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# NEW INSIGHTS INTO THE ROLE OF THE UDP-GLUCOSE: GLYCOPROTEIN GLUCOSYLTRANSFERASE 1 IN THE ENDOPLASMIC RETICULUM QUALITY CONTROL

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**NEW INSIGHTS INTO THE ROLE OF THE UDP-GLUCOSE:  
GLYCOPROTEIN GLUCOSYLTRANSFERASE 1 IN THE ENDOPLASMIC  
RETICULUM QUALITY CONTROL**

A Dissertation Presented

by

ABLA TANNOUS

Submitted to the Graduate School of the  
University of Massachusetts Amherst in partial fulfillment  
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Molecular and Cellular Biology

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## DEDICATION

To my wonderful mother Jacqueline Haidar  
and my loving Husband Hadi-El Farr

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## **ABSTRACT**

### **NEW INSIGHTS INTO THE ROLE OF THE UDP-GLUCOSE: GLYCOPROTEIN GLUCOSYLTRANSFERASE 1 IN THE ENDOPLASMIC RETICULUM QUALITY CONTROL**

SEPTEMBER 2015

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The UDP-glucose:glycoprotein glucosyltransferase 1 (UGT1) is a central quality control factor in the Endoplasmic Reticulum (ER). It surveys the folding status of proteins in the ER and redirects them, via its reglucosylation activity, to bind to the ER carbohydrate binding (lectin) chaperones calreticulin (CRT) and calnexin (CNX). However, the cellular mechanism of UGT1 is not completely understood. Using a cell based reglucosylation assay, we found that UGT1 reglucosylated proteins that eventually fold. This modification was transient and resulted in delay of protein trafficking in the secretory pathway and prolonged binding to lectin chaperones in the ER. In addition, terminally misfolded substrates, disease associated mutants or proteins with reduced disulfides were reglucosylated by UGT1. Yet, this reglucosylation, despite being efficient and persistent, did not affect their degradation by the ER-associated degradation pathway. This suggested that degradation occurred independently of the glucose containing branch on the substrate's glycan. Further investigation showed that trimming mannose residues of the substrate's glycan is responsible for terminating the substrate reglucosylation by UGT1 in the ER. Moreover, our preliminary data suggests that the free cysteines of the



substrate might aid in its recognition and persistent reglucosylation by UGT1. Thus, our strategy unraveled new details of the role of UGT1 in ER quality control that further highlight its importance in protein maturation.

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# CHAPTER 1

## INTRODUCTION

*(Sections from this chapter are adapted from (Tannous et al., 2015b))*

### **Cellular folding**

The fundamental principles of folding described by Anfinsen state that the native structure of a protein is its most thermodynamically stable form and is dictated by its linear amino-acid sequence (Anfinsen, 1973). While many of these principles were acquired by studying the refolding of denatured proteins in the test tube, understanding how proteins fold in the cellular environment is complicated by many factors. Proteins fold in a highly crowded cellular environment, which may result in less efficient folding, and high cellular protein concentrations can lead to aggregation. Moreover, cellular stresses such as extreme temperatures or mutations contribute to hindering folding efficiency. However, other cellular factors can increase folding efficiency, such as folding co-translationally and the presence of chaperones. (Powers et al., 2009).

Folding can commence as the protein is being synthesized, thus the rate of translation can be a determining factor in the folding pathway (Fedorov and Baldwin, 1997) (Figure 1.1). Co-translational folding limits the conformations that a protein may sample due to its tethering to the ribosome, thus assisting the protein to more rapidly and efficiently reach its native state (Pearse and Hebert, 2010). Additionally, chaperones and folding enzymes largely assist cellular folding. As a result of the abovementioned factors, even when proteins fail to fold on their own in the test tube, they could fold more efficiently in the complex cellular environment (Rothman and Schekman, 2011).

## **Protein homeostasis and diseases**

Maintaining protein homeostasis is critical for retaining normal cellular functions and its disruption can lead to disease. Some of these can be described as loss of function diseases (Powers et al., 2009). A well-studied example is cystic fibrosis, which is caused by reduced targeting of functional cystic fibrosis transmembrane conductance regulator (CFTR) to the plasma membrane due to a mutation in CFTR that disrupts its folding pathway resulting in its retention in the endoplasmic reticulum (ER) and subsequent degradation (Jensen et al., 1995). In other instances, disease results from the abnormal accumulation of misfolded proteins that evade the cellular degradation pathways and result in buildup of toxic aggregates. Neurodegenerative diseases such Alzheimer's, Parkinson's and Huntington's are described as conformational diseases that are the consequence of different forms of aggregates or cellular inclusions (Takalo et al., 2013).

## **Targeting to the ER and signal sequence cleavage**

Approximately one third of the proteome traverses the secretory pathway, and this begins with targeting to the ER (Stolz and Wolf, 2010). The ER is a highly specialized and organized organelle where protein folding and quality control occur, amongst a multitude of other vital functions. The ER harbors an arsenal of folding and quality control factors essential to maintaining the cellular protein homeostasis network (Figure 1.1). These factors include carbohydrate binding chaperones or lectin chaperones, classical chaperones, oxidoreductases and peptidyl prolyl isomerases, all of which optimize folding efficiency. While properly folded proteins are targeted for ER exit to traffic through the remainder of the secretory pathway, proteins that are deemed

misfolded are usually retained in the ER and are degraded through the process of ER associated degradation (ERAD) involving the ubiquitin-proteasome system (Figure 1.1) (Tamura et al., 2010).

Proteins that are targeted to the ER possess an N-terminal signal sequence, constituted of approximately the first 25 amino acids (Hortsch et al., 1986; Migliaccio et al., 1992; Braakman and Hebert, 2013). Signal sequences are recognized by the signal recognition particle (SRP), a cytosolic ribonucleoprotein. SRP targets the ribosome/mRNA complex to the ER by binding to the SRP receptor, an integral membrane protein of the ER (Walter et al., 1984). The ribosome associates with the Sec61 translocon pore (Figures 1.1 and 1.3), which allows proteins to translocate through the pore and enter the ER lumen. Signal sequence cleavage is critical for the maturation of a protein and is generally accepted to occur co-translationally, but may also occur post-translationally (Braakman and Hebert, 2013; Daniels et al., 2003; Tamura et al., 2011). The timing of signal sequence cleavage may also influence folding, as tethering a protein to the membrane can constrain the nascent chain and help in directing the folding process (Braakman and Hebert, 2013; Daniels et al., 2003; Tamura et al., 2011).

### **N-Glycans direct protein folding**

Proteins that contain the consensus glycosylation site Asn-X-Ser/Thr are modified in the ER by the addition of a 14-membered carbohydrate composed of three glucoses, nine mannoses and two N-acetyl glucosamines ( $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ ) (Figure 1.2). These N-linked glycans are added *en bloc* by the oligosaccharyltransferase (OST) complex, from dolichol pyrophosphate lipid carriers (Burda and Aeby, 1999) (Figure 1.3, Step 1).



The OST is a large multimeric complex, and the catalytic subunit of the eukaryotic OST is STT3, which in mammals has two forms: STT3A and STT3B (Mohorko et al., 2011). Glycans are usually added co-translationally by an OST complex containing STT3A; however, the STT3B isoform can add glycans post-translationally on skipped sites, usually found at the C-termini of glycoproteins (Sato et al., 2012). Of note, defects in the glycan synthesis pathway or decreased levels of the catalytic subunit STT3B have been associated with hypoglycosylation leading to congenital disorders of glycosylation (CDG) (Mohorko et al., 2011; Shrimal and Gilmore, 2015).

Addition of glycans has a direct impact on the proper folding of proteins (O'Connor and Imperiali, 1998; Hebert et al., 2014). Glycans are large hydrophilic groups that can change the biophysical properties of the modified protein. Glycan addition may cause the protein to sample conformations that are more compact or more closely resemble the native structure (O'Connor and Imperiali, 1998; Imperiali and O'Connor, 1999; Chen et al., 2010). Glycans can also increase a protein's solubility and prevent it from forming aggregates (Sola and Griebenow, 2009). In addition, glycans can protect proteins against proteolysis. This is proposed to be accomplished either by shielding proteolysis sites from proteases by the glycan, or increasing the overall protein stability (Russell et al., 2009). These properties make glycans crucial elements in the folding and maturation of secretory proteins.

### **Chaperones of the ER**

Chaperones that are resident in the ER are classified into two families: classical chaperones and lectin or carbohydrate-binding chaperones. While classical chaperones

function independently of glycans, lectin chaperones require their presence. Classical chaperones recognize hydrophobic regions that are usually buried in the folded form of the native substrate. The ER contains classical chaperones from the Hsp70 and the Hsp 90 families (Brodsky and Skach, 2011). One of the most abundant chaperones in the ER is the Hsp70 BiP (Immunoglobulin heavy chain binding protein). Hsp 70s contain a substrate binding domain connected to a nucleotide binding domain via a linker region, and thus their activity is regulated by binding to nucleotides, in addition to other factors (Bukau and Horwich, 1998; Zhuravleva et al., 2012). BiP not only assists in folding but also functions in protein translocation into the ER, activation of the unfolded protein response (UPR) and ERAD (Hendershot, 2004) (Figure 1.1). BiP can collaborate with chaperones from the lectin family and is proposed to act earlier in the folding process than lectin chaperones (Hammond and Helenius, 1994; Molinari and Helenius, 2000; Wang et al., 2005). It has also been proposed that BiP can act as a backup chaperone when one of the lectin chaperones is absent (Pieren et al., 2005).

In contrast to classical chaperones, lectin chaperones associate with maturing glycoproteins based on their glycan composition. The composition of the substrate's glycan is dynamic and incurs modifications throughout the lifetime of the protein in the ER (Brodsky and Skach, 2011). These modifications report on the structure of the protein and direct it to different routes. Thus, glycans are considered as a code that could determine the fate of proteins (Figure 1.2).

### **Early trimming events and entry into the CNX/CRT cycle**

As soon as the glycan is added to nascent chains, the first glucose is trimmed by  $\alpha$ -glucosidase I (Figure 1.3, Step 2) (Kornfeld and Kornfeld, 1985; Parodi, 2000; Deprez et al., 2005). For a long time, the di-glucosylated form of protein-bound oligosaccharides generated by the action of  $\alpha$ -glucosidase I was considered an extremely short-lived trimming intermediate lacking biological significance. The discovery of malectin, a membrane-bound ER-resident lectin that specifically binds di-glucosylated glycans, changed this view (Schallus et al., 2010). Malectin is induced under conditions of ER stress (Galli et al., 2011) and is proposed to preferentially associate with off-pathway non-native conformers of well-studied glycoproteins like *influenza* hemagglutinin (HA) and *null* Hong Kong (NHK), a folding-defective variant of the secretory protein  $\alpha$ -1-antitrypsin (A1AT) (Galli et al., 2011; Chen et al., 2011; Qin et al., 2012). However, the role of malectin in ER quality control is not yet well understood.

Subsequently,  $\alpha$ -glucosidase II trims the second and the third glucose of the N-linked glycan (Figure 1.3, steps 3 and 5). The two trimming events play opposing roles. After the first trimming step, maturing substrates associate with the lectin chaperones calnexin (CNX) and calreticulin (CRT), which recognize mono-glucosylated glycans. The second trimming step releases the protein from the lectin chaperones due to the reduced affinity of these chaperones to glycans lacking the terminal glucose residue (Figure 1.3, step 5) (Hammond et al., 1994; Ora and Helenius, 1995; Hebert et al., 1995). A series of de-glucosylations by  $\alpha$ -glucosidase II (step 5) and re-glucosylations by a glucosyltransferase, the UGT1 (step 6) can occur (Hebert et al., 1995; Cannon and

Helenius, 1999). Re-glucosylation by UGT1 directs the rebinding of CNX or CRT to the mono-glucosylated substrate, hence the name CNX/CRT binding cycle.

### **The role of the lectin chaperones CNX and CRT**

CNX is a type I membrane protein. CRT is its soluble paralogue possessing 39% sequence homology (Wada et al., 1991). CNX and CRT share similar structural properties: they both have an N-terminal globular domain that contains the lectin binding site, a long arm domain and require  $\text{Ca}^{2+}$  to function. The globular domain is a  $\beta$ -sandwich of concave and convex  $\beta$ -sheets and the arm domain, shaped in an overall hairpin like structure, is termed the P-domain (Figure 1.4). The latter protrudes 140 Å away from the globular domain and has two different proline rich motifs named 1 and 2, of four copies each (Schrage et al., 2001). The four copies of motif 1 on one strand interact in a head-to-tail fashion with the four copies of motif 2 on the other strand of the hairpin (Schrage et al., 2001). In CRT, the P-domain is similar but shorter than CNX and has three instead of four tandem repeats (Ellgaard et al., 2002).

Despite the similarities between CNX and CRT, striking differences have been attributed to their roles or binding specificities. The location and the number of glycans recognized by each of the lectins could be quite distinct (Hebert et al., 1997; Harris et al., 1998; Molinari et al., 2004). Furthermore, even though CNX and CRT share some of the same substrates, some proteins are exclusively clients of CNX or CRT. These differences could be assigned to their distinct topologies, as it was observed that CRT, when fused to the membrane segment of CNX, acquired the substrate specificity of CNX (Wada et al., 1995). Consistent with different substrate specificity and/or with specific functions in the

ER lumen is the fact that CNX clients seem to associate with BiP rather than with CRT upon CNX deletion (Pieren et al., 2005) and that CNX and CRT knockouts (KO), which are well tolerated in cultured cells, have different phenotypes in mice. CRT KO is embryonic lethal due to defective heart development (Mesaeli et al., 1999), whereas CNX KO mice are viable and show motor disorders, associated with a dramatic loss of large myelinated nerve fibers (Denzel et al., 2002). Finally, the depletion of either of the lectins may have opposing effects on folding. Depletion of CRT enhances the maturation of a subset of viral and cellular proteins with a minor decrease in folding efficiency. To the contrary, CNX depletion prevents the maturation of a substrate like HA, while its absence does not affect the maturation of other proteins (Molinari et al., 2004). These differences highlight the unique roles of each of these lectins despite their similarities.

### **The lectin chaperones CNX and CRT recruit folding enzymes**

The lectins CNX and CRT have been demonstrated to recruit other folding factors. One of these folding factors is ERp57, which belongs to the family of protein disulfide isomerases (PDIs) (Figures 1.1 and 1.3). PDIs can catalyze the oxidation or isomerization of disulfides in proteins until they reach their final correct disulfide pattern exhibited in the native state (Oka and Bulleid, 2013; Wang et al., 2012). PDIs usually contain thioredoxin-like domains characterized by a CXXC motif, which is responsible for the catalytic activity. The interaction between ERp57 and lectin chaperones is independent of the presence of client proteins further confirming that this interaction is direct (Oliver et al., 1999). Additionally, it was suggested that ERp57 interacts with the tip of the P-domain of the lectin chaperones (Frickel et al., 2002). Substrate folding

assisted by CNX and CRT is enhanced significantly in the presence of ERp57 (Zapun et al., 1998).

Cyclophilin B (CyB), which is a member of the peptidyl prolyl isomerases (PPIs) family (Kozlov et al., 2010), is another folding assistant that is recruited by the lectin chaperones (Figures 1.1 and 1.3). The peptide bond of a proline can adopt a *cis* or *trans* conformation (Di Martino et al., 2014), yet the *trans* conformation is slightly more energetically favorable. An energy barrier of ~20 kcal/mol is required to allow inter-conversion and maintain equilibrium. PPIs assist in lowering this barrier, which otherwise could be a rate limiting step in the folding process (Di Martino et al., 2014). CyB, like ERp57, interacts with the tip of the CNX and CRT P-domain (Kozlov et al., 2010). Hence, it was proposed that CyB functions in concert with the lectin chaperones and ERp57, and is part of the CNX/CRT cycle.

### **The role of the folding sensor UGT1**

Release from the lectin chaperones CNX and CRT is followed by trimming of the innermost glucose residue by  $\alpha$ -glucosidase II, which prevents immediate re-association of the newly synthesized polypeptide to CNX and CRT. Here, a decision has to be made whether a protein is properly folded and should be targeted for anterograde trafficking or whether its forward transport should be prevented in order to retain it longer in the folding environment or to select it for clearance from the ER lumen. The UDP-glucose: glycoprotein glucosyltransferase 1 (UGT1) has been proposed to be central in this decision making, and is described as a folding sensor because it can recognize structural imperfections such as exposed hydrophobic domains on maturing proteins, a function that

is performed by its N-terminal folding sensor domain (Sousa and Parodi, 1995; Arnold and Kaufman, 2003).

Upon recognition of structural defects on cargo proteins, the UGT1 transfers a glucose residue onto the A branch of the glycans via its C-terminal catalytic domain (Figure 1.3, step 6). This forces immature proteins to rebind to CNX and CRT for another round of folding attempts under the assistance of the associated enzymes ERp57 and CyB. Cycles of de-glucosylation and re-glucosylation occur (Hammond et al., 1994), indicative of a role of UGT1 in retaining incompletely folded proteins in the ER (Hebert et al., 1995; Cannon and Helenius, 1999; Wada et al., 1997; Tannous et al., 2015a). UGT1 also contributes to the luminal retention of unassembled subunits of multimeric complexes, thus promoting efficient and complete assembly (Keith et al., 2005).

### **The conservation, organization and expression of UGT1**

The reglucosylation activity of UGT1 was discovered in the parasite *Trypanosoma cruzi* (Parodi and Cazzulo, 1982; Trombetta et al., 1989). UGT1 is highly conserved amongst eukaryotes including mammals, plants, fungi and protozoans. Interestingly, UGT1 is absent from *S. cerevisiae* (Fernandez et al., 1994). It is a large protein of approximately 170 kDa that possesses a signal sequence and an ER retention sequence whose specific residues vary depending on the species (Ito et al., 2015). The human UGT1 is highly homologous with *Drosophila*, *C. elegans* and *S. pombe* UGTs, especially within the last 300 amino acid residues (Arnold et al., 2000). The homology of the last 300 amino acids, which comprise the catalytic domain (Figure 1.5), is shared with

bacterial glycosyltransferases (Arnold et al., 2000). The rest of UGT1 is less conserved, and is hypothesized to be the substrate recognition or folding sensor domain (Figure 1.5).

The glycosylation status of UGT1 has not been studied extensively. While more than one glycosylation site has been predicted, a recent study relying on mass spectrometry suggested that UGT1 is only glycosylated on N269 (Daikoku et al., 2014) (Figure 1.5). Although the functional relevance of this glycosylation site is not clear, its conservation among several species, including human UGT1, points to an important role of this glycan for the function of UGT1 (Daikoku et al., 2014). The expression pattern of UGT1 further reinforces its physiological importance, as human UGT1 mRNA is expressed in several tissues, but is highest in pancreatic and lowest in lungs and heart tissues (Arnold et al., 2000). It is upregulated approximately three to four fold upon ER stress, supporting its role in quality control of incompletely folded or unfolded proteins that accumulate under similar ER stress conditions (Arnold et al., 2000).

### **The catalytic domain of UGT1**

The catalytic activity of UGT1 requires high calcium concentrations (D'Alessio et al., 2010) and implicates four residues that are essential for the glucosyltransferase activity, Asp (D) 1452, Gln (Q) 1453, Asp (D) 1454 and Leu (L) 1455 (Arnold and Kaufman, 2003). A DXD motif is proposed to bind to cations that are required for the coordination of UDP-sugars in other glycosyltransferases; as a result, the DQD motif in human UGT1 is hypothesized to be involved in a similar mechanism (Parodi, 1999) (Figure 1.5).



### **The folding sensor domain of UGT1**

There have been few insights into the structure of the folding sensor or substrate recognition domain of UGT1. Since UGT1 recognizes substrates in a manner akin to that of classical chaperones, it can be hypothesized that they may share similar structural properties. Surprisingly, a recent study found that the folding sensor domain shares structural characteristics with the class of oxidoreductases that feature domains with a thioredoxin-like fold (Zhu et al., 2014). Homology modeling on *Chaetomium thermophilum* UGT1, which shares 35% identity with human UGT1, predicted that its folding sensor domain contains three tandem thioredoxin-like sub-domains (Zhu et al., 2014) (Figure 1.5). The crystal structure of the third predicted thioredoxin-like domain was solved, revealing a typical thioredoxin-like fold characterized by five  $\beta$ -sheets surrounded by six helices (Zhu et al., 2014). Active thioredoxin-like domains are characterized by a CXXC motif involved in oxidation and isomerization of disulfides. Yet, the thioredoxin-like domains of UGT1 lack a CXXC motif, and contain only one cysteine, which makes the functional relevance of these domains unclear. Inactive thioredoxin-like domains have been proposed in some cases to be involved in substrate recognition, rather than modulating the redox state of the protein (Denisov et al., 2009; Pirneskoski et al., 2004). Thus, the inactive thioredoxin domain of UGT1 could potentially serve a similar purpose.

