 is coexpressed. UGGT1

cotransfection significantly shifts the pool sizes of both NHK and ATZ towards the soluble intracellular fraction (Chapter 2, Figures 2.1A, 2.1B). However, closer inspection of the outcome for these two substrates reveals that UGGT1 complementation shifts these pool sizes in different ways. For NHK, UGGT1-mediated enrichment of the intracellular soluble pool derives from a combination of decreased escape to the endoglycosidase H-resistant, extracellular (secreted) fraction (indicative of transport to and through the Golgi complex) and from decreased entry to the insoluble fraction (Figure 2.1A, middle panel; Figure 2.4B). For ATZ, the activity of UGGT1 promotes a decrease in the detergent-insoluble fraction—that is thought to represent loop-sheet polymerized ATZ—but no change in ATZ secretion (Figure 2.1B, middle panel). Thus, UGGT1 acts as an ERQC factor in both cases, enhancing solubility, but the consequences of that solubility are different for the two substrates.

The mechanism(s) underlying the different consequences of UGGT1-mediated reglucosylation on these two glycoprotein substrates is not clear, but it is likely to involve intrinsic differences in substrate folding states. Consider that the two substrate pools, extracellular and insoluble intracellular, represent the end point of two different exit paths from the soluble intracellular pool. Then, consider that UGGT1 acts as a folding sensor for molecules traveling on either of these two distinct pathways. And finally, consider that UGGT1 has optimal recognition of near-native intermediate folding forms in comparison to other distinct folding forms on opposite ends of the folding spectrum, i.e., native or severely misfolded[11, 12].

Thus, it is tempting to speculate that at ER exit sites, near-native (but nevertheless mutant) NHK molecules are recognized by UGGT1, reglucosylated, and returned to the ER pool through iterative interaction with CRT/CNX[13]. It is similarly tempting to speculate that NHK folding forms approaching the ERQC/ERAC compartment are badly misfolded, so that only a small percentage of these molecules can be recognized by UGGT1 and reglucosylated, limiting

ERAD. Furthermore, I hypothesize that the majority of NHK molecules that are already engaged in protein aggregates are not recognized by UGGT1 at all.

For ATZ, the situation might be that molecules delivered to ER exit sites appear as well-folded substrate such that they pass through the UGGT1-mediated ERQC checkpoint (this idea is supported by the fact that exported ATZ has biological activity[14]), and once beyond the ER, ATZ is allowed to be secreted. But the ATZ folding forms approaching the ERQC/ERAC—which could include small (dimer, trimer, oligomer) forms of ATZ—are recognized by UGGT1, reglucosylated, and returned to the soluble fraction, presumably via renewed interaction with CRT/CNX.

Thus, I propose that the difference in UGGT1-mediated trafficking of NHK and ATZ is based on the folding status of subpopulations of each substrate when they encounter UGGT1. However, I must acknowledge an alternative explanation, namely, that NHK expression causes ER stress and UPR activation whereas ATZ expression does not. This could result in different amounts of ER chaperones in cells expressing the different substrates. As a consequence, when NHK or ATZ molecules are reglucosylated by UGGT1 and associate with CRT/CNX, there could be different subsets of additional chaperones recruited to this complex, which could mediate decreased secretion or decreased polymerization, respectively.

This alternative explanation would likely benefit from testing UGGT1-mediated trafficking effects on other luminal ER glycoproteins, to determine if the trafficking differences between NHK and ATZ are limited to these two substrates or are a more general phenomenon. The hepatic toxicity due to ATZ accumulation in alpha-1 antitrypsin disease has been suggested to be analogous to the brain toxicity caused by accumulation of neuroserpin mutants in the familial dementia known as FENIB (familial encephalopathy with neuroserpin inclusion bodies). These mutant neuroserpins are ER luminal glycoproteins that form ordered polymers similar to those of ATZ and as such, could represent another good model for testing UGGT1-dependent trafficking changes[15]. For NHK,

another well-studied ER luminal substrate that is also glycosylated, but not thought to form ordered polymers, is an engineered soluble form of beta-site amyloid precursor protein-cleaving enzyme 1 (BACE)[16]. Studies on these and other model glycoprotein substrates would allow for an understanding of whether the differences in UGGT1-mediated trafficking of NHK and ATZ are unique to these two molecules.

Finally, in my analysis, I assume that once these substrates are released to the media, they do not return to either intracellular pool. Importantly, at the conclusion of pulse-labeling, the entirety of newly-synthesized NHK and ATZ begins in the soluble fraction. Thus, over the course of time as iterative rounds of newly-made NHK and ATZ progress towards their steady state distributions, a fraction of these substrates must transition to the detergent-insoluble pool. However, it remains unknown if substrate from the insoluble pool can be resolubilized and return to the soluble pool. Such a thing is possible, as there is precedent for aggregate dissolution, both in bacteria and in eukaryotic cells[17-19].