UGT1 binds to a protein named Sep15, which belongs to the family of selenocysteine containing proteins, and is the only protein identified so far to bind directly to UGT1 (Korotkov et al., 2001; Labunskyy et al., 2005). It does not seem to be a coincidence that Sep15 also assumes a thioredoxin-like fold. Sep15 has been proposed to

be an active oxidoreductase (Labunskyy et al., 2007). Yet, whether Sep15 affects the UGT1 activity has not been studied. Consequently, identifying the role of the thioredoxin-like domains of UGT1 might unravel a previously unforeseen mechanism of UGT1 in ER quality control that could involve Sep15.

### **Proposed models for substrate recognition by UGT1**

Two models have been described for the mechanism of UGT1 substrate recognition. The first model states that UGT1 is directly influenced by the structural and biophysical properties of the peptide backbone that is proximal or local to the reglucosylated glycan of the substrate. It was noted that UGT1 favors an alternating peptide hydrophobicity pattern located at one to three amino acids C-terminal to the glycan that was reglucosylated (Taylor et al., 2003). The study assayed the reglucosylation of 24 peptides obtained from trypsin digestion of a full-length protein. In another study, analysis of reglucosylation activity using synthetic penta-peptides indicated that a proximal serine, C-terminal to the glycosylation site, is important for UGT1 recognition (Kudo et al., 2014). It was anticipated that the hydroxyl-residue of the serine might be involved in hydrogen bonding with residues in the substrate recognition domain of UGT1, which may help stabilize the UGT1-substrate interaction. (Kudo et al., 2014).

An investigation relying on a glycan modified with BODIPY (Boron-dipyrrromethene), which was shown to be a good UGT1 substrate, further supported that the neighboring glycan environment may affect UGT1 activity. In this analysis, BODIPY consisted of the “aglycon” instead of the peptide backbone. The study demonstrated that

the distance between BODIPY and the glycan affects UGT1 activity (Totani et al., 2009). Upon varying the distance between the glycan and BODIPY, using increasing lengths of polyethylene glycol (PEG) linkers, the activity of UGT1 decreased as the length of the PEG linker increased. Moreover, other synthetic “aglycons” were tested and it was observed that the hydrophobicity and the orientation of the aglycon portion neighboring the glycan significantly affected the UGT1 recognition of the substrate (Totani et al., 2009).

In contrast with the model proposing that UGT1 is directly influenced by the properties of the peptide backbone proximal to the glycan, a study proposed that UGT1 can reglucosylate glycans that are distant from the site where UGT1 binds to the peptide backbone of the substrate (Taylor et al., 2004). Using a substrate with a known crystal structure, it was shown that UGT1 can recognize glycans that are 40 Å away from the defect site, arguing for a “reach and grab” model, where UGT1 can extend to modify glycans in folded regions (Taylor et al., 2004). These two models may not necessarily be mutually exclusive and UGT1 may use both mechanisms to allow optimal recognition, although this still remains to be demonstrated.

### **Glycan elements recognized by UGT1**

Pioneering work that assessed which parts of the glycans are recognized by UGT1 proposed that UGT1 recognizes the innermost N-acetylglucosamine (GlcNAc) residue (Sousa and Parodi, 1995). This was concluded by comparing inhibition of UGT1 activity by EndoH or PNGase deglycosylated substrates that compete with UGT1 glycosylated substrates. Endo-H is a deglycosylating enzyme that cleaves the bond between the two

GlcNAc residues, leaving a protein with one GlcNAc on the asparagine. PNGase F cleaves the bond between the innermost GlcNAc and the asparagine residue. EndoH-deglycosylated substrates were able to inhibit UGT1 activity towards glycosylated substrates by out-competing them, suggesting high affinity for UGT1, while PNGase treated substrates did not (Sousa and Parodi, 1995), indicating that the innermost GlcNAc residue is the minimum glycan recognition motif for UGT1.

These observations were later supported using glycan-methotrexate conjugates as artificial UGT1 substrates (Totani et al., 2005). When these glycans lacked the innermost GlcNAc, UGT1 could not modify them by glucose addition (Totani et al., 2005). Additional work using these glycan conjugates proposed that mannose residues could be important for UGT1 recognition, as UGT1 most efficiently recognized the core pentasaccharide Man<sub>3</sub>GlcNAc<sub>2</sub> compared to non-mannose containing glycans (Totani et al., 2009). The role of mannose sugars of the substrate's glycan in UGT1 activity will be discussed further in this manuscript.

### **The influence of the UGT1 localization on its role**

A few studies have attempted to address the localization of UGT1 within the ER. The ER is highly compartmentalized. A simplified classification divides the ER into rough and smooth ER. Yet, more evidence is arising that there are additional levels of compartmentalization, which may be mediated by complex formation, or simply by the concentration of ER machinery in distinct regions of the ER (Leitman et al., 2013). Based on immunofluorescence and immunogold labeling microscopy, as well as proteomic studies, it seems as though UGT1 is more concentrated or localized in an ER

compartment beyond the rough ER, such as the smooth ER, ER exit sites, or pre-Golgi intermediates, and it is therefore active later in the protein maturation pathway.

Immunofluorescence microscopy showed that UGT1 was distributed throughout the ER, but interestingly was concentrated in foci near ER exit sites, hinting that the activity of UGT1 can occur or persist until later maturation steps (Cannon and Helenius, 1999; Arnold et al., 2000). Immunogold microscopy found that although some labeling of UGT1 was detected in the rough ER, the most intense labeling of UGT1 was in the pre-Golgi intermediate compartment, with twice as much found as in the rough ER (Zuber et al., 2001). Glucosidase II and CRT's presence was also noted in pre-Golgi intermediates. The presence of the elements of the CNX/CRT cycle in a later ER compartment reinforces that UGT1 is most active in later steps in the maturation of secretory proteins (Zuber et al., 2001). Additionally, a proteomic approach relying on fractionation procedures to separate the rough from the smooth ER determined that UGT1 is present predominantly in smooth ER microsomes. This result validates partially the notion that UGT1 is situated deeper in the ER lumen (Gilchrist et al., 2006).

Agreeing with a later involvement of UGT1 in protein maturation, Pearse et al., 2008, showed that UGT1 reglucosylates proteins only post-translationally. This was tested by performing a reglucosylation assay on nascent chains arrested on ribosomes (Pearse et al., 2008). Reglucosylation was only observed when the arrested chains were released from the ribosomes. The exclusive posttranslational reglucosylation of proteins could be explained by the localization of UGT1 in a distant ER compartment, supportive of the previous microscopy and proteomics studies.

In addition, UGT1 was found in a complex with a number of other ER quality control proteins, including but not restricted to BiP, PDI and CyB (Meunier et al., 2002). These data also point to a particular spatial organization of the ER, and suggest that UGT1 is sequestered in a specific ER location.

Despite these observations, some discrepancies were detected such that other components of the reglucosylation cycle, CNX and CRT, were excluded from the complex that contained UGT1 (Meunier et al., 2002), which raises the question of how the localization of UGT1 relates to its role in the CNX/CRT cycle. Accordingly, further studies are required to clarify the spatial distribution of UGT1 and how this distribution affects its function.

### **The role of UGT1 in physiological processes**

UGT1 is essential in mice as knocking out UGT1 is embryonic lethal (Molinari et al., 2005), and results in activation of ER stress responses (Rutkowski et al., 2006). This supports that UGT1 plays important physiological roles since its absence had severe consequences on mice development. However, the activity of UGT1 appears to be substrate specific, and does not impact equally all maturing proteins. In an investigation from the Molinari lab, the absence of UGT1 caused either a delay, an acceleration or had no effect on the maturation of the substrates studied (Solda et al., 2007). The significance of UGT1 activity is further highlighted by the roles it plays in several physiological processes including antigen presentation and plant development as shown in the case studies below.

UGT1 has been implicated in antigen presentation because in its absence, the surface expression of the major histocompatibility (MHC) class I molecules is decreased. In addition, the maturation and peptide loading of MHC class I is defective (Zhang et al., 2011). Similarly, UGT1 is involved in the maturation of CD1d, the lipid antigen presentation molecules. In absence of UGT1, CD1d prematurely associates with  $\beta$ 2-microglobulin ( $\beta$ 2-m), and some of these CD1d- $\beta$ 2m heterodimers are unstable. Interestingly, despite that the levels of CD1d- $\beta$ 2m at the surface were unchanged in absence of UGT1, the cells exhibited altered antigenicity (Kunte et al., 2013). The implication of UGT1 in antigen presentation further stresses the importance of studying its role in other physiological processes. UGT1 recognizes and aids in the folding of transferrin, which regulates iron levels (Wada et al., 1997), and prosaposin, which is involved in lysosomal storage diseases (Pearse et al., 2010). Yet, how UGT1 affects iron levels or lysosomal storage disease progression has never been addressed. Addressing these questions could make UGT1 a potential therapeutic target.

The *Arabidopsis thaliana* (*A. thaliana*) UDP-glucose:glycoprotein glucosyltransferase, named UGT, also appears to play critical roles in plant physiology. A recent study showed that UGT is required for the biogenesis of SOBIR I (Suppressor of BIR 1), which is responsible for the activation of immune responses in *A. thaliana* (Zhang et al., 2015). Moreover, UGT has been implicated in the biogenesis of *A. thaliana* transmembrane receptors and is essential for the retention of the defective brassinosteroid receptor *bri1-9* in the ER, which is essential for growth. (Jin et al., 2007). These observations emphasize the importance of UGT in activation of plant immune responses and plant development.

In conclusion, it is very clear that UGT1 is an essential factor in various kingdoms of life, making it crucial to define its detailed mechanism in ER quality control.

### **Conclusion**

In summary, although we know a great deal about UGT1 from previous studies, many questions concerning its role and mechanism in ER quality control remain unanswered. This lack of a complete understanding stems from the adoption of a reductionist approach that relies on purified components in the assays used to study UGT1 activity. Studying the activity of UGT1 in the complex environment of the ER would require more intricately designed approaches. A main hurdle in studying the mechanism of UGT1 in intact cells is that the product of reglucosylation and that of the early trimming events by glucosidases I and II are indistinguishable. Moreover, the spatial organization and compartmentalization of the ER adds another level of complexity, which cannot be addressed by cell free assays. Our lab has developed a cell based reglucosylation assay that overcomes this hurdle, and is a powerful tool to advance our understanding of the reglucosylation or CNX/CRT cycle.

Being such a central and prominent player in the ER quality control, we will pose and answer, in the next three chapters, fundamental questions concerning the role and mechanism of UGT1, which can be summarized as follows:

- 1) What types of substrates are recognized by UGT1 and how does reglucosylation affect the fate of these substrates?
- 2) How are reglucosylation-chaperone binding cycles terminated?



- 3) Are free thiols recognized as a hallmark of misfoldedness and used as a handle by UGT1 to retain misfolded substrates in the ER?

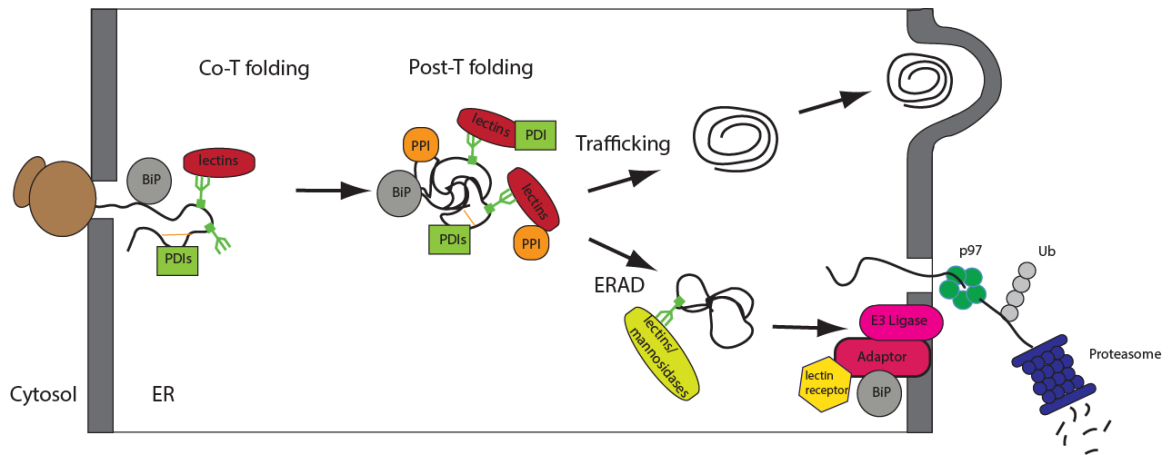


Figure 1.1 Quality control in the ER

Nascent proteins are translocated into the ER via the Sec61 translocon. Glycans are generally added co-translationally. A set of folding factors such as the Hsp70 BiP, lectin chaperones and PDIs can assist in the co-translational (co-T) folding of proteins. Some PDIs directly recognize proteins; others are recruited by lectin chaperones. Additional folding factors such as peptidyl prolyl isomerases (PPIs) also assist in folding by directly recognizing proteins or by being recruited by the lectin chaperones. Folding continues post-translationally (Post-T). A properly folded protein will be targeted to exit out of the ER via vesicular trafficking to the Golgi. Misfolded proteins are destined for degradation via the process of ERAD. Early steps of ERAD include recognition of proteins by ERAD lectins and mannoside trimming. Other lectins that recognize mannoside-trimmed substrates target the protein to a retro-translocation complex nucleated by an adaptor protein and contains BiP, ubiquitin ligases and other factors. Misfolded proteins are retro-translocated to the cytosol. The AAA ATPase p97 assists in the extraction process. Proteins are ubiquitinated and degraded by the proteasome.

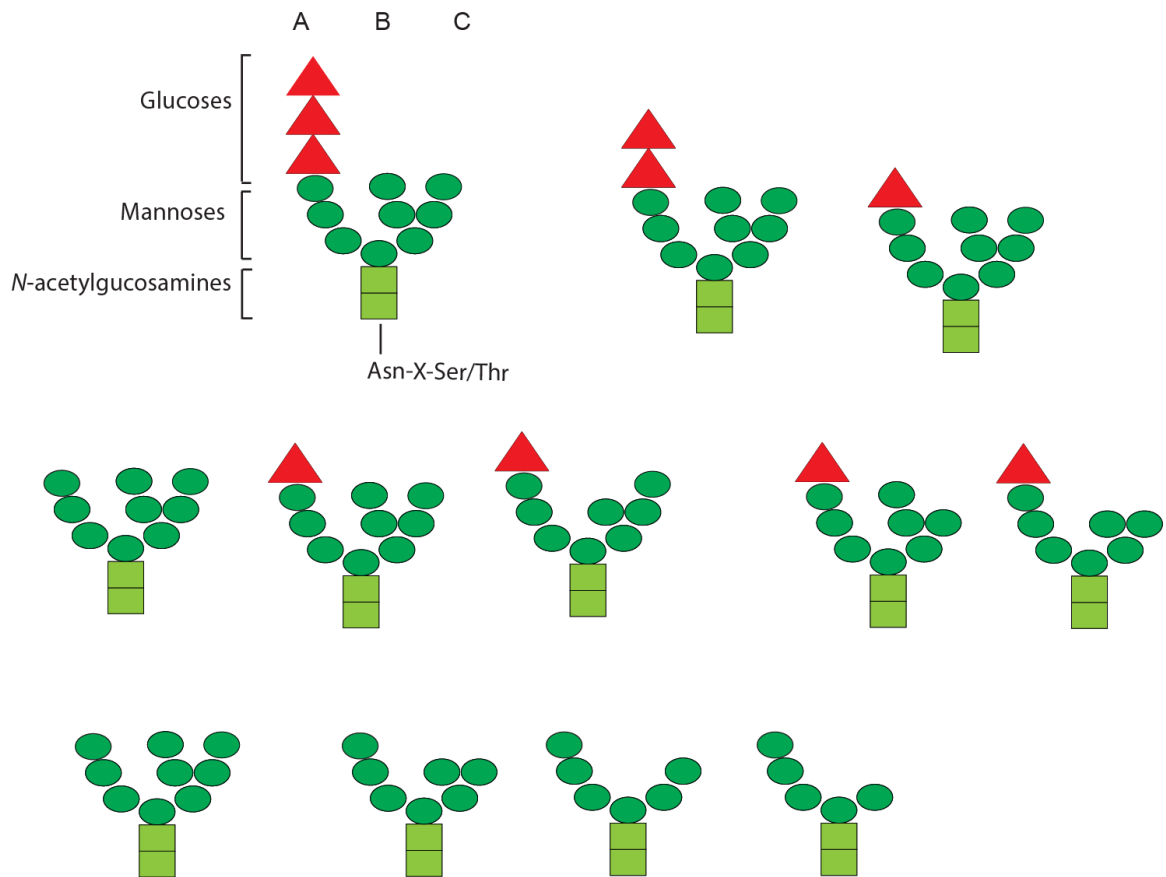


Figure 1.2 The glycan code

Glycans are added onto Asn in consensus sites consisting of Asn-X-Ser/Thr. They are comprised of 2 N-acetylglucosamines, 9 mannoses and 3 glucoses, and are distributed over the A, B and C branches of the glycan. Early modifications of the glycan composition in the ER involves sequential trimmings of the first and the second glucoses. Each of these steps can recruit different ER lectin chaperones. After trimming of the last glucose, the branch A of the glycan can be reglucosylated by a glucosyltransferase. These modification steps are usually associated with folding events. Subsequent modifications involve trimming of mannose residues which could target the protein to exit out of the ER for vesicular trafficking. More extensive mannose trimming targets the protein for degradation.

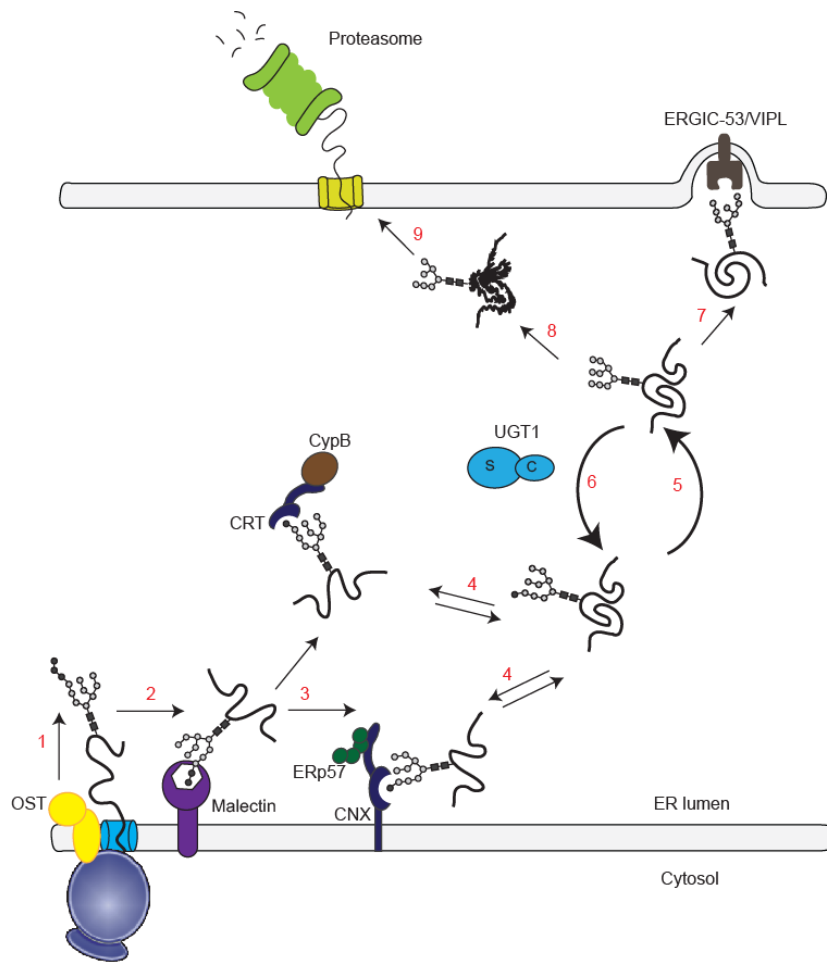


Figure 1.3 The CNX/CRT cycle

(Tannous et al., 2015b)

The OST complex catalyzes the addition of glycans onto nascent chains (step 1). The first glucose is trimmed by  $\alpha$ -glucosidase I (step 2). Di-glucosylated proteins can bind to malectin.  $\alpha$ -glucosidase II cleaves the second glucose (step 3) forming mono-glucosylated glycans, which interact with CNX and CRT. Binding and release from lectin chaperones could occur (step 4). The second trimming by  $\alpha$ -glucosidase II, which removes the last glucose, triggers the release from the lectin chaperones (step 5). Re-glucosylation by UGT1 directs the re-association of the proteins with CNX or CRT (step 6). Properly folded proteins are exported from the ER (step 7). Export from the ER could involve the binding of substrates to sorting receptors such as ERGIC53 and VIPL. Glycans of misfolded proteins are further processed by mannosidases (step 8) and targeted for proteasomal degradation (step 9). The substrate-binding and catalytic domains of UGT1 are shown with s and c, respectively.

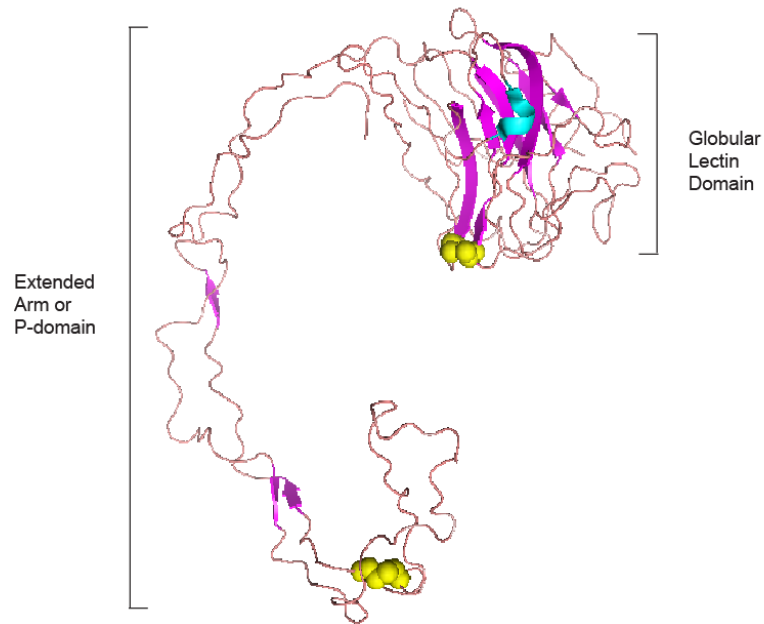


Figure 1.4 Crystal structure of the calnexin luminal domain. PDB 1JHN

(Schrage et al., 2001)

Calnexin consists of a globular lectin  $\beta$ -sandwich domain from which extends a 140Å arm domain, also termed the P-domain, which contains repeats of proline rich motifs. In yellow are shown disulfide bonds. The rest of the structure is disordered and was not modeled (Schrage et al., 2001).

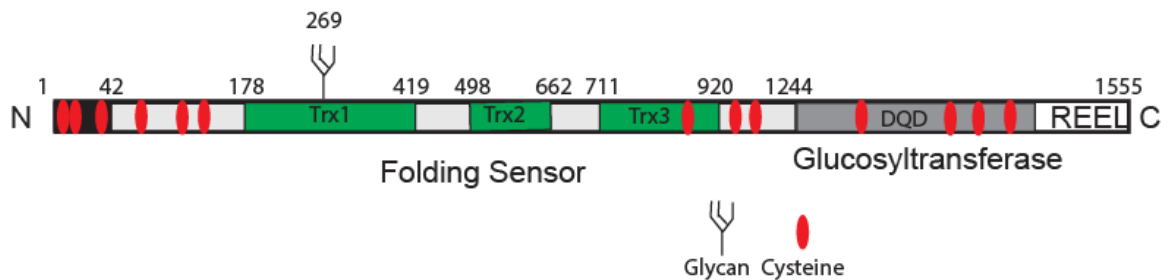


Figure 1.5 Human UGT1

Human UGT1 has an N-terminal cleavable signal sequence (black rectangle) and a C-terminal REEL ER retention sequence. It appears to be glycosylated at one site, Asn269. The N-terminal ~80% of UGT1 is composed of the folding sensor domain (light gray), which contains three thioredoxin-like (Trx) domains predicted from the *C. thermophilum* homolog of UGT1, denoted by the green rectangles. The C-terminal domain is the glucosyltransferase domain (dark gray) where the Asp (D) 1452, Gln (Q) 1453 and Asp (D) 1454 are essential for the catalytic activity of UGT1.

## CHAPTER 2

### REGLUCOSYLATION BY UGT1 DELAYS GLYCOPROTEIN SECRETION BUT NOT DEGRADATION

*(This chapter is adapted from (Tannous et al., 2015a))*

#### **Introduction**

Misfolded proteins can expose hydrophobic regions that are usually buried in the folded form. They can also contain unpaired cysteine residues resulting from the failure to form disulfides. These hallmarks may be recognized by the quality control machinery to distinguish native from misfolded substrates and to retain misfolded substrates in the ER and target them for degradation (Ellgaard et al., 1999; Isidoro et al., 1996; Nishikawa et al., 2005). However, folding intermediates could exhibit similar structural characteristics to misfolded proteins. Thus, it is more difficult to conceptualize how the quality control machinery is able to distinguish folding intermediates from misfolded proteins. Despite that, the ER quality control machinery is able to make that distinction, thus retaining intermediates in the ER until they attain their native state, and preventing their premature degradation. Previous work in the literature has proposed that UGT1 could make such a distinction (Tannous et al., 2015b; Tamura et al., 2010; D'Alessio et al., 2010). Yet, most of these conclusions were drawn from cell free assays, and the ability of UGT1 to discriminate between foldable intermediates and terminally misfolded proteins remained an open question.

A pioneering study relied on ER microsomal extracts as the source for UGT1, and purified native or denatured thyroglobulin as the substrate (Trombetta et al., 1989).

Interestingly, UGT1 was only able to efficiently recognize denatured thyroglobulin (Trombetta et al., 1989). While this was the first study showing that UGT1 favors denatured over native substrates, it is worth stating that thyroglobulin is a large dimer of 660 kDa, containing 14 glycosylation sites and 30 disulfide bonds. The large size, and the large number of glycans and disulfides could make it difficult to generalize this mechanism of UGT1 for secretory proteins as most do not possess such complicated features. Several studies that followed used purified UGT1 from different sources such as *Drosophila*, rat liver and *S. pombe* to study reglucosylation by UGT1. Some of the substrates investigated were soybean agglutinin, phytohemagglutinin and ribonuclease B, which are smaller substrates with fewer glycosylation sites and disulfides than thyroglobulin (Fernandez et al., 1994; Trombetta and Parodi, 1992; Parker et al., 1995). All these studies agreed that UGT1 preferentially recognized the denatured over the native form as previously noted with thyroglobulin. Building from these conclusions, one might hypothesize that UGT1 favors proteins that are deemed misfolded in the ER over proteins that will ultimately fold. Nevertheless, the affinity of UGT1 to any intermediate folding states, or to various levels of structural perturbations was not assessed in these reports. As a result, the hypothesis that UGT1 could favor intermediate folding states of proteins that will eventually reach the native state could not be excluded.

The abovementioned ribonuclease B (RNase B) substrate was used later more extensively to determine if purified UGT1 can recognize different levels of substrate structural perturbations (Trombetta and Helenius, 2000). Cleavage of the last 20 amino acids of RNase B results in a partially structured state, which UGT1 favored over the fully denatured or the native state. The study concluded that UGT1 is able to sense

variations in the level of structural defect (Trombetta and Helenius, 2000). In addition, exo (1,3)- $\beta$ -glucanase ( $\beta$ -Glc) was used as another model substrate to further support the latter observation (Taylor et al., 2004). When the F280S mutation was introduced in  $\beta$ -Glc, it caused only a minor loss in  $\beta$ -Glc enzymatic activity, yet reglucosylation by purified UGT1 increased substantially relative to wild type (Taylor et al., 2004), corroborating the hypothesis that UGT1 is sensitive to even slight alterations in the structure of glycoproteins.

Further evidence demonstrating that UGT1 favors near native folding intermediates came from a study that employed purified UGT1 and fragments of different lengths and structure of chymotrypsin inhibitor 2 (Caramelo et al., 2003). The fragments ranged from a short truncation that is fully unstructured, to the native folded full length protein, and included a longer fragment shown to adopt a near native conformation. The assay showed that UGT1 preferentially reglucosylated the nearly native long fragment over the short unstructured fragment or the full length native protein (Caramelo et al., 2003). Not only did UGT1 distinguish between the various folding states of the substrates, it appeared to favor late folding intermediates. If these observations were to be extrapolated to the context of the ER environment, it can be hypothesized that UGT1 favors proteins on the pathway to fold or “on-pathway substrates” and ignores those that are extensively misfolded or “off-pathway” substrates.

Although the abovementioned studies helped to better delineate the mechanism of UGT1, they were performed under non-physiological conditions, hence the importance of studying UGT1 mechanism in live cells. We investigated the ability of UGT1 to distinguish between “on-pathway” and “off-pathway” glycoproteins and the effect of



UGT1 reglucosylation on the fate of these proteins in intact cells. We found that UGT1 was able to reglucosylate off-pathway maturation substrates more efficiently and persistently than wild type maturing secretory pathway clients. Trapping wild type UGT1 substrates in the monoglucosylated state delayed their secretion, whereas trapping off-pathway substrates in the monoglucosylated state did not affect their rate of degradation. This suggested that terminally misfolded substrates were efficiently extracted from the lectin chaperone binding cycle and targeted for degradation through a dominant process that was unaffected by the presence of monoglucosylated side chains on the substrate.

## **Results**

### **The reglucosylation of ERAD substrates**

To explore the substrate specificity of UGT1 in live cells, the reglucosylation of model maturation and quality control substrates was analyzed using a cellular reglucosylation assay. The MI8-5 Chinese hamster ovary (CHO) cells are deficient in the dolichol-pyrophosphate glucose-dependent glycosyltransferase termed Alg6, that is responsible for glucose transfer during glycan synthesis (Quellhorst et al., 1999). Truncated unglucosylated N-linked glycans lacking glucoses ( $\text{Man}_9\text{GlcNAc}_2$ ) are transferred instead of the normal triglucosylated species ( $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ ). Reglucosylation is the sole mechanism through which glycoproteins can reach the monoglucosylated state in these cells (Figure 2.1A, bottom scheme). The reglucosylated or monoglucosylated proteins can be isolated based on their affinity for GST-calreticulin (GST-CRT) (Pearse et al., 2008; Pearse et al., 2010). Glucosidase inhibition traps reglucosylated glycans in their monoglucosylated state in MI8-5 CHO cells since these CHO derived cells lack an

endomannosidase activity (Karaivanova et al., 1998), further facilitating the ability to monitor the reglucosylation reaction.

Alpha-1-antitrypsin (A1AT) was chosen as the initial substrate to evaluate for reglucosylation since it has a number of mutations with varying levels of structural perturbations and is associated with disease. A1AT is a soluble secretory glycoprotein synthesized in hepatocytes that belongs to the serine protease inhibitor or serpin family of proteins. Serpins are monomeric proteins composed of three sheets and nine helices. Three different variants of A1AT were expressed in MI8-5 cells including wild type and two mutant variants termed *null* Hong Kong (NHK) and A1ATZ. NHK is a frame-shift and premature truncation mutation that is associated with gross misfolding, ER retention and rapid subsequent turnover by ER-associated degradation (ERAD) (Sifers et al., 1988; Liu et al., 1999). It was named *null* as it was found to be absent from serum. A common pathological variant of A1AT is A1ATZ caused by a single site mutation (E342K) (Blanco et al., 2006). A1ATZ contains structural perturbations that slow its folding and favor polymerization (Bottomley, 2011; Kass et al., 2012). While ~15% of A1ATZ is secreted, the remainder is ER retained and degraded by both ERAD and autophagy (Lomas et al., 1992; Hidvegi et al., 2010). The retention of polymerized A1ATZ in the ER of hepatocytes is associated with cirrhosis.

Cells transfected with A1AT were pulsed for 1-hr with [<sup>35</sup>S]-Met/Cys and either analyzed directly after the pulse or a 1-hr chase (Figure 2.1B). The pulse and chase were performed in the presence of the glucosidase inhibitor DNJ to trap reglucosylated glycans. Cell lines defective in assembling the complete dolichol precursor such as MI8-5 are commonly associated with the hypoglycosylation of nascent proteins since the

oligosaccharyltransferase frequently transfers the incompletely assembled glycan inefficiently (Huffaker and Robbins, 1983). The hypoglycosylation of A1AT was observed for all three forms after immunoprecipitation of the samples with anti-A1AT sera, resulting in the formation of a ladder of glycosylation states ranging from the faster migrating unglycosylated protein to the slower migrating fully glycosylated protein containing all three glycans (Figure 2.1B, lanes 11-16). To confirm that the multiple bands of A1AT were caused by hypoglycosylation, samples were treated with Peptide N-glycosidase F (PNGase F) to remove any heterogeneity attributed to N-linked carbohydrates. All protein bands observed collapsed into a single band migrating with the same mobility as unglycosylated A1AT demonstrating that the different protein bands resolved were due to different levels of glycosylation (Figure 2.2A, lanes 1-6).

Reglucosylation or the presence of monoglucosylated glycans was probed by monitoring binding to GST-CRT, a bacterial expressed and purified lectin fusion protein (Pearse et al., 2008; Pearse et al., 2010). A large number of proteins of a variety of sizes were found to be monoglucosylated (Figure 2.1B, lanes 1-8). To characterize A1AT reglucosylation, the GST-CRT pull down was followed by the immunoprecipitation with anti-A1AT sera (Figure 2.1B, lanes 17-24). Twenty percent of wild type A1AT was reglucosylated immediately after the pulse and this level diminished to 11% after a 1-hr chase. Interestingly, the level of reglucosylation doubled for A1AT mutants, NHK and A1ATZ, and this level of ~40% reglucosylation persisted after 1-hr of chase. These values were calculated using A1AT possessing all three of its glycans, however similar reglucosylation efficiencies were obtained when all A1AT glycoforms were grouped. The fastest migrating protein bands (unglycosylated A1AT) were not recognized by GST-

CRT. Therefore, while all glycoforms of A1AT were reglucosylated, the terminally misfolded mutant forms were more efficiently reglucosylated than the wild type protein (See the legend of figure 2.1 for statistical analysis), and the level of reglucosylation of the two mutant variants was similar even though they possessed different types of abnormalities.

To determine if UGT1 efficiently modified other ERAD or disease-associated substrates, the reglucosylation of several additional proteins was studied. These included proteins with varying topologies such as the soluble  $\alpha$ -N-acetylgalactosaminidase ( $\alpha$ -NAGAL), as well as two membrane proteins, T-cell receptor alpha-subunit (TCR $\alpha$ ) and tyrosinase.

$\alpha$ -NAGAL is a lysosomal protein that possesses five N-linked glycans (Clark and Garman, 2009). There are a number of loss-of-function mutations in  $\alpha$ -NAGAL that are associated with Schindler/Kanzaki disease (Clark et al., 2012). The reglucosylation of C-terminally tagged FLAG constructs of wild type  $\alpha$ -NAGAL and the missense E367K mutant was studied as described above. Samples were treated with Endoglycosidase H (EndoH) to differentiate EndoH sensitive ER glycoforms of  $\alpha$ -NAGAL from protein that had trafficked through the Golgi and received complex sugars rendering them EndoH resistant. During the timeframe studied, all forms of  $\alpha$ -NAGAL appeared to be EndoH sensitive consistent with their ER residency. The level of reglucosylation for wild type protein (7%) was lower than that observed for the E367K mutant (10%), immediately after the pulse (Figure 2.1C). This trend continued after the 1-hr chase, where WT  $\alpha$ -NAGAL reglucosylation was 10% and the mutant was 15%. While the difference between reglucosylation levels of the WT and the mutant  $\alpha$ -NAGAL was not statistically

significant at the 0-hr time point, it was after 1-hr of chase (See the legend of figure 2.1 for statistical analysis). Therefore, the  $\alpha$ -NAGAL E367K mutant was more efficiently modified compared to the wild type protein after 1-hr of chase.

TCR $\alpha$  is a membrane glycoprotein that forms a larger heterocomplex in T lymphocytes, however when it is unable to assemble, it is rapidly degraded through the ERAD pathway (Bonifacino et al., 1990; Huppa and Ploegh, 1997; Call et al., 2002). MI8-5 cells were transfected with a TCR $\alpha$  construct containing a C-terminal HA tag and pulsed for 1-hr in [<sup>35</sup>S]-Met/Cys followed by a chase for the indicated times in the presence of a glucosidase inhibitor. TCR $\alpha$  was found to be efficiently (23-25%) and persistently reglucosylated regardless of the duration of the chase period (Figure 2.1D).

Tyrosinase is a type I membrane glycoprotein with seven N-glycans that is involved in melanin biosynthesis (Ujvari et al., 2001). A C89R missense mutation for tyrosinase is an ERAD substrate and this loss-of-function mutation is associated with albinism (Halaban et al., 1997). MI8-5 cells were transfected with C-terminal FLAG constructs of either human wild type tyrosinase or a C89R mutant, and a pulse-chase procedure was performed as described above. Both wild type and the C89R mutant of tyrosinase were efficiently reglucosylated, with only a very slight increase in the level for reglucosylation observed for C89R over wild type immediately after 1-hr of chase (Figure 2.1E).

MI8-5 cells supported the efficient glycosylation and reglucosylation of a variety of glycoproteins. Three of four glycoproteins studied were efficiently glycosylated while a fourth protein (A1AT) was found to be hypoglycosylated, indicating that hypoglycosylation of glycoproteins in the MI8-5 cells was protein dependent. All four

proteins were efficiently reglucosylated. The level for reglucosylation for the two mutants of A1AT was doubled that observed for wild type A1AT. Wild type  $\alpha$ -NAGAL and tyrosinase were efficiently reglucosylated, but the reglucosylation of their mutants was associated with only a modest increase in modification, and in the case of tyrosinase it was seen only after a 1-hr chase.

### **Trapping proteins in their monoglucosylated state delays their secretion**

Reglucosylation of proteins by UGT1 supports their rebinding to the lectin chaperones CNX and CRT (Caramelo and Parodi, 2008). To determine if trapping glycoproteins in their monoglucosylated state would affect their secretion, a pulse chase experiment was performed using MI8-5 cells transfected with wild type A1AT in the absence and presence of DNJ. Cell lysates and the culture media were collected and analyzed.

The ability of glucosidase inhibition to trap wild type A1AT in a monoglucosylated state was verified, as the addition of DNJ greatly increased the level of reglucosylation observed with the lysate samples after sequential precipitation with GST-CRT and anti-A1AT sera (Figure 2.2A, compare lanes 7-9 to 25-27). Trapping A1AT in the monoglucosylated state was also associated with a significant decrease in secretion, as less than half the amount of A1AT was found in the culture media after a 2-hr chase when DNJ was present compared to in its absence (Figure 2.2A compare lanes 10-12 to 28-30, and Figure 2.2C). The secreted protein found in the media was largely not glucosylated as probed by GST-CRT binding (Figure 2.2A, lanes 16-18 and 34-36). These results demonstrated that the inhibition of deglucosylation of reglucosylated protein delayed the secretion of ectopically expressed wild type A1AT.

Since quality control pathways can be saturated by protein overexpression, the effect of trapping a reglucosylated substrate on the secretion of an endogenous UGT1 substrate was analyzed. Previously, we found that prosaposin was an obligate substrate for UGT1 as it was efficiently reglucosylated in MI8-5 cells and its maturation was inefficient in *ugt1*<sup>-/-</sup> mouse embryonic fibroblast cells (Pearse et al., 2010). As previously observed, glucosidase inhibition trapped a significant fraction of the prosaposin in the monoglucosylated state (Figure 2.2B, lanes 13-15). Furthermore, glucosidase inhibition and monoglucosylation trapping was associated with less than half the amount of prosaposin being secreted into the culture media after 2-hr of chase (Figures 2.2B, lanes 9 and 18, and 2C). The delay in secretion of prosaposin in the presence of DNJ appeared to be due to CRT and CNX binding to prosaposin as chaperone binding levels increased in the presence of the glucosidase inhibitor (Figure 2.2D, compare lanes 3 to 6, and 21 to 24). Altogether, these results demonstrated that trapping either ectopically expressed wild type A1AT or endogenously expressed prosaposin in the monoglucosylated state significantly inhibited their secretion.