3.3 UGGT1 mediates a major fraction of the N-glycosylation solubility effect

Experiments with N-glycosylation site mutants of NHK demonstrate that a major portion of the solubility increase of NHK on N-glycan addition is UGGT1-dependent. Additionally, each of the NHK N-glycans contribute to solubility, and each provides a fairly similar contribution (Chapter 2, Figure 2.6). These results are analogous to those found in a study of secretion of BACE variants with combinations of four N-glycosylation sites mutated; each of which showed similar effects[16]. One study showed that in isolated microsomes, two N-glycans in close proximity were necessary for efficient formation of monoglucosylated N-glycans[20]. Both the study of BACEs and the present study of NHK N-glycosylation mutants show that in cells, singly-glycosylated substrates can still

enter the calnexin cycle, strongly suggesting UGGT1-dependent monoglucosylation of solo N-glycans.

The finding relating N-glycan addition to substrate solubility may have important implications for strategies to improve secretion of protein therapeutics by adding new N-glycosylation sites into naïve sites[21, 22]. Studies to date that have used Sf9 insect cells to produce model substrates are concentrated on mainly Man₃GlcNAc₂ N-glycans—the major oligosaccharide form transferred by Sf9 OST—for which re-entry into the calnexin cycle is not possible, because they lack the terminal A-chain mannose necessary for UGGT1-mediated reglucosylation[23]. Although addition of novel N-glycosylation sites does increase glycoprotein stability and decrease propensity to aggregate in vitro, it is unclear what the effect of adding novel N-glycosylation sites to glycoproteins would have in the context of the CNX/CRT/UGGT1 ERQC system in mammalian cells.

3.4 Future directions

One of the challenges in the field of glycoprotein ERQC is forming general theories about trafficking from separate studies on disparate model proteins, as it has been shown repeatedly that different substrates can meet quite different fates in the same cell[24] and the same substrate can meet different fates in different cells[25]. This is especially apparent for our understanding of aggregate handling and degradation of ER glycoproteins. A strategy used by Kopito's group to understand cytoplasmic protein misfolding and aggregation made use of multiple substrates with different properties: 1) a mutant protein that misfolds at nonpermissive temperatures, 2) a protein subunit lacking its usual partner, and 3) amyloidogenic substrates[26]. Each of these substrates was tagged with a fluorescent label, conditionally expressed in cells, with intracellular localization followed by microscopy. By expressing the different substrates simultaneously in the same cell, they were able to show that the amyloidogenic substrates

accumulated only in one cytosolic subregion (IPODs in which the substrates were largely immobile) while other misfolding substrates could accumulate in IPODs or another cytosolic subregion, the JUNQ compartment (in which the substrates underwent rapid exchange with the cytosol)[26].

A similar strategy could be employed to better understand glycoprotein misfolding and aggregation in the ER. Continuing with the same substrates used in this thesis, fluorescent tags could be added to NHK and ATZ, and then expressed using a conditional expression system to provide more control over the timing of expression than with transient transfection. These two substrates could then be expressed in the same cells, and the fluorescent signal observed by microscopy to determine if the two substrates are accumulating in the same compartment, possibly the ERQC/ERAC compartment. This experimental paradigm could then be manipulated with proteasome or autophagy inhibitors to determine whether blockade of either of these degradation pathways results in a build-up of substrate in any specific cytoplasmic location, or in the ER. Also, the same method could also be used in *Uggt1*^{-/-} MEFs with and without UGGT1 cotransfection, to determine if UGGT1 affects the localization of these substrates in the ER, qualitatively and quantitatively. Additionally, luminal, transmembrane or multi-subunit proteins could be utilized here, as well as proteins with N-glycosylation sites mutated.

3.5 Closing remarks

Production of correctly folded glycoproteins is a highly regulated process, centered on modification and recognition of protein bound N-glycans. In this thesis, I have shown that the folding sensor enzyme UDP-glucose:glycoprotein glucosyltransferase (UGGT1) plays a critical role in this process for two misfolded alpha-1 antitrypsin variants, NHK and ATZ. Importantly, I have provided evidence that at least for NHK, simple addition of N-glycans to the protein provides some increased solubility, but engagement of the calnexin cycle

chaperone system by UGGT1 maximizes solubility. UGGT1-mediated monoglucosylation of these substrates appears to play a significant role in enhancing intracellular protein solubility, as detergent-insoluble fractions were found to completely lack monoglucosylated glycans. These findings and further studies of glycoprotein ERQC should help us to better understand ER storage diseases like alpha-1 antitrypsin deficiency, and produce new therapeutic avenues for treatment.