### **Trapping ERAD substrates in their monoglucosylated state does not delay their turnover**

Next, we determined if trapping an efficiently reglucosylated ERAD substrate in the monoglucosylated state would influence its turnover. First, the reglucosylation and secretion of NHK was followed in MI8-5 cells in the absence and presence of DNJ by pulse-chase analysis. NHK was not secreted regardless of whether DNJ was excluded or included in the pulse and chase conditions, demonstrating the effectiveness of the quality control process in retaining and degrading this terminally misfolded protein and

recapitulating the *null* phenotype (Figure 2.3A, lanes 9-12 and 29-32). A fraction of NHK was also observed in the Triton X-100 insoluble material (Figure 2.3A, lanes 13-16 and 33-36). This fraction was included in the quantification of the amount of NHK remaining before and after trapping in the monoglucosylated state. Glucosidase inhibition efficiently captured NHK in the monoglucosylated state as observed by sequential pull-downs with GST-CRT and anti-A1AT sera (Figure 2.3A, compare lanes 25-28 to 5-8). Interestingly, trapping NHK in the monoglucosylated glycoforms did not influence the half-life of NHK regardless of the conditions employed (Figure 2.3B). Monoglucosylated NHK was detected in the detergent insoluble fraction even after 6-hr of chase indicating that insoluble NHK was also reglucosylated (Figure 2.3A, lanes 37-40). Yet, it is not clear if reglucosylation of insoluble NHK occurred before or after the accumulation of NHK in aggregates. The increase in NHK levels observed after the addition of proteasome (MG132) or the mannosidase (kifunensine) inhibitors, demonstrated that NHK continued to be degraded by ERAD in the absence or presence of glucosidase inhibition (Figure 2.3C and D).

To test the generality of this result, we investigated whether trapping another ERAD substrate, TCR $\alpha$ , in the monoglucosylated state would affect its disposal by ERAD. Persistent monoglucosylation of TCR $\alpha$  was observed in the presence of the glucosidase inhibitor as observed by the pull-down with GST-CRT followed by immunoprecipitation with anti-HA serum (Figure 2.4A, compare lanes 13-16 to 5-8). The half-life of TCR $\alpha$  was not significantly affected by the presence of the glucosidase inhibitor, as the half-life of TCR $\alpha$  was ~1 hr in the absence or the presence of DNJ (Figure 2.4B). TCR $\alpha$  was not detected in the Triton X-100 insoluble material (data not



shown), indicating that TCR $\alpha$  analyzed using the detergent soluble fraction was representative of the total amount of the protein remaining. TCR $\alpha$  was degraded by ERAD as MG132 inhibited its turnover; however, mannosidase inhibition stabilization was less efficient (Figure 2.4C and D). Altogether, these results demonstrated that the degradation of the ERAD substrates NHK and TCR $\alpha$  was unaffected by being trapped in the monoglucosylated state.

### **The timing and persistency of reglucosylation**

The previous experimental strategy trapped reglucosylated substrates in their monoglucosylated state to query the influence on secretion or degradation. Since the persistent trap did not allow the determination of the timing for reglucosylation, a transient 15 min window of glucosidase inhibition was applied at various time points in respect to the radioactive pulse and the cold chase to trap reglucosylated substrates over a range of times. Reglucosylated substrates were isolated using GST-CRT followed by the immunoprecipitation of the specific substrate.

After maturing properly in the ER, prosaposin has two separate fates: it can traffic to lysosomes where it is processed by cathepsin D into four separate proteins that act as co-factors for different lipid hydrolases, or it can be secreted as a full-length protein with only its signal sequence removed (O'Brien et al., 1994; Lefrancois et al., 2003). To differentiate ER resident prosaposin in cell lysates from lysosomal prosaposin, prosaposin immunoprecipitated from cell lysates was treated with EndoH. High-mannose glycoforms consistent with ER residency remain EndoH sensitive, while complex sugar glycoforms that have trafficked through the Golgi become EndoH resistant (Halaban et

al., 1997). Most of the prosaposin from cell lysates displayed an EndoH sensitive profile throughout the experiment, however after 15 min of chase a fraction of prosaposin that increased slightly with time was found to be EndoH resistant (Figure 2.5A, lanes 6 and 8).

The reglucosylation of prosaposin was transient as monoglucosylated protein was isolated by GST-CRT most effectively when DNJ was added during the 15 min pulse (Figure 2.5A, lanes 17 and 18, and Figure 2.5B). The amount of prosaposin trapped in the monoglucosylated state with the 15 min of DNJ treatment greatly decreased during the chase even though a significant fraction of prosaposin continued to be EndoH sensitive signifying ER residence. A similar profile was observed for transiently expressed wild type A1AT (Figure 2.5C and D). Monoglucosylated A1AT was generated only during the 15 min pulse period and did not accumulate after a chase period of 15 min or more.

In contrast, reglucosylation of the ERAD substrate NHK A1AT was more efficient during the pulse period and reglucosylation persisted for the next hour (Figure 2.5C and D). After 105 min of chase, the reglucosylation level dropped to roughly a third of its maximum. While the triton-insoluble fraction with wild type A1AT remained empty throughout the chase period, the triton-insoluble fraction for NHK increased in a time dependent fashion. The persistent reglucosylation of NHK appeared to contribute to its ER retention and the lack of appearance in the media to create the *null* phenotype. Overall, these results indicated that for both prosaposin and wild type A1AT, reglucosylation occurred early in the maturation process. Though protein remained in the ER during the extended chase period, it did not appear to be monoglucosylated. For the A1AT NHK mutant variant, reglucosylation occurred at a higher level and persisted,

consistent with the conclusion that UGT1 reglucosylates off-pathway aberrant structures most efficiently.

### **Efficient reglucosylation of reduced protein**

Some of the previous results have suggested that UGT1 does not modify grossly misfolded proteins. This was in part supported by the observation that in some cases ER glucosyltransferases did not recognize reduced substrates (Pearse et al., 2008; Fernandez et al., 1998; Ritter and Helenius, 2000). The timing of DTT addition, its activation of the UPR and the employment of different species complicate the interpretation of these results (Travers et al., 2000). We determined if DTT affected the reglucosylation of the obligate UGT1 substrate prosaposin using our cell-based reglucosylation assay. Cells were pulsed with [<sup>35</sup>S]-Met/Cys for 30 min in the presence or absence of DTT and either processed immediately after the pulse or after an oxidative chase for 1 hr. DNJ was added to trap reglucosylated substrates and the samples were processed by immunoprecipitation and using GST-CRT pull downs.

Reduced prosaposin migrated more slowly than prosaposin that accumulated under oxidizing conditions as free thiols were modified by the alkylating agent N-ethyl maleimide included in the lysis buffer (Figure 2.6A, lanes 1-4). The amount of prosaposin was also diminished by DTT treatment likely due to the activation of UPR and translation attenuation. Maximal reglucosylation of prosaposin was observed immediately after the pulse when glucosidases were inhibited by DNJ (Figure 2.6A, lane 10 and 2.6B), and this level dropped significantly after a 1-hr oxidative chase (Figure 2.6A, lane 30 and 6B). Optimal reglucosylation also occurred in the presence of DTT

immediately after the pulse (Figure 2.6A, lane 12 and 2.6B), but this level of reglucosylation remained elevated even after the chase. When oxidation was initiated post-translationally, prosaposin was more efficiently reglucosylated compared to commencing oxidation during translation. This suggested that the post-translation oxidation of prosaposin was not as efficient and created aberrant conformations that continued to be recognized by UGT1 even after 1 hr of oxidation. Therefore, optimal reglucosylation occurred immediately after the pulse regardless of whether DTT was present; however, DTT supported efficient and persistent reglucosylation consistent with UGT1 preference for non-native substrates.

### **Discussion**

A main question concerning the biological role of UGT1 as a central decision maker in the fate of glycoproteins in the ER is whether UGT1 is able to recognize productively folding immature and/or terminally misfolded proteins, and how it affects the subsequent cellular fates of the proteins it modifies. Using a cell based reglucosylation assay, we found that UGT1 generally glucosylated mutant, off-pathway substrates more efficiently than wild type substrates. Secretion of wild type substrates was slowed and less efficient when they were trapped in the monoglucosylated state. Surprisingly, the turnover of off-pathway substrates was not affected by locking them in monoglucosylated states. These results suggested that UGT1 was capable of differentiating between native and non-native proteins, but downstream factors appeared to play a dominant role in determining the fate of terminally misfolded proteins.

The initial studies using cell-free or purified protein assays showed that UGT1 favored the modification of non-native compared to native conformations (Trombetta et al., 1989; Fernandez et al., 1994; Trombetta and Parodi, 1992; Parker et al., 1995; Sousa et al., 1992). More recent and detailed studies using biophysically characterized and engineered substrates further examined the specificity of UGT1 and found the preferential reglucosylation of proteins that are partially structured or possessed minor local folding defects (Taylor et al., 2004; Trombetta and Helenius, 2000; Caramelo et al., 2003; Ritter and Helenius, 2000; Caramelo et al., 2004; Ritter et al., 2005). In some of these reports, grossly misfolded structures were poorly modified. The glucosylation of biophysically characterized neoglycoproteins derived from C-terminal truncation fragments of chymotrypsin inhibitor 2 showed that UGT1 had a preference for fragments with molten globule-like conformations when compared to random coil or native conformations (Caramelo et al., 2003; Caramelo et al., 2004). Consistent with the preference of ER glucosyltransferases for near native targets, in some cases they did not efficiently modify reduced proteins (Pearse et al., 2008; Fernandez et al., 1998; Ritter and Helenius, 2000). The reglucosylation of cruzipain in *Trypanosoma cruzi* was directed towards later oxidative intermediates that enabled binding by its lone lectin chaperone calreticulin (Labriola et al., 1999). An earlier study using MI8-5 cells found that late but not early oxidative intermediates of hemagglutinin were reglucosylated on membrane proximal glycans, which supported persistent CNX binding (Pearse et al., 2008). From these results, it was concluded that UGT1 recognizes near native substrates more efficiently than native or severely misfolded substrates. This suggested that UGT1 might possess the ability to redirect the binding of lectin chaperones to maturing substrates that

are expected to be salvageable for eventual proper on-pathway folding and trafficking (D'Alessio et al., 2010).

Our cellular results are indicative of a less discriminatory role for UGT1 in live cells as UGT1 generally reglucosylated terminally misfolded mutant proteins more efficiently than immature wild type proteins. UGT1 was capable of modifying both on-pathway and off-pathway targets. The Z-variant involves a missense mutation and self-associates to form ER-retained polymers (Lomas et al., 1992). The less common NHK mutation consists of a frame-shift at position Leu318 that creates a premature termination codon at position 334 producing a more severe disruption to A1AT (Sifers et al., 1988). NHK is ER retained and subsequently degraded by ERAD. As A1AT is comprised of 3 sheets and 9 helices, NHK is missing or has mutations in one or two of the strands from each of the three sheets. Though Z and NHK likely have very different levels of structural perturbations, they are both reglucosylated at approximately twice that observed for wild type A1AT indicative of the ability of UGT1 to recognize terminally misfolded substrates. Ferris *et al.*, found that the level of mutant A1AT in triton-insoluble fractions was increased in MEF cells lacking UGT1 suggestive of a role for UGT1 in maintaining the solubility for glycoproteins (Ferris et al., 2013). However, in our study reglucosylated mutant A1AT was also found in the triton-insoluble fractions suggesting that reglucosylation did not ablate aggregation. These differences might be due to the hypoglycosylation or the constitutive activation of UPR found in MI8-5 cells (Pearse et al., 2008). Although UPR is activated in MI8-5 cells, the total levels of UGT1, CNX, CRT and BiP are unchanged (Pearse et al., 2008), which suggests that any difference, if existent, in their role in ER quality of MI8 compared to WT CHO cells, is not due to

changes in their abundance. Whether the activation of UPR has other side effects remains unknown. The hypoglycosylation does not seem to affect the fate of the protein. When fully glycosylated and hypoglycosylated A1AT were quantified (data not shown), similar trends in the extent of reglucosylation, secretion and degradation were observed. Yet, it remains to be determined if hypoglycosylation could have other consequences that have not been addressed yet, such as its effect on protein solubility or impact on interaction of the substrates with the lectin chaperones.

The ERAD substrate TCR $\alpha$  was also efficiently reglucosylated, as were the disease associated mutants for  $\alpha$ -NAGAL and tyrosinase. However, the increase in the reglucosylation of the mutant for  $\alpha$ -NAGAL compared to its wild type counterpart was not as high in magnitude as was observed for A1AT, and no increase in reglucosylation was observed for the mutant of tyrosinase compared to wild type. The reglucosylation assay traps reglucosylated glycans after a single reglucosylation event. Therefore, it cannot differentiate between the reglucosylation of folding intermediates that are transiently glucosylated from a terminally misfolded protein that might be persistently reglucosylated. Wild type tyrosinase appears to be a slow and inefficient folder (Halaban et al., 1997).  $\alpha$ -NAGAL also appears to fold inefficiently in CHO cells. This likely also helps to favor the efficient modification of their wild type proteins, dampening the difference in reglucosylation between wild type and mutants. Maturation of these difficult folders maybe further derailed in MI8-5 cells since the transfer of Man<sub>9</sub>GlcNAc<sub>2</sub> glycans bypasses the initial binding to CNX and CRT initiated by glucosidases I and II trimming of the triglucosylated glycan. Lectin chaperone binding is solely directed by UGT1 reglucosylation in MI8-5 cells (Figures 2.1A and 2.7A).

Earlier studies using a CHO-derived cell line that transfers Man<sub>5</sub>GlcNAc<sub>2</sub> carbohydrates (MadIA214 cells) found that maturing HA was reglucosylated, as well as a mutant of ribophorin that is an ERAD substrate (Ermonval et al., 2000; Ermonval et al., 2001). The fate of trapped monoglucosylated proteins was not followed and the efficiency for the reglucosylation of the mutant of ribophorin was not compared to the native ER resident protein. These studies displayed the scope of proteins modified by the glucosyltransferase in live cells and that proteins possessing Man<sub>5</sub> side chains are suitable substrates for modification. As mannosidase inhibitors stabilized mutant ribophorin expressed in MadIA214 cells that lacked B and C branch mannose residues, these results also suggest that mannose trimming of the A-branch might be required for ERAD. Alternatively, these mannose derivatives might also inhibit the binding of ERAD factors to the downstream ERAD adapter SEL1L, as has been found for EDEM1, EDEM3 and OS-9 (Christianson et al., 2008; Cormier et al., 2009; Saeed et al., 2011).

Proteomic and morphological studies indicated that UGT1 is largely situated in the smooth ER near ER-exit sites (Zuber et al., 2001; Gilchrist et al., 2006). This positioning is consistent with the observation that reglucosylation occurs post-translationally after the nascent chain has been released from the ribosome-associated translocon as ribosome-arrested nascent chains were unable to be reglucosylated (Pearse et al., 2008). This provides an explanation for the observation that UGT1 displayed a preference for late oxidative intermediates or folding domains in MI8-5 CHO cells and *T. cruzi* for *influenza* hemagglutinin and cruzipain, respectively (Pearse et al., 2008; Labriola et al., 1999). Therefore, the localization and accessibility of the enzyme rather than an inherent specificity towards more mature substrates likely explain these findings.



Conflicting results have been obtained for the ability of UGT to modify reduced proteins. Several studies found that UGT did not modify reduced proteins favoring the conclusion that UGT does not recognize grossly misfolded substrates (Pearse et al., 2008; Trombetta and Helenius, 2000; Fernandez et al., 1998; Ritter and Helenius, 2000). In some instances, reglucosylation was dependent on the alkylating agent employed (Trombetta and Helenius, 2000) while in other cases, UGT1 was found to modify reduced proteins (Taylor et al., 2004; Zapun et al., 1997). The absence of reglucosylation in microsomes or cells treated with DTT could be explained by either the timing being soon after synthesis as reglucosylation appears to occur post-translationally, or perhaps problems with the protein bookkeeping in cells as DTT induces the UPR that leads to protein translation attenuation. We found that DTT supported the efficient and persistent reglucosylation of endogenous prosaposin in cells, favoring the conclusion that UGT1 can modify severely misfolded non-native substrates.

UGT1 can also recognize slight structural perturbations as it efficiently modified the orphan subunit of the heteromeric T-cell receptor, TCR $\alpha$ . A recent study found that unassembled TCR $\alpha$  caused the release of its single transmembrane region into the ER lumen where it was then targeted for ERAD (Feige and Hendershot, 2013). The ability of UGT1 to recognize slight alterations is also exploited for the regulation of cellular processes such as antigen presentation and calcium homeostasis by supporting the recruitment of lectin chaperones and their associated oxidoreductase ERp57 (Kunte et al., 2013; Camacho and Lechleiter, 1995; John et al., 1998; Li and Camacho, 2004). The reglucosylation of fully folded MHC class I heavy chain or SERCA2b modulates their function and localization. It is clear that UGT1 can recognize subtle aberrations in protein

structure but it also efficiently recognizes terminally misfolded substrates. UGT1 does not appear to have the ability to preferentially select on-pathway or repairable substrates for further lectin chaperone intervention but rather simply modified non-native substrates.

The secretion of glucosylated trapped wild type proteins was significantly delayed. A similar finding was previously observed for endogenous cruzipain in *T. cruzi* that naturally transfers high mannose glycans (Labriola et al., 1995). That secretion of wild type glucosylated proteins in cells treated with DNJ would ultimately saturate at the same level of proteins secreted in cells non-treated with DNJ remains to be investigated. This can be done by examining secretion in both conditions at longer time points. In sharp contrast, the more efficient reglucosylation of ERAD substrates did not appear to influence their fate, or the turnover of terminally misfolded proteins when glucosylated glycans were trapped on the substrate. This was suggestive of a dominant downstream ERAD sorting receptor playing an important role that was unaffected by the presence of glucosylated glycans or lectin chaperone binding. Mannose trimming exposes  $\alpha$ 1,6-linked mannoses side chains on C-chains that act as degradation tags for recognition by the luminal ERAD receptors (Quan et al., 2008; Clerc et al., 2009). Alternatively, A-branch demannosylation would remove a glycoprotein from being a substrate for reglucosylation and subsequent lectin chaperone binding to possibly favor recognition by ERAD machiner (Olivari et al., 2006).

Therefore, mannose trimming likely plays a key role in rapidly sorting the trapped glucosylated proteins for destruction. Understanding the precise mechanism by which mannose trimming contributes to the ERAD process is complicated by the dual role that N-linked glycans play in the secretory pathway as they act as sorting and quality control

tags, as well as docking tags for ER machinery complex formation and targeting (Hebert and Molinari, 2012).

The recognition and modification of ERAD substrates by UGT1 appears to be more a consequence of the ability of UGT1 to recognize structural imperfections akin to molecular chaperones rather than of functional significance that directly impacts the fate for defective proteins. Downstream ERAD receptors evidently efficiently sort defective cargo for destruction regardless of their glucosylation status. Our results favor the model that UGT1 modifies non-native proteins regardless of whether they are on- or off-pathway (Figure 2.7A and 2.7B). This query likely occurs after an initial period of maturation that is dictated by the localization of UGT1 and its access to the maturing nascent chain. Importantly, reglucosylation inhibits secretion of on-pathway substrates either by supporting persistent chaperone binding or inhibiting binding to anterograde targeting receptors (Wieland et al., 1987; Balch et al., 1994; Aridor and Balch, 1996). The significance of the more efficient reglucosylation of off-pathway terminally misfolded substrates is uncertain as they evidently display a signal that is efficiently recognized by a downstream ERAD receptor. Perhaps persistent lectin chaperone binding of the monoglucosylated substrate localizes the ERAD substrate to a location in the ER where ERAD receptors or mannosidases are concentrated such as the proposed ER quality control compartment (ERQC) where CNX, CRT, ER ManI and EDEM1 have been localized but not UGT1 and ERp57 (Avezov et al., 2008). Likely candidates to shepherd glycosylated proteins through this retrograde trafficking route include ER ManI/Man1B1, EDEM1-3, Os-9 and XTP3-B (Hosokawa et al., 2003; Molinari et al., 2003; Oda et al., 2003; Hosokawa et al., 2009; Groisman et al., 2011; Aikawa et al.,

2014). Currently there is vigorous debate over the location, roles and substrate selectivity for some of these factors.

Some diseases are caused by the ER retention of an otherwise active protein by an apparent overzealous quality control process (Guerriero and Brodsky, 2012), which is capable of recognizing slight imperfections found even associated with active proteins. If the quality control process could be relaxed for these substrates, proper trafficking could alleviate the loss-of-function disease. UGT1 is a prime candidate for modulation for diseases of this category as the reglucosylation of wild type on-pathway targets supported ER retention, and delayed the secretion of potentially active substrates. The fact that the terminally misfolded substrates were still cleared efficiently and rapidly when proteins were trapped with monoglucosylated glycans suggests that the modulation of the activity of UGT1 might not disrupt the ERAD process and might provide an effective therapy for the treatment of some of these loss-of-function diseases. A recent study showed that recombinant ER ManI/Man1B1 was capable of trimming glucosylated glycans, and favored the trimming of denatured proteins (Aikawa et al., 2014); however, there is current debate on whether this exo-mannosidase is localized to the ER or Golgi, and the necessity of its activity for ERAD (Avezov et al., 2008; Pan et al., 2013; Ninagawa et al., 2014). Further studies will be required to identify the mechanism by which reglucosylated ERAD substrates are efficiently recognized and targeted for degradation. Sorting out these concerns and the mechanism by which reglucosylated ERAD substrates are efficiently recognized and targeted for degradation will require further cellular experiments, as well as approaches using purified components. However, in the next chapter, we will discuss how mannose trimming is involved in termination of

reglucosylation cycles, and potentially mannosidases could be extracting proteins from the CNX cycle for degradation.

### **Materials and Methods**

**Reagents-** MI8-5 Chinese hamster ovary (CHO) cells were a gift from S. Krag (Johns Hopkins University, Baltimore, MD) (Quellhorst et al., 1999). Plasmids for pGEX-3X GST-calreticulin, T cell receptor  $\alpha$  (TCR $\alpha$ ) with a HA tag at its C terminus, human  $\alpha$ 1-antitrypsin and  $\alpha$ -NAGAL were from M. Michalak (University of Alberta), S. Fang (University of Maryland), M. Ziak and J. Roth (University of Zurich), and S. Garman (University of Massachusetts, Amherst), respectively. Polyclonal rabbit  $\alpha$ 1-antitrypsin (Dako, Denmark) and calreticulin antisera (Thermo Fisher Scientific), monoclonal mouse HA antibodies (Roche, Mannheim, Germany) and polyclonal goat antisera against murine prosaposin (G. Grabowski, University of Cincinnati, College of Medicine) were obtained as indicated. Cell culture material and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA) and polyethylenimine (PEI) from Polysciences Inc. (Warrington, PA). [ $^{35}$ S]-Met/Cys was acquired from PerkinElmer (Waltham, MA). Reduced glutathione Sepharose 4B and protein- A Sepharose 4B were from GE Healthcare (Uppsala, Sweden) and protein G-plus agarose beads from Santa-Cruz Biotechnology (Santa Cruz, CA). N-butyl deoxynojirimycin (DNJ) and kifunensine (KIF) were obtained from Toronto Research Chemicals (Toronto, Canada). PNGase F and EndoH were acquired from New England Biolabs (Ipswich, MA). All other reagents were purchased from Sigma-Aldrich.

**Transfection and metabolic labeling-** MI8-5 CHO cells were cultured in minimum essential media alpha supplemented with 5 or 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin at 34 °C in 5% CO<sub>2</sub>. Nearly confluent cells were transfected with the indicated plasmid using Lipofectamine 2000 or polyethyleneimine according to manufacturer's instructions.

Cells were starved for 30 min or 1 hr in Cys/Met free media with 0.5 mM DNJ where indicated, then pulse labeled with 60 µCi of [<sup>35</sup>S]-Cys/Met in 3.5 cm plates for 30 min or 1 hr as indicated. When analyzing degradation of TCRα or NHK, no prior amino acid starvation was performed. Instead, cells were pre-incubated with 0.5 mM DNJ for 30 min before pulse labeling. Immediately after the pulse, cells were washed with phosphate buffer solution (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.4 mM KH<sub>2</sub>PO<sub>4</sub>) and chased with regular growth media for the indicated times. DNJ was present throughout the chase where indicated except for figure 2.5 where DNJ was added at the indicated times relative to the start of chase. Radiolabeled cells were washed twice with PBS on ice followed by lysis with MNT buffer (20 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), 100 mM NaCl, 0.5% triton X-100, 30 mM Tris-HCl pH 7.5) containing 50 µM calpain inhibitor I, 1 µM pepstatin, 10 µg/mL aprotinin, 10 µg/ml leupeptin, 0.4 mM phenylmethyl-sulfonyl fluoride (PMSF) and 20 mM N-ethyl maleimide (NEM). Media and the triton X-100 insoluble pellet were collected where indicated. Cells were incubated as designated with 20 µM MG132 or 100 µM KIF for 4 or 2 hr before the pulse, respectively.

**Immunoprecipitations and SDS-PAGE-** The post-nuclear fraction was pre-cleared with 10% zysorbin for 1 hr at 4 °C then incubated with antibody and protein A-sepharose

beads and rotated end-over-end for 14 hr at 4 °C, except with the prosaposin antibody where protein G-agarose was used. The triton X-100 insoluble pellet was solubilized in 1% SDS in 100 mM Tris-HCl pH 8 by high-speed vortexing at 22 °C for 5 min, followed by heating for 10 min at 100 °C then sonication. The SDS was quenched by dilution with excess MNT buffer. The media and triton X-100 insoluble fractions were also pre-cleared with 10% zysorbin and immunoprecipitations were conducted as described above for the post-nuclear supernatant. Immunopellets were washed with 100 mM Tris pH 8.6, 300 mM NaCl, 0.1% SDS and 0.05% triton X-100. Proteins were eluted from beads with reducing sample buffer then SDS-PAGE was performed. Radiolabeled samples were visualized by phosphorimaging (FLA-500; Fujifilm) and quantified using MultiGauge software or ImageQuant (Fujifilm).

**GST-calreticulin pull-down-** Recombinant GST-calreticulin was expressed in *E. coli* and purified as previously described (Pearse et al., 2008; Baksh and Michalak, 1991). A fraction of the cell lysate was incubated with 8 µg of purified GST-calreticulin pre-bound to reduced glutathione beads at 4 °C and rotated end-over-end for 14 hr. Samples were washed with 100 mM Tris pH 8.6, 300 mM NaCl, 0.1% SDS and 0.05% triton X-100, and eluted with reducing sample buffer. If followed by immunoprecipitation, samples were eluted with 1% SDS in 10 mM Tris pH 7.5 and 150 mM NaCl at 100 °C and quenched with excess MNT buffer followed by incubation with the corresponding antisera and sepharose beads overnight and washing as described above.

**Co-immunoprecipitation studies with calnexin and calreticulin-** Cells were grown in 10 cm plates, lysed with 2% CHAPS in 50 mM HEPES, 200 mM NaCl pH 7.5 (HBS) and incubated with the respective antibody for 3 hr. Immunopellets were washed with

0.5% CHAPS in HBS and eluted from beads with reducing sample buffer. When followed by a second immunoprecipitation, samples were eluted with 1% SDS in 10 mM Tris pH 7.5, 150 mM NaCl at 100 °C, quenched with excess 2% CHAPS in HBS followed by incubation with the corresponding antisera and sepharose beads overnight and washing with 0.5% CHAPS in HBS.

**Calculation of percentages of reglucosylation-** The percentage of reglucosylation was calculated by dividing the number obtained from quantifying the bands in the lanes with the GST-CRT pull down followed by immunoprecipitation, by the number obtained from quantifying the bands in the lanes with the corresponding immunoprecipitation directed against the given substrate. A larger fraction of the cell lysate was used for the GST-CRT pull down followed by immunoprecipitation. Since different percentages of lysate were used to perform the GST-CRT pull down followed by immunoprecipitation and the immunoprecipitation alone, this number was accounted for in the division. A linear correlation is assumed between the signal and the fraction of cell lysate used. Yet, due to an expected partial loss of signal as a consequence of multiple washes, the actual percentages of reglucosylation may be higher. The fully glycosylated bands were used for the quantification when they were well resolved. Otherwise, all bands were quantified.



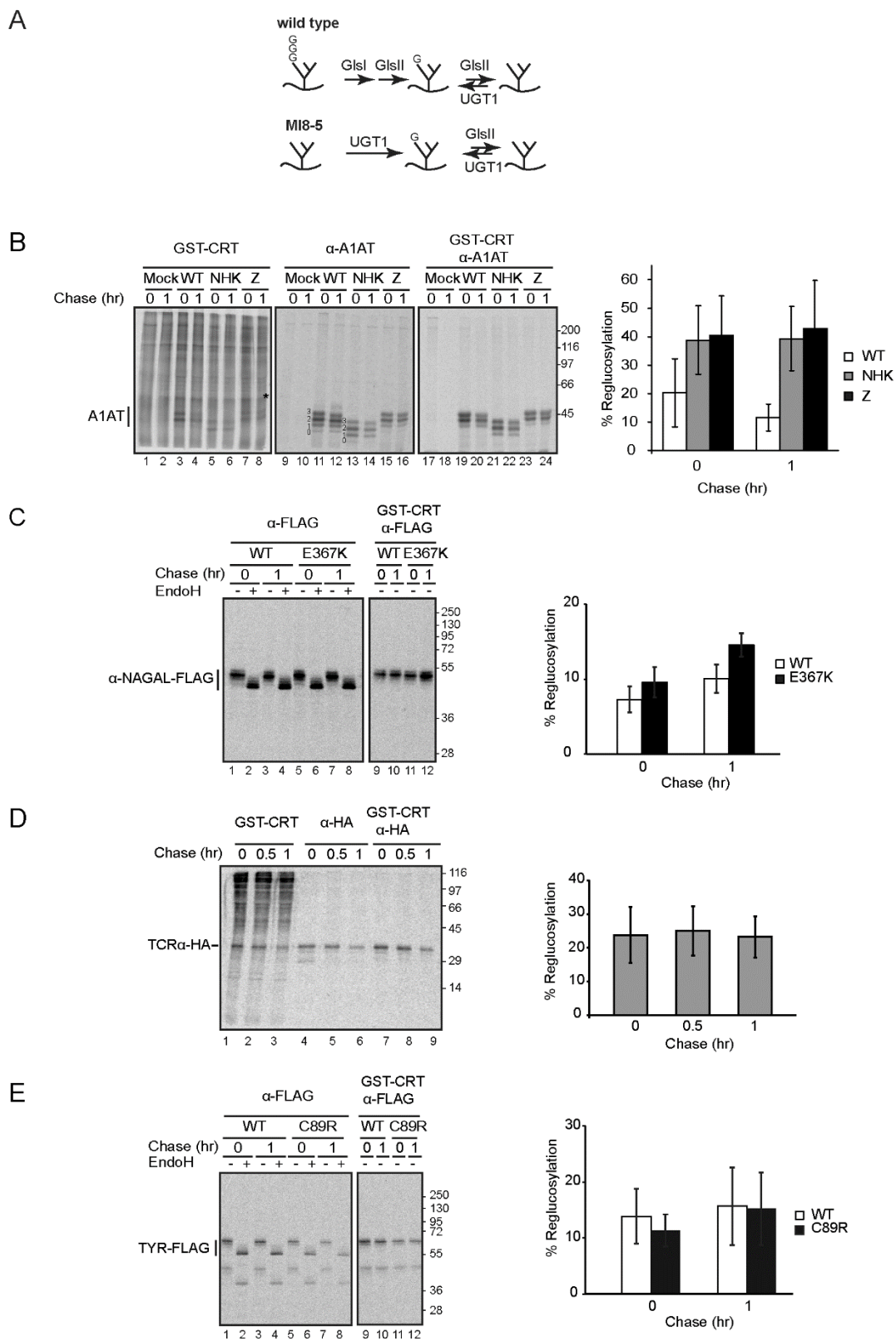


Figure 2.1 Reglucosylation of ERAD and disease associated mutants is efficient.

(A) Schematic of glycan processing in MI8-5 and wild type cells. While wild type cells allow the transfer of triglucosylated glycans onto the nascent chain, unglucosylated glycans are transferred in MI8-5 cells. Thus, monoglucosylated glycans can only be generated through reglucosylation by UGT1 in MI8-5 cells. G, glucose; GlsI, glucosidase I; and GlsII, glucosidase II. (B) Right- MI8-5 CHO cells were transiently transfected either with an empty vector (mock) or with wild type alpha-1-antitrypsin (WT A1AT), alpha-1-antitrypsin *null* Hong Kong (NHK) and alpha-1-antitrypsin Z (A1ATZ). Cells were radiolabeled for 1 hr and chased for the indicated times. 0.5 mM DNJ was added to the pulse and chase media. Monoglucosylated proteins were isolated by GST-calreticulin (CRT) pull down from 10% of the cell lysate each and WT A1AT, NHK and A1ATZ were isolated with A1AT antisera. Monoglucosylated WT A1AT, NHK and A1ATZ were isolated by GST-CRT pull down followed by immunoprecipitation with A1AT antisera from 80% of the cell lysate. All samples were resolved on 9% SDS-PAGE reducing gels. The numbers next to the bands indicate the number of glycans on A1AT. The asterisk indicates the previously identified endogenous UGT1 substrate prosaposin. Left- Quantification of the percentage of reglucosylation of WT A1AT, NHK, and A1ATZ. The quantifications of the fully glycosylated (top band) from lanes 19-24 were divided by the quantifications from lanes 11-16 that were multiplied by 8 to account for lower percentage of sample used. The error bars are representative of the standard deviation of five independent experiments. (C) Right- MI8-5 CHO cells were transiently transfected with FLAG tagged  $\alpha$ -NAGAL, wild type and E367K mutant and treated as in B.  $\alpha$ -NAGAL was isolated with antisera recognizing the FLAG epitope from 10% of the cell lysate and an equal fraction was followed by EndoH treatment where indicated. Monoglucosylated  $\alpha$ -NAGAL was isolated by GST-CRT pull down followed by immunoprecipitation with FLAG antisera from 70% of the cell lysate. All samples were resolved on a 9% SDS-PAGE reducing gel. Left- Quantification of the percentage of reglucosylation of  $\alpha$ -NAGAL was performed as in B. The error bars are representative of the standard deviation of three independent experiments. (D) Right- MI8-5 CHO cells were transiently transfected with HA tagged TCR $\alpha$  and treated as in B. TCR $\alpha$  was isolated with antisera recognizing the HA epitope and total radiolabeled monoglucosylated proteins with GST-CRT from 10% of the cell lysate. Monoglucosylated TCR $\alpha$  was isolated by GST-CRT pull down followed by immunoprecipitation with HA antisera from 80% of the cell lysate. All samples were resolved on a 12% SDS-PAGE reducing gel. Left- Quantification of the percentage of reglucosylation of TCR $\alpha$  was performed as in B. The error bars are representative of the standard deviation of three independent experiments. (E) Right- MI8-5 CHO cells were transiently transfected with FLAG tagged tyrosinase, wild type and C89R mutant and treated as in B. Total and monoglucosylated tyrosinase were isolated as in C. Left- Quantification of the percentage of reglucosylation of tyrosinase was performed as in B. Statistical analysis using single factor anova test gave a P value of  $0.04 < 0.05$  for WT A1AT compared to NHK and Z mutants at 0 hr, P values of 0.001 and 0.004  $< 0.01$  for

WT A1AT compared to NHK and Z mutant respectively at 1 hr and a P value of 0.03 <0.05 for WT  $\alpha$ -NAGAL compared to the E367K at 1 hr indicating that the increase in reglucosylation of the mutants relative to WT at the indicated time points is statistically significant.

Panels B and D are experiments performed by Nishant Patel

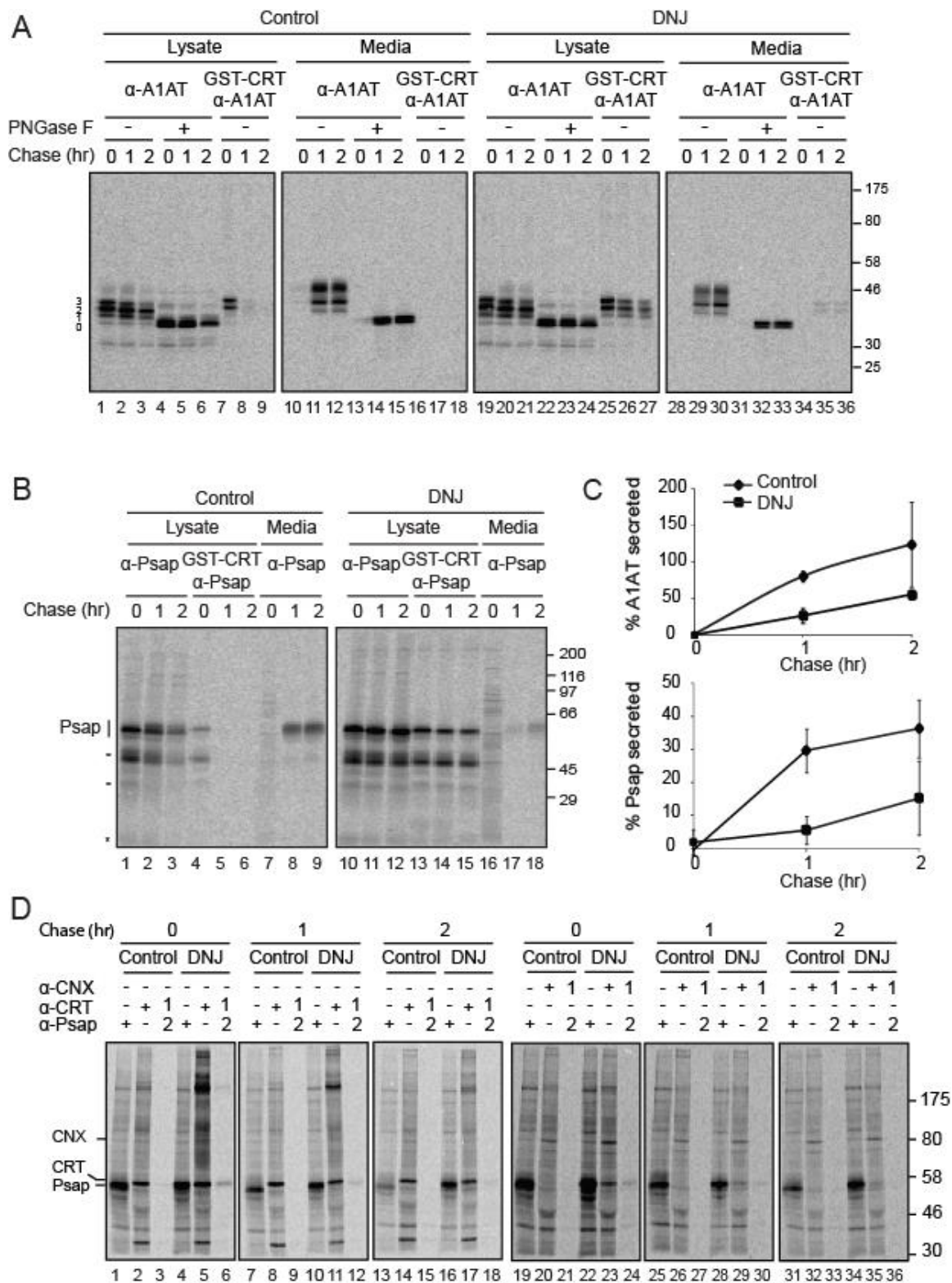


Figure 2.2 Trapping WT A1AT and the endogenous substrate prosaposin (Psap) in the monoglucosylated state reduced their secretion efficiency and prolonged chaperone binding.