3.6 Figures

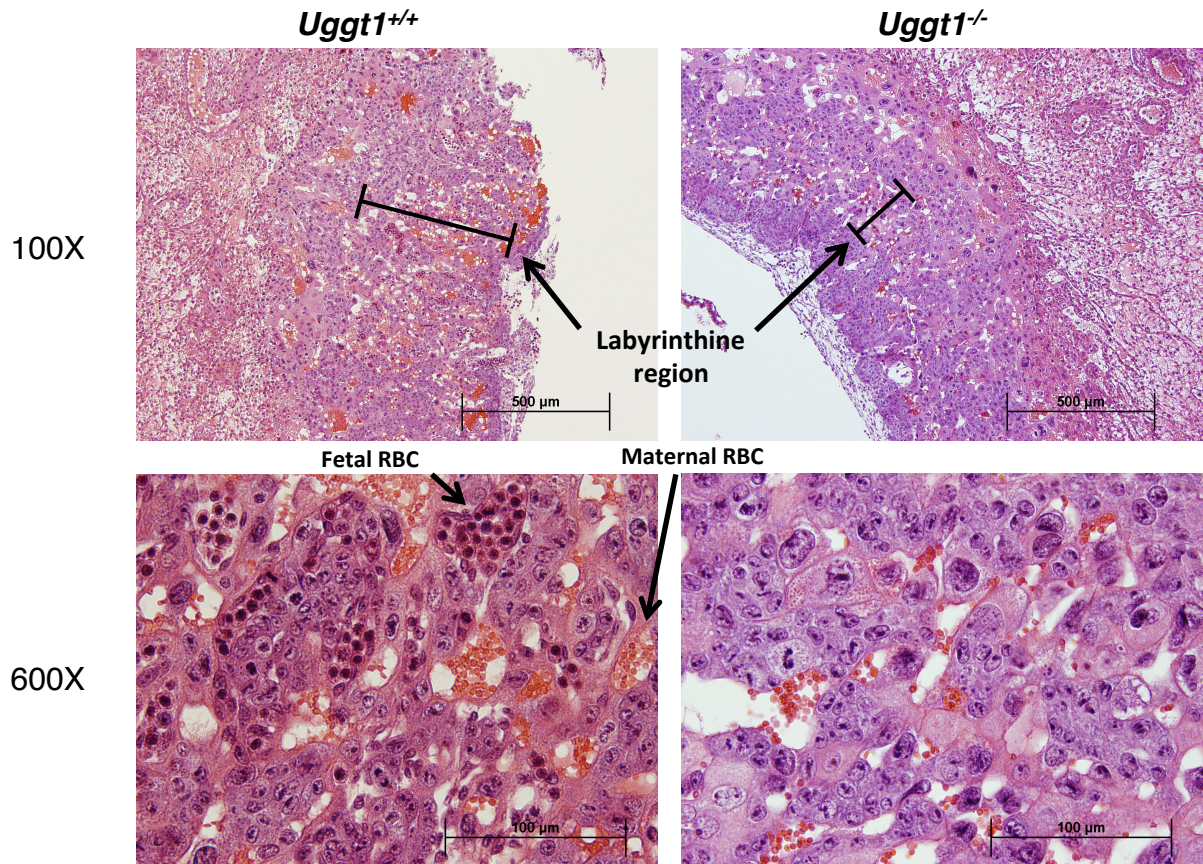


Figure 3.1 Vascular insufficiency in *Ugg1^{-/-}* placenta

Hematoxylin and eosin (H&E) staining of placental sections associated with E11.0 *Ugg1^{+/+}* or *Ugg1^{-/-}* mouse embryos. The labyrinthine region of *Ugg1^{-/-}* placenta is small and underdeveloped, with a significant reduction in fetal blood vessels, observed through specific lack of nucleated fetal red blood cells.