(A) MI8-5 CHO cells were transiently transfected with WT A1AT, radiolabeled for 30 min and chased for the indicated times. 0.5 mM DNJ was added to the pulse and chase media where indicated. WT A1AT was isolated with A1AT antisera from 12% of the cell lysate or the media and an equal fraction was treated with PNGase F. Monoglucosylated WT A1AT was isolated by GST-CRT pull down followed by immunoprecipitation with A1AT antisera from 50% of the cell lysate or the media. All samples were resolved on a 9% SDS-PAGE reducing gel. (B) MI8-5 CHO cells were radiolabeled for 30 min and chased for the indicated times. 0.5 mM DNJ was added to the pulse and chase media where indicated. Psap was isolated with Psap antisera from 30% of the lysate or the media. Monoglucosylated Psap was isolated by GST-CRT pull down followed by immunoprecipitation with Psap antisera from 60% of the cell lysate. All samples were resolved on a 9% SDS-PAGE reducing gel. The asterisk (\*) indicates cleaved Psap products. (C) Quantification of the percentage of the fully glycosylated (top band) WT A1AT (Top panel) and endogenous prosaposin (Bottom panel) secreted into the media as a percentage of synthesis. The error bars are representative of the standard deviation from three independent experiments. (D) MI8-5 CHO cells were radiolabeled for 1 hr and chased for the indicated times. Prosaposin (Psap) was isolated from 5% of the lysate with Psap antisera, calreticulin (CRT) and calnexin (CNX) bound species from 15% of the lysate with their respective antisera. For sequential immunoprecipitations, 65% of the lysate was sequentially immunoprecipitated as indicated. All samples were resolved via 7.5% reducing SDS-PAGE

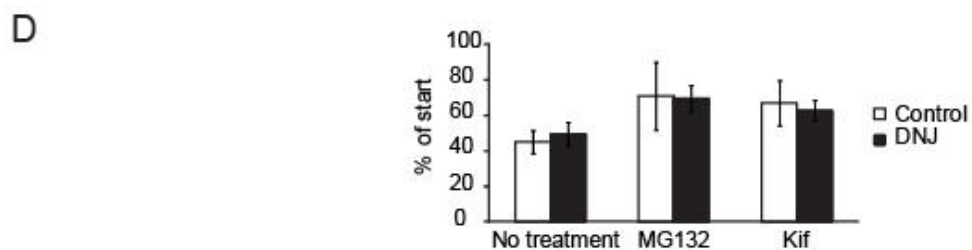
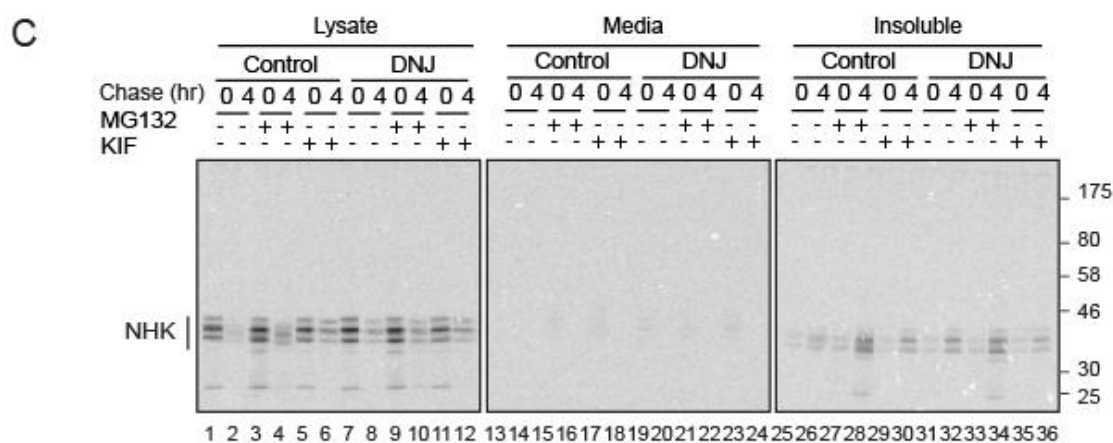
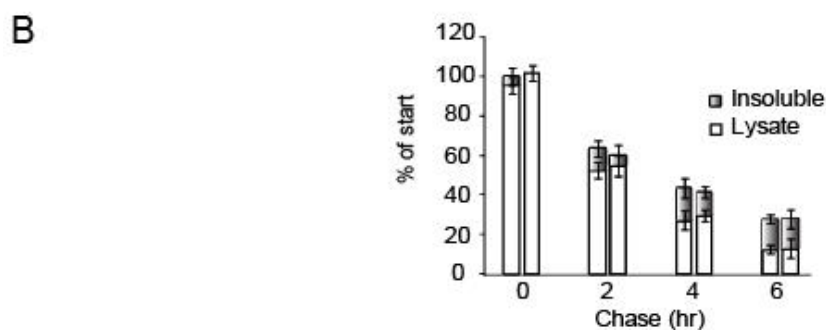
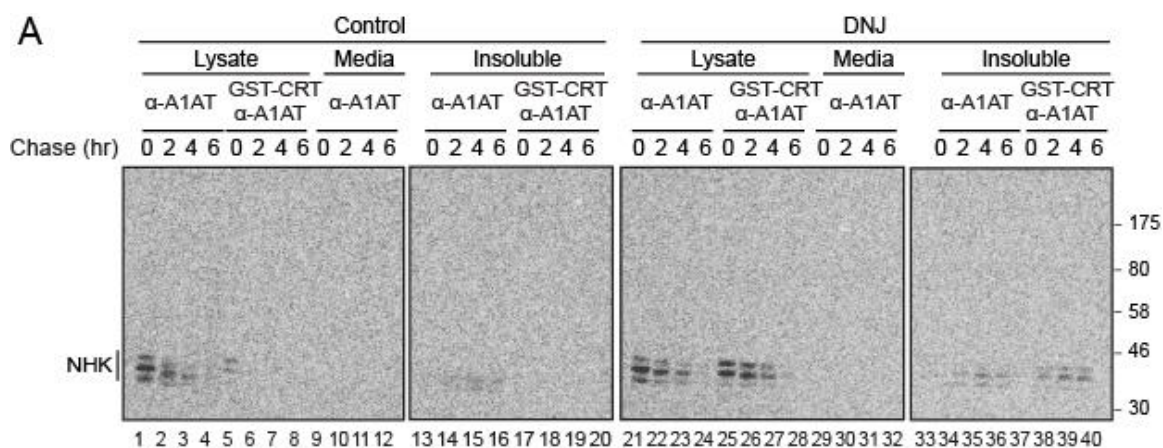


Figure 2.3 Trapping NHK in the monoglucosylated state did not change its degradation rate.

(A) MI8-5 CHO cells were transiently transfected with NHK, radiolabeled for 30 min and chased for the indicated times. 0.5 mM DNJ was added to the pulse and chase media where indicated. NHK was isolated with A1AT antisera from 10% of the lysate, the media or the triton X-100 insoluble fraction. Monoglucosylated NHK was isolated by GST-CRT pull down followed by immunoprecipitation with A1AT antisera from 80% of the cell lysate or the triton X-100 insoluble fraction. All samples were resolved on a 9% SDS-PAGE reducing gel. (B) Quantification of the percentage of NHK from the lysate and the triton X-100 insoluble fraction as a percentage of synthesis. The first bar for each time point corresponds to control conditions whereas the second corresponds to DNJ treatment. All bands were quantified because the fully glycosylated (top band) was not always well resolved in the later time points or the insoluble fraction. The error bars are representative of the standard deviation of three independent experiments. (C) Cells were treated as in panel A. 20  $\mu$ M of MG132 or 100  $\mu$ M kifunensine was added 4 and 2 hr, respectively, before the pulse, and included in the pulse and the chase where indicated. All samples were resolved on a 9% reducing SDS-PAGE gel. (D) Quantification of the percentage of the sum of NHK from the cell lysate and the triton X-100 insoluble fraction as a percentage of synthesis (4 hr chase). The error bars are representative of the standard deviation of three independent experiments.

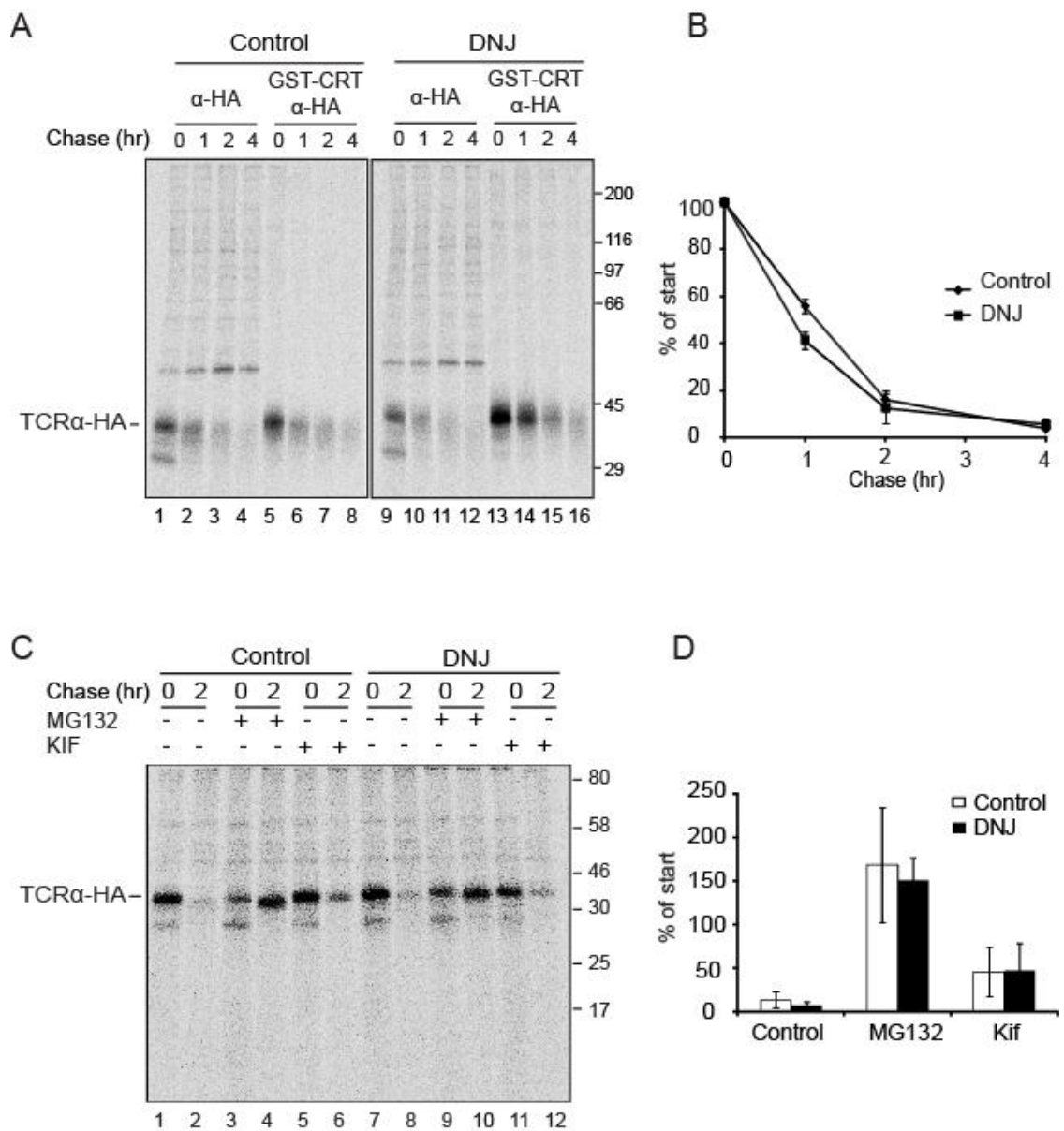
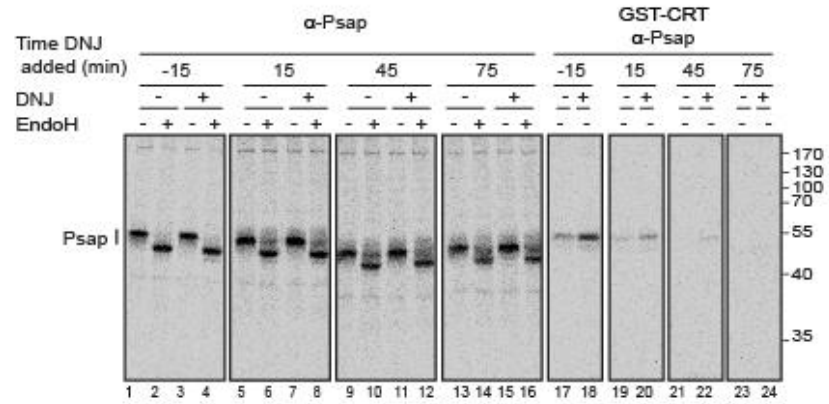




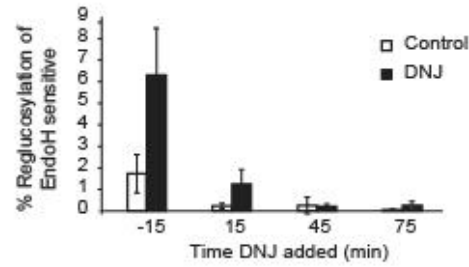
Figure 2.4 Trapping TCR $\alpha$  in the monoglucosylated state did not change its degradation rate.

(A) MI8-5 CHO cells were transiently transfected with TCR $\alpha$ , radiolabeled for 30 min and chased for the indicated times. 0.5 mM DNJ was added to the pulse and chase media where indicated. TCR $\alpha$  was isolated with HA antisera from 40% of the lysate. Monoglucosylated TCR $\alpha$  was isolated by GST-CRT pull down followed by immunoprecipitation with HA antisera from 50% of the cell lysate. All samples were resolved on a 9% SDS-PAGE reducing gel. (B) Quantification of the percentage of TCR $\alpha$  from the lysate as a percentage of synthesis. The error bars are representative of the standard deviation of three independent experiments. (C) Cells were treated as in panel A. MG132 (20  $\mu$ M) or kifunensine (100  $\mu$ M) was added 4 and 2 hr, respectively before the pulse, and included in the pulse and the chase where indicated. (D) Quantification of the percentage of TCR $\alpha$  from the cell lysate as a percentage of synthesis (2 hr chase). The error bars are representative of the standard deviation of four independent experiments for no treatment and three independent experiments for MG132 and kifunensine treatments.

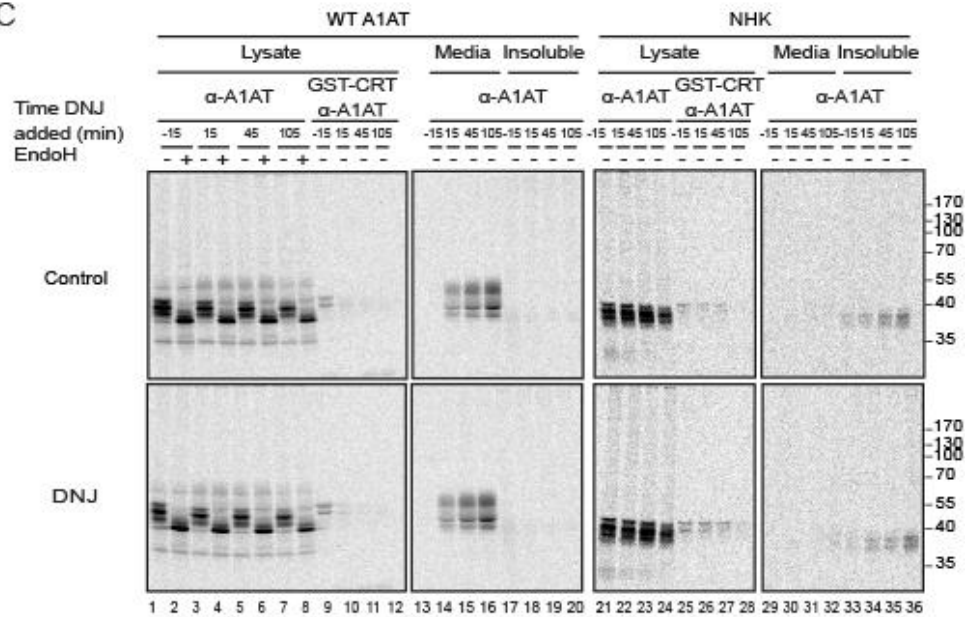
A



B



C



D

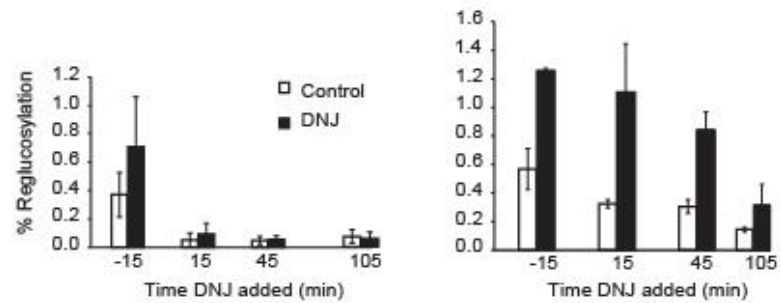


Figure 2.5 WT A1AT and prosaposin are transiently reglucosylated by UGT1 while reglucosylation of NHK is more persistent.

(A) MI8-5 CHO cells were radiolabeled for 30 min. Where indicated, 0.5 mM DNJ was added 15 min before the end of the pulse. DNJ was also added at the indicated times of the chase, followed by 15 min incubation. Prosaposin was isolated with prosaposin antisera followed by treatment with EndoH where indicated. Monoglucosylated prosaposin was isolated by GST-CRT pull-down followed by immunoprecipitation with prosaposin antisera. All samples were resolved on a 9% SDS-PAGE reducing gel. (B) The quantification of the percentage of reglucosylation of prosaposin was performed as in figure 2.1 accounting for differences in amounts of sample used for each treatment as a percentage of EndoH-sensitive prosaposin since this represents ER localized protein compared to EndoH-resistant protein localized to the lysosome. The error bars are representative of the standard deviation of three or more independent experiments. (C) MI8-5 CHO cells were transfected with either WT or NHK A1AT and treated as in A. (D) The quantification of the percentage of reglucosylation of all bands was performed as in figure 2.1 accounting for differences in amounts of sample used for each treatment. The error bars are representative of the standard deviation of three or more independent experiments.

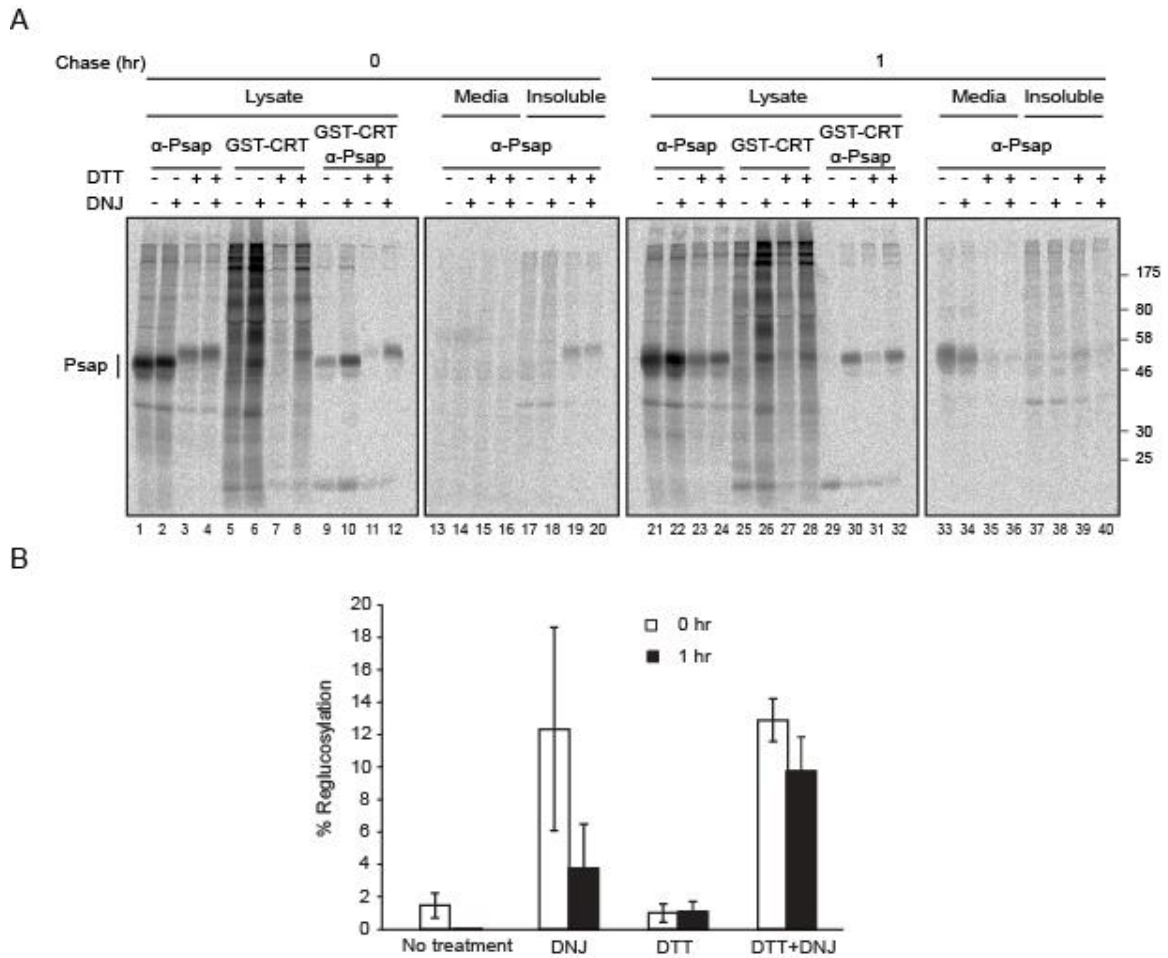
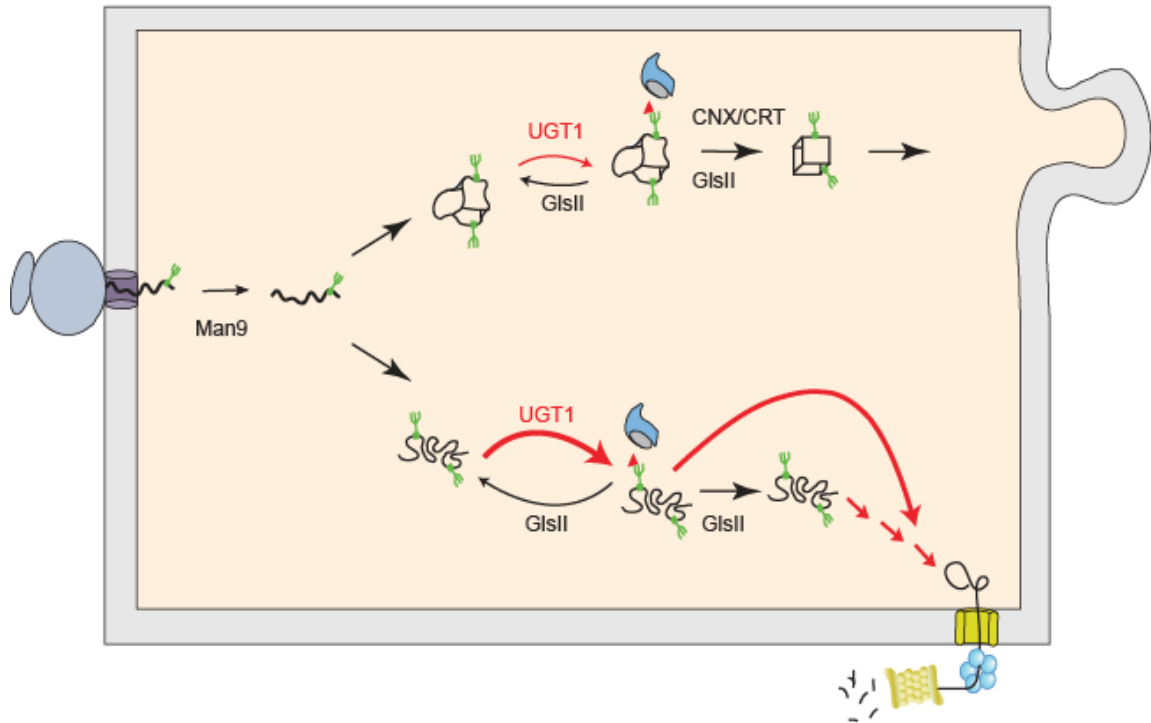


Figure 2.6 Reduced prosaposin is efficiently reglucosylated by UGT1.

Cells were radiolabeled for 30 min and chased for 1 hr. Where indicated, 0.5 mM DNJ was added in the pulse and chase media. 5 mM DTT was added in the pulse media where indicated and excluded from the chase media. Prosaposin was isolated with prosaposin antisera. Monoglucosylated prosaposin was isolated by GST-CRT pull-down followed by immunoprecipitation with prosaposin antisera. All samples were resolved on a 9% SDS-PAGE reducing gel. (B) The quantification of the percentage of reglucosylation of prosaposin was performed as in figure 2.1 accounting for differences in amounts of sample used for each treatment. The error bars are representative of the standard deviation of three or more independent experiments.

A



B

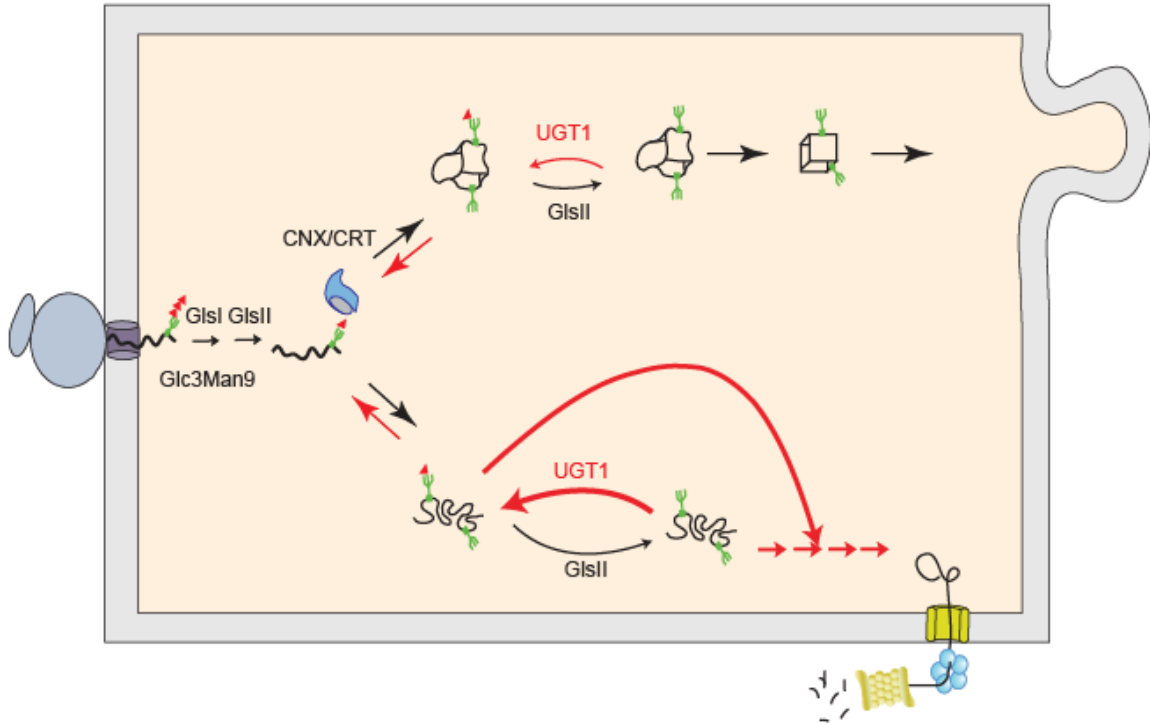


Figure 2.7 Model for UGT1 reglucosylation of on- and off-pathway substrates.

(A) In MI8-5 CHO cells, unglucosylated glycans are transferred onto the protein. On-pathway substrates in the near-native conformations are transiently reglucosylated by UGT1 until folding is completed (top route). DNJ addition favors accumulation of these substrates in the UGT1 cycle supporting prolonged chaperone binding and secretion delay (represented by the red arrows). In contrast, aberrant off-pathway substrates are more efficiently and persistently reglucosylated by UGT1 (as indicated by the more prominent arrows on the bottom route). The addition of the glucosidase inhibitor DNJ, despite trapping misfolded proteins in their monoglucosylated state, does not protect substrates from ERAD as they are efficiently extracted for degradation by a dominant ERAD selection process (exhibited by the long red arrows). (B) In WT CHO cells, monoglucosylated substrates generated by GlcI and GlcII trimming bind to the lectin chaperones. After trimming of the last glucose by GlcII, substrates are released from the lectin chaperones and continue to be recognized by UGT1 and proceed in the top or bottom routes as described in part A.

## **CHAPTER 3**

### **THE ROLE OF MANNOSE TRIMMING IN TERMINATING REGLUCOYLATION CYCLES**

#### **Introduction**

Glycoproteins that have gone through several reglucosylation and deglucosylation rounds will ultimately exit the reglucosylation cycles. Those that have folded successfully will leave the ER via vesicular trafficking, while those that have failed to fold will be sentenced for destruction by ERAD. Nevertheless, it is still not understood how the reglucosylation/deglucosylation cycles are terminated. One possibility is that UGT1 will keep recognizing substrates as long as they have not folded properly. While this could be the case, even misfolded proteins cease being recognized by UGT1, which hints that there is another mechanism independent of the folding status of the substrate that ultimately shuns misfolded substrates from futile and endless cycles of reglucosylation. The failure of proteins to exit the reglucosylation cycles could have deleterious consequences on the cell as it may result in accumulation of improperly folded substrates in the ER which can stress the organelle and could lead to aggregation or apoptosis.

Mannose trimming has been hypothesized to be the event that results in the exit of glycoproteins from reglucosylation cycles (Sousa et al., 1992). Trimming of mannose residues may terminate reglucosylation by UGT1 in two ways: First, trimming of the mannose residue that receives the glucose added by UGT1 on the A branch of the glycan will stop reglucosylation. Second, the trimming of the mannose residues of the B and C

branches of the glycan may decrease the affinity of UGT1 for glycans, which would reduce the reglucosylation activity (Figure 3.1).

Evidence from previous work that mannose trimming contributes to terminating reglucosylation cycles has been unsatisfactory and sometimes conflicting. An early study showed that UGT1 from rat liver microsomal extracts recognized Mannose (Man)<sub>8</sub> and Man<sub>7</sub> glycans at a much slower rate than Man<sub>9</sub> (Sousa et al., 1992). The recognized glycans still retained the glucose acceptor mannose residue of the A branch, suggesting that the reduced number of mannose residues in the B and C branches diminishes UGT1's ability to recognize the modified glycan. However, the results of this assay are inconclusive since they were performed using a cell free approach, and it is not certain that it recapitulates the nuances of UGT1 inside the ER.

Cellular assays presented conflicting data that mannose trimming may not be the main factor to reduce the glucose transfer by UGT1. A study using a mutant CHO cell line MadIA214, which only transfers Man<sub>5</sub>GlcNAc<sub>2</sub> missing most of the mannose residues in the B and C branches, found that a major fraction of the glycans in this cell line are glucosylated (Ermonval et al., 2001). The only way glucose containing glycans can be formed in MadIA214 cells is via reglucosylation by UGT1. This suggests that mammalian UGT1 may efficiently recognize glycans with low mannose content in the B and C branches, unlike what has been previously proposed (Ermonval et al., 2001). Nonetheless, the study did not provide a quantitative approach to directly compare the levels of reglucosylation of high mannose to low mannose glycans.



Support for this observation is an investigation in *Saccharomyces pombe* using different strains with mutations in the glycan synthesis pathway. These strains allowed the transfer of truncated glycans with various mannose residues content of Man9, Man7, Man6 and Man5, and showed that UGT1 activity is not influenced by reducing the number of mannose residues in yeast (Stigliano et al., 2011). In agreement with this, a study suggested that the glucosyltransferase of *Trypanosoma brucei* may not only reglucosylate Man9 but also Man5 containing glycans, hinting that the *T. brucei* glucosyltransferase may recognize glycans with lower mannose content (Izquierdo et al., 2009). In conclusion, it is apparent that cellular assays do not always agree with cell free assays. Thus, it remains to be determined if mannose trimming is responsible for triggering the release of glycoproteins from the reglucosylation cycles.

Using our previously described cell based reglucosylation assay (Chapter 2), we investigated the hypothesis that mannose trimming prevents UGT1 mediated reglucosylation. We found that inhibiting mannose trimming significantly enhanced the accumulation of a reglucosylated misfolded substrate. We concluded that mannose trimming hinders glucose transfer by UGT1 in live cells, thus causing the termination of reglucosylation cycles. Similarly, an endogenous substrate that eventually folds also accumulated in the reglucosylated form upon inhibition of mannose trimming, although to a less extent than the misfolded substrate. Thus, the extent of the contribution of mannose trimming to terminating reglucosylation cycles depends on the properties of the substrate.

## **Results**

To investigate the length of persistence of substrates in the reglucosylation cycles, we employed the cell based reglucosylation assay relying on MI8-5 CHO cells that transfer unglucosylated glycans. Therefore, monoglucosylated substrates in MI8-5 are generated exclusively via UGT1 reglucosylation. To trap substrates in the monoglucosylated state, deglucosylation by glucosidase II was inhibited by addition of the inhibitor DNJ. Cells were radiolabeled with  $^{35}\text{S}$ -cysteine/methionine, chased for the indicated time, and monoglucosylated substrates were isolated by performing a GST-CRT pull down. If DNJ was to be added throughout the pulse and the chase, monoglucosylated substrates generated by one round of reglucosylation are trapped in that state for the length of the experiment, thus making the timing of the reglucosylation cycles indiscernible. In the interest of determining the timing of UGT1 cycles, DNJ is added only shortly before lysing the cells at the indicated time points (Figure 3.2).

To inhibit mannose trimming, the ER mannosidase inhibitors DMJ and kifunensine were added throughout the pulse and the chase where indicated (Figure 3.2). Cells were transfected with the misfolded ERAD substrate NHK A1AT, a truncated form of A1AT resulting from a frame shift that inserts a premature stop codon. The cells were radiolabeled for 30 min and chased for the indicated time. DNJ was added only at 15 min before lysing the cells and performing immunoprecipitation with A1AT antibody. Mannose trimming can clearly be seen after 1 hr of chase by the faster migrating bands, and continues to 2 hr of chase (Figure 3.3, lanes 7, 8 and 13, 14). Upon addition of DMJ and KIF, slower migrating bands were observed due to inhibition of mannose trimming at 1 and 2 hr (Figure 3.3, lanes 9, 10 and 15, 16). When DNJ was combined with either of

the two drugs, A1AT NHK migrated slower (Figure 3.3, lanes 11, 12 and 17, 18). To isolate only monoglucosylated NHK, a pull down with GST-CRT followed by immunoprecipitation with A1AT antibody was performed. Upon addition of DNJ at each of the indicated time points, an increase in the amount of isolated monoglucosylated NHK can be clearly seen (Figure 3.3, compare lanes 20 to 19, 26 to 25 and 32 to 31). A threefold increase in monoglucosylated NHK was measured upon addition of DNJ with no chase (Figure 3.3, panel B), showing that DNJ trapped NHK in the monoglucosylated state by inhibiting glucosidase II trimming.

The increase persisted when DNJ was added in the later time points (45 min and 105 min), indicating that reglucosylation cycles persisted up to 2 hr (Figure 3.3, panel B). Interestingly, an increase in monoglucosylated substrates was also observed upon addition of DMJ or KIF alone at all the time points, giving a first line of evidence that inhibiting mannose trimming contributes to prolonging the life of NHK in the monoglucosylated form (Figure 3.3 compare lanes 27 and 28 to 25, and lanes 33 and 34 to 31, and Figure 3.3, panel B).

Upon combining DNJ with either DMJ or KIF, a striking increase in the amount of isolated monoglucosylated NHK was noted (Figure 3.3, compare lanes 29 and 30 to 26, and lanes 35 and 36 to 32, and Figure 3.3 panel B). The quantifications from three independent experiments revealed an average increase of reglucosylated NHK between eight to twelve fold approximately when DNJ was added at 45 or 105 min in the presence of KIF or DMJ (Figure 3.4), providing a clear indication that, upon inhibiting mannose trimming, the misfolded substrate NHK lingered longer in the monoglucosylated state, pointing to a more persistent reglucosylation of NHK by UGT1.

To gain further insight into how reglucosylation by UGT1 in its native environment is terminated, we asked if mannose trimming affects reglucosylation of an endogenous substrate, allowing us to circumvent any issues that could be associated with protein overexpression. To that end, we investigated reglucosylation levels of the endogenous substrate prosaposin in presence of DMJ and KIF in MI8-5 CHO cells. The same approach was used as described above, where DNJ was added 15 min before collecting the cells so reglucosylation cycles can be detected at the different time points. Additionally, DMJ and KIF were added throughout the pulse and the chase. Because prosaposin matures in the Golgi by receiving complex sugars and gets targeted to the lysosome, we sought to distinguish between its mature and its ER resident form by using an EndoH digestion assay (Refer to Chapter 2 for more details on the assay). The EndoH sensitive band indicates ER resident prosaposin while the EndoH resistant band indicates mature prosaposin that has gone through the Golgi.

Cells were radiolabeled for 30 min and chased up to 90 min, followed by lysis and immunoprecipitation with prosaposin antibody. At the earliest time point, cells were collected at the end of the pulse. Prosaposin was fully EndoH sensitive, indicating it is ER resident (Figure 3.5, lanes 1 to 12). In the later time points, an EndoH resistant band appeared indicating a fraction of prosaposin had trafficked to the Golgi, although most prosaposin was still EndoH sensitive. GST-CRT pull down was performed and followed by immunoprecipitation to isolate reglucosylated prosaposin. The highest level of reglucosylated prosaposin was observed at the first time point right after the pulse, and this level increased upon DNJ addition (Figure 3.5, compare lane 50 to 49). Reglucosylation of prosaposin was transient as small amounts of reglucosylated

prosaposin were isolated at 1 hr chase despite the persistence of a large fraction of prosaposin in the ER (Figure 3.5, lanes 61 and 62). No differences were observed at the first time point in reglucosylation levels upon addition of DMJ or KIF together with DNJ (Figure 3.5, compare lanes 53 and 54 to 50, and Figure 3.5 panel B). However, upon addition of DNJ at 15 min of chase, in the presence of DMJ and KIF, almost a doubling in reglucosylation levels of prosaposin was noted (Figure 3.5, compare lanes 59 and 60 to 56 and Figure 3.5, Panel B). This increase was even higher at the later times point, as total reglucosylated levels of prosaposin dropped in presence of DNJ alone, while it persisted upon mannosidase inhibition (Figure 3.5, compare lanes 65 and 66 to 62, and lanes 71 and 72 to 68, Figure 3.5, Panel B). In conclusion, mannose trimming appears to be a general mechanism to reduce or stop reglucosylation cycles of glycoproteins. Moreover, our approach of using an endogenous substrate, without the need for cell transfection, represented a less invasive method to uncover a fundamental process at a normal physiological substrate concentration.

## **Discussion**

The process by which reglucosylation cycles are ended has been obscure for a long time. We found that mannose trimming contributes to ending reglucosylation cycles of both folding and misfolded substrates, but more so for misfolded substrates. It was not surprising to see this difference between the two types of substrates as reglucosylation of on-pathway substrates is generally more transient than that of misfolded substrates (See Chapter 2). In addition, substrates that eventually fold undergo less extensive mannose trimming than misfolded or ERAD substrates. Consequently, mannose trimming may not

be the only contributor to ending their reglucosylation cycles. An alternative mechanism is that, once these substrates reach their native or near-native state, they lose the structural defect that UGT1 recognizes. This cannot be the case for substrates like NHK that are terminally misfolded, and thus would require an additional mechanism such as mannose trimming to be able to put an end to futile cycles. Thus, our data strongly points to mannose trimming reducing the affinity of UGT1 to the substrate's glycan.

In addition to mannose trimming reducing glucose transfer by UGT1, it could be hypothesized that mannose trimming increases the activity of glucosidase II, which would also result in faster exit from the reglucosylation cycles. As a result, upon inhibiting mannose trimming, the cleavage rate by glucosidase II of the glucose residue added by UGT1 would decrease, causing an accumulation of monoglucosylated substrates as we observed with DMJ and KIF. However, strong evidence in the literature suggests that this is highly unlikely, as reduced mannose content has been shown to reduce glucosidase II activity rather than increasing it, resulting in a lengthening of the half-life of the substrates in the monoglucosylated form. The data comes from both cell free as well as cellular assays (Stigliano et al., 2011; Grinna and Robbins, 1980; Totani et al., 2006; Stigliano et al., 2009; Olson et al., 2013).

Glucosidase II (Gls) is composed of catalytic (GlsII- $\alpha$ ) and regulatory (GlsII- $\beta$ ,) subunits (Stigliano et al., 2009; Trombetta et al., 1996). GlSII- $\beta$  contains a mannose-6-phosphate (MRH) recognition domain that binds mannose residues, which enhances the catalytic activity of GlSII- $\alpha$ . An intact terminal mannose residue on the C branch of the substrate's glycans has been shown to be essential for the glucosidase II activity (Totani et al., 2006; Olson et al., 2013). Thus, one of the models presented for the mechanism of

glucosidase II is that the MRH domain of Glc-II  $\beta$  binds to the terminal mannose residue of the B and/or C branch of the substrate glycan. This would position GlcII- $\alpha$  to bind to the A branch and cleave the glucose residue (Olson et al., 2013). In short, it appears that the driving force to ending reglucosylation cycles is reducing the activity of UGT1, which is accomplished via mannose trimming, rather than increasing the activity of glucosidase II.

It is intriguing that mannose trimming could have opposite consequences where, on one hand, it would lead to longer residence in the reglucosylation cycle due to the decreased activity of glucosidase II, but on the other hand, it can trigger the exit from reglucosylation cycles due to the decreased reglucosylation by UGT1. A possible explanation is the sequence of trimming of mannose residues in the different branches of the glycan. We are proposing a model where UGT1 reglucosylates complete glycans on glycoproteins, redirecting them to bind to the lectin chaperones. Mannose trimming of the B and C could occur first, reducing glucosidase II activity but not affecting that of UGT1, which would result in prolonged binding to the lectin chaperones and give more chances for the protein to attempt to fold. Yet, after few attempts to fold, a protein may still be released from the lectin chaperones by the residual activity of glucosidase II. Subsequently, trimming of the mannose residue, which accepts the glucose of the A branch, would inhibit UGT1 reglucosylation putting an end to the cycles. This is supported by the major impact we observed of mannose trimming on the reglucosylation of the substrates. Such strong correlation is expected to be due to the loss of the glucose acceptor mannose residue of the A branch, rather than the B and C branches.