3.7 References

1. Ellis, R.J., *Molecular chaperones: assisting assembly in addition to folding*. Trends Biochem Sci, 2006. **31**(7): p. 395-401.
2. Ellis, R.J. and S.M. Hemmingsen, *Molecular chaperones: proteins essential for the biogenesis of some macromolecular structures*. Trends Biochem Sci, 1989. **14**(8): p. 339-42.
3. Vendruscolo, M., *Proteome folding and aggregation*. Curr Opin Struct Biol, 2012. **22**(2): p. 138-43.
4. Kamimoto, T., et al., *Intracellular inclusions containing mutant alpha1-antitrypsin Z are propagated in the absence of autophagic activity*. J Biol Chem, 2006. **281**(7): p. 4467-76.
5. Sifers, R.N., et al., *A frameshift mutation results in a truncated alpha 1-antitrypsin that is retained within the rough endoplasmic reticulum*. J Biol Chem, 1988. **263**(15): p. 7330-5.
6. Greenblatt, E.J., J.A. Olzmann, and R.R. Kopito, *Derlin-1 is a rhomboid pseudoprotease required for the dislocation of mutant alpha-1 antitrypsin from the endoplasmic reticulum*. Nat Struct Mol Biol, 2011. **18**(10): p. 1147-52.
7. Svedine, S., et al., *Carbohydrates act as sorting determinants in ER-associated degradation of tyrosinase*. J Cell Sci, 2004. **117**(Pt 14): p. 2937-49.
8. Mesaeli, N., et al., *Calreticulin is essential for cardiac development*. J Cell Biol, 1999. **144**(5): p. 857-68.
9. Denzel, A., et al., *Early postnatal death and motor disorders in mice congenitally deficient in calnexin expression*. Mol Cell Biol, 2002. **22**(21): p. 7398-404.
10. Coe, H., et al., *ERp57 modulates STAT3 signaling from the lumen of the endoplasmic reticulum*. J Biol Chem, 2010. **285**(9): p. 6725-38.
11. Pearce, B.R., et al., *A cell-based reglucosylation assay demonstrates the role of GT1 in the quality control of a maturing glycoprotein*. J Cell Biol, 2008. **181**(2): p. 309-20.
12. Caramelo, J.J., et al., *The endoplasmic reticulum glucosyltransferase recognizes nearly native glycoprotein folding intermediates*. J Biol Chem, 2004. **279**(44): p. 46280-5.
13. Avezov, E., et al., *Endoplasmic reticulum (ER) mannosidase I is compartmentalized and required for N-glycan trimming to Man5-6GlcNAc2 in glycoprotein ER-associated degradation*. Mol Biol Cell, 2008. **19**(1): p. 216-25.
14. Lomas, D.A., et al., *Effect of the Z mutation on the physical and inhibitory properties of alpha 1-antitrypsin*. Biochemistry, 1993. **32**(2): p. 500-8.
15. Kroeger, H., et al., *Endoplasmic reticulum-associated degradation (ERAD) and autophagy cooperate to degrade polymerogenic mutant serpins*. J Biol Chem, 2009. **284**(34): p. 22793-802.

16. Vanoni, O., P. Paganetti, and M. Molinari, *Consequences of individual N-glycan deletions and of proteasomal inhibition on secretion of active BACE*. *Mol Biol Cell*, 2008. **19**(10): p. 4086-98.
17. Tagliavacca, L., Q. Wang, and R.J. Kaufman, *ATP-dependent dissociation of non-disulfide-linked aggregates of coagulation factor VIII is a rate-limiting step for secretion*. *Biochemistry*, 2000. **39**(8): p. 1973-81.
18. Tkach, J.M. and J.R. Glover, *Amino acid substitutions in the C-terminal AAA+ module of Hsp104 prevent substrate recognition by disrupting oligomerization and cause high temperature inactivation*. *J Biol Chem*, 2004. **279**(34): p. 35692-701.
19. Rosenzweig, R., et al., *Unraveling the mechanism of protein disaggregation through a ClpB-DnaK interaction*. *Science*, 2013. **339**(6123): p. 1080-3.
20. Deprez, P., M. Gautschi, and A. Helenius, *More than one glycan is needed for ER glucosidase II to allow entry of glycoproteins into the calnexin/calreticulin cycle*. *Mol Cell*, 2005. **19**(2): p. 183-95.
21. Culyba, E.K., et al., *Protein native-state stabilization by placing aromatic side chains in N-glycosylated reverse turns*. *Science*, 2011. **331**(6017): p. 571-5.
22. Price, J.L., et al., *N-glycosylation of enhanced aromatic sequons to increase glycoprotein stability*. *Biopolymers*, 2012. **98**(3): p. 195-211.
23. Aeed, P.A. and A.P. Elhammer, *Glycosylation of recombinant prorenin in insect cells: the insect cell line Sf9 does not express the mannose 6-phosphate recognition signal*. *Biochemistry*, 1994. **33**(29): p. 8793-7.
24. Helenius, A. and M. Aebi, *Roles of N-linked glycans in the endoplasmic reticulum*. *Annu Rev Biochem*, 2004. **73**: p. 1019-49.
25. Cabral, C.M., et al., *Organizational diversity among distinct glycoprotein endoplasmic reticulum-associated degradation programs*. *Mol Biol Cell*, 2002. **13**(8): p. 2639-50.
26. Kaganovich, D., R. Kopito, and J. Frydman, *Misfolded proteins partition between two distinct quality control compartments*. *Nature*, 2008. **454**(7208): p. 1088-95.