Hence, it is essential to unravel the identity of the mannosidases responsible for ending the reglucosylation cycles. Mannose trimming has been long known to be the signal for targeting proteins to degradation by ERAD (Tamura et al., 2010; Liu et al., 1999; Hosokawa et al., 2003; Hosokawa et al., 2001). Several mannosidases have been identified in the ER that play essential roles in ERAD, though none of these mannosidases have been studied for their role in terminating glucose transfer by UGT1. As a consequence, any of these mannosidases is a potential candidate for playing this role. These mannosidases are the ER  $\alpha$ -1,2 mannosidase-I (ER manI) and its homologs that are members of the ER degradation enhancing  $\alpha$ -mannosidase like family (EDEM1, 2 and 3 (Olivari et al., 2006; Hosokawa et al., 2003; Hosokawa et al., 2001; Mast et al., 2005; Hirao et al., 2006). All these mannosidases and mannosidase-like proteins have been demonstrated to accelerate degradation of misfolded substrates upon their overexpression in mammalian cells and this overexpression is associated with increased mannose trimming. Potentially, these mannosidases could all cooperate or act as a backup for each other in terminating reglucosylation cycles. Alternatively, it is possible that the different mannosidases have unique substrate preferences.

Nevertheless, evidence points that EDEM1 is the most likely candidate for rendering glycans incompatible with UGT1 recognition site (s). In past work, a shorter persistence of monoglucosylated substrates was noted upon EDEM1 overexpression supporting that EDEM1 could prevent persistent reglucosylation by UGT1 (Molinari et al., 2003). The latter observation may not necessarily be due to the mannosidase activity of EDEM1, but simply due to EDEM1 capturing the substrate, which prevents its reglucosylation. However, our data that mannose trimming inhibits reglucosylation



cycles strongly hints that EDEM1 acts as a mannosidase to terminate reglucosylation. This report is the first concrete evidence for the mechanism that terminates reglucosylation cycles, and is not only relevant to advancing our basic understanding of the ER quality control but also could hold the basis for designing potential therapies. UGT1 appears to be a stringent quality control factor that retains substrates in the ER. Yet, relieving this stringency could result in beneficial outcomes for substrates that are retained in the ER due to minor structural defects but are otherwise functional. Since mannose trimming reduces the reglucosylation activity of UGT1, finding drugs that could specifically activate mannosidases responsible for trimming of the branch A of glycans appears to be promising.

### **Materials and Methods**

Refer to the Materials and Methods section in Chapter 2.

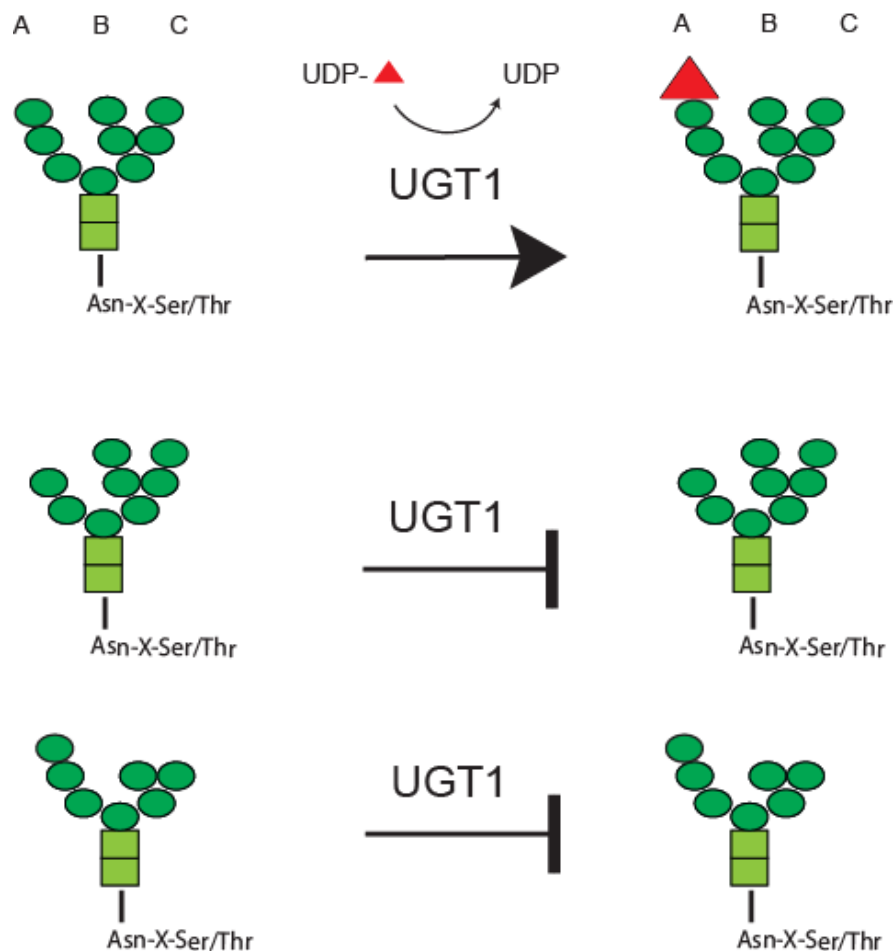


Figure 3.1 Possible mechanisms of mannose trimming in terminating reglucosylation by UGT1.

Mannose trimming can result in ending reglucosylation by UGT1 in two ways: 1) Trimming of the glucose acceptor mannose residue on the A branch stops reglucosylation (Upper scheme). 2) Trimming of mannose residues of the B and/or C branches reduces the affinity of UGT1 to the glycan, which is more likely to diminish the reglucosylation by UGT1 than stopping it (Lower scheme).

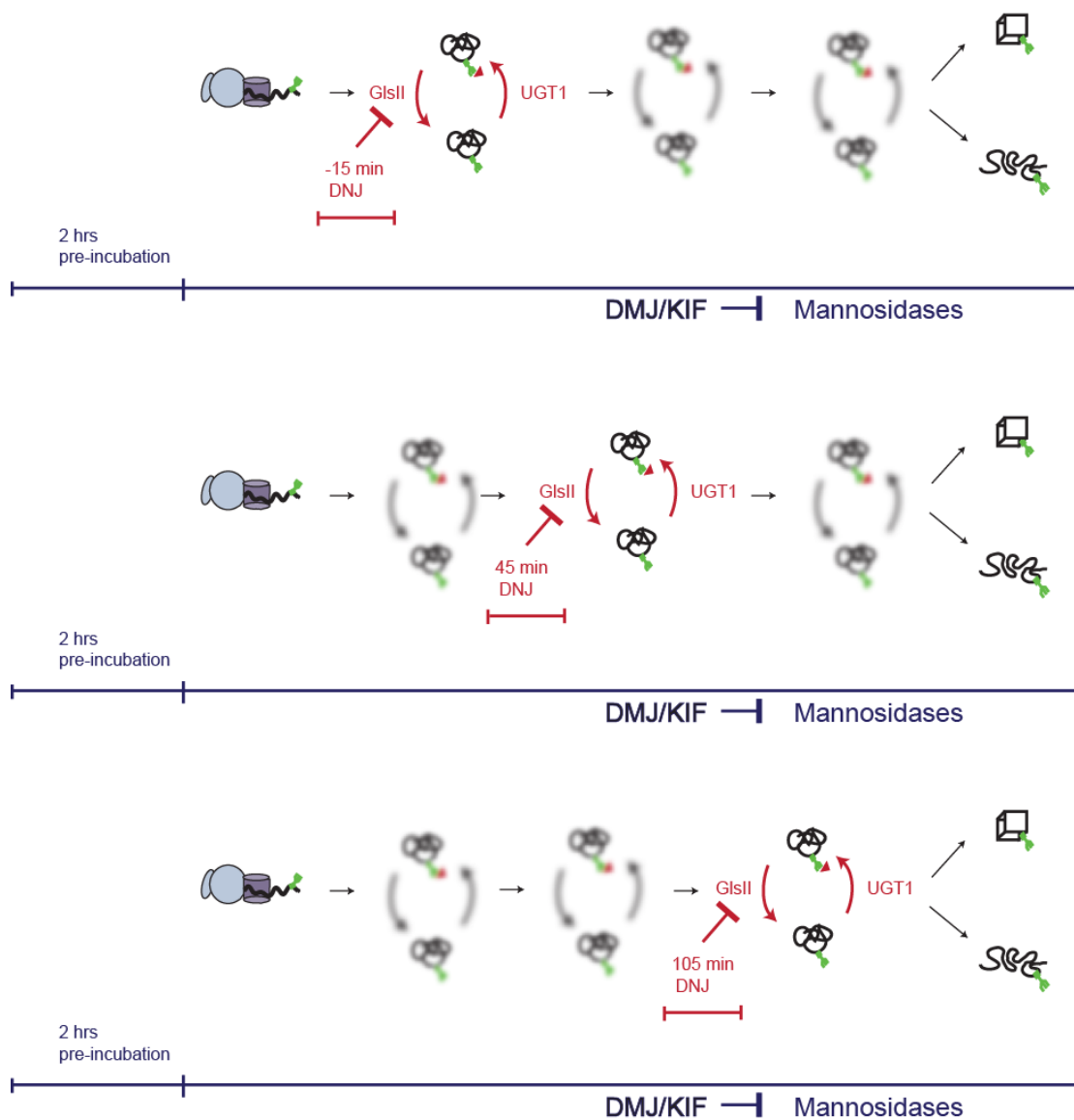


Figure 3.2 Experimental design to investigate the role of mannose trimming in terminating reglucosylation cycles.

To evaluate reglucosylation by UGT1 at separate time points, DNJ is added to the cells at only 15 min before lysis, to inhibit glucosidase II activity, and trap substrates in the monoglucosylated state, which are later pulled-down by GST-CRT. When DNJ was added 15 min before the pulse, it is indicated by -15 min. The rest of the time points indicate the time DNJ was added after the start of the chase. DMJ and KIF were present in the cells 2 hrs before the pulse, and during the pulse and the chase.

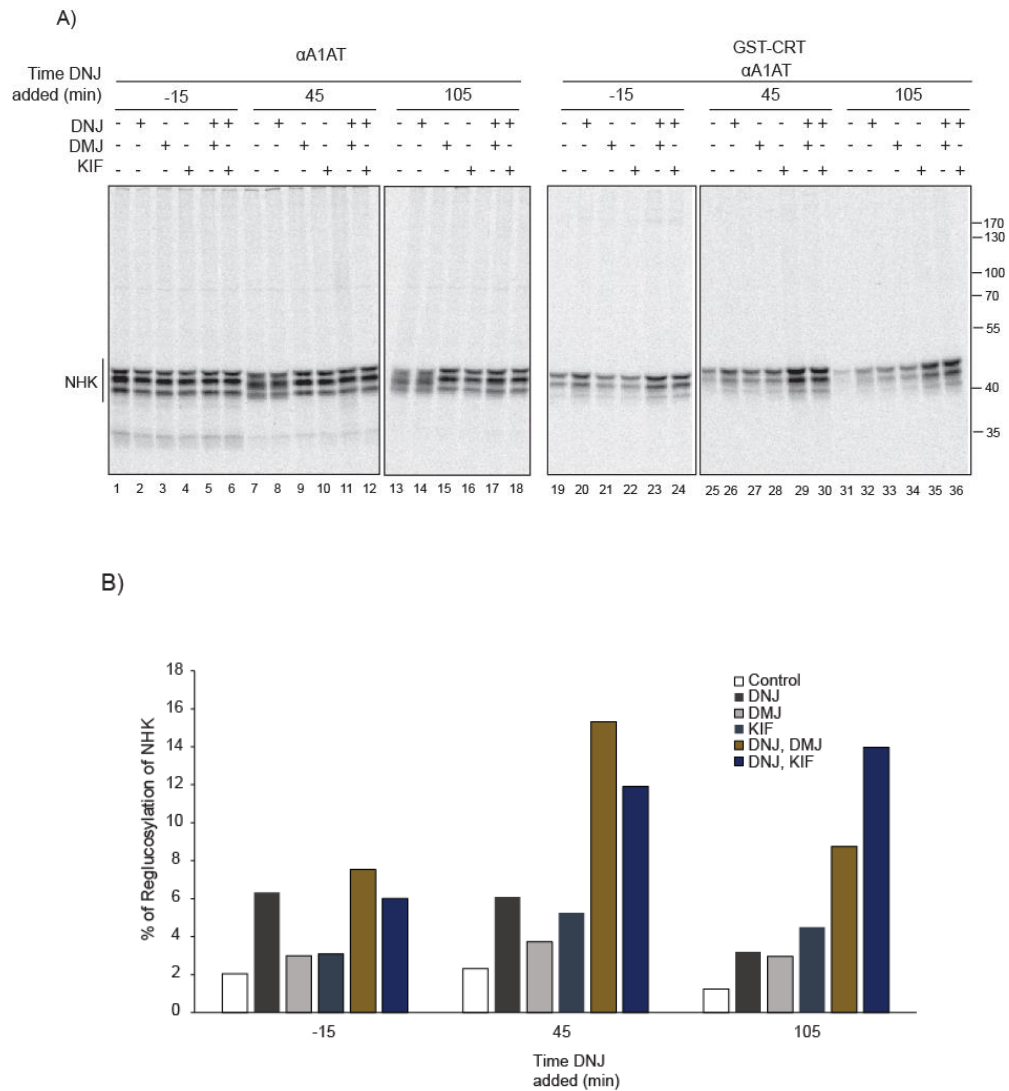


Figure 3.3 Inhibiting mannose trimming leads to the accumulation of NHK in the monoglucosylated state.

A) MI8-5 CHO cells were transfected with NHK and radiolabeled for 30 min. Cells were pre-treated for 2 hrs before the pulse with 1 mM DMJ and 0.1 mM KIF, as well as during the pulse and the chase, and 0.5 mM DNJ was added 15 min before the end of the pulse where indicated. DNJ was also added at the indicated time of the chase, followed by 15 min incubation. NHK was isolated with A1AT antisera from 10% of the total lysate. Monoglucosylated NHK was isolated by GST-CRT pull-down from 80% of the total lysate followed by immunoprecipitation with A1AT antisera. All samples were resolved on a 9% SDS-PAGE reducing gel. B) The quantification of the percentage of reglucosylation of NHK was performed by dividing the lanes 19 to 36 from the gel in Panel A, by the corresponding lanes 1 to 18, which were multiplied by 8 to account for the difference of sample used. The bar graphs are representative of one experiment.

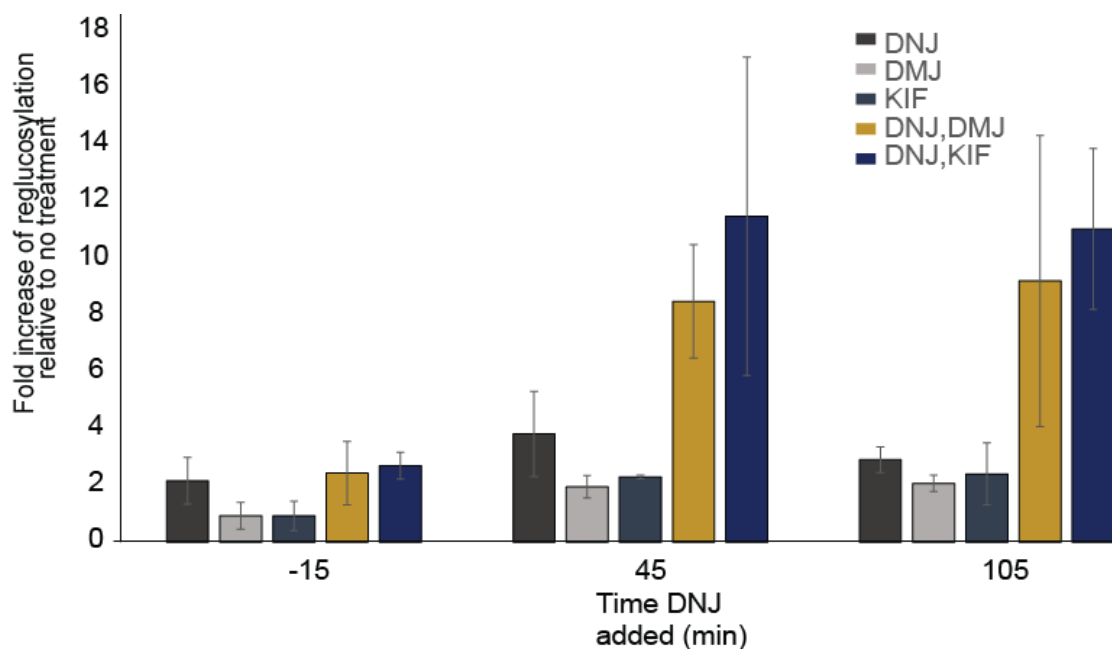
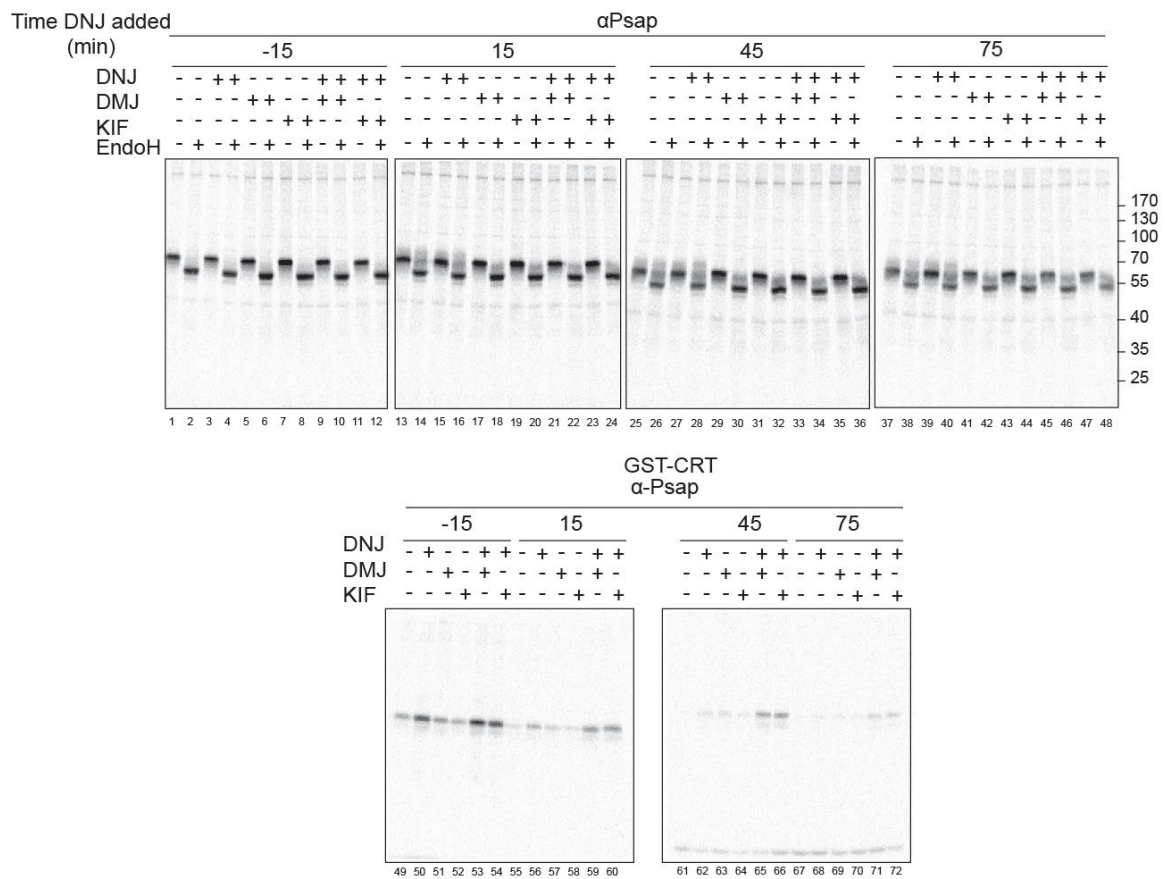


Figure 3.4 Quantification of the increase in the reglucosylation levels upon inhibiting glucosidase II trimming, mannose trimming, or both.

The increase in reglucosylation levels was obtained by dividing the percentage of monoglucosylated NHK isolated from cells treated with the indicated drug(s), by the percentage of monoglucosylated NHK isolated from non-treated cells. The error bars are the standard deviation from three independent experiments, including the results from figure 3.3.

A)



B)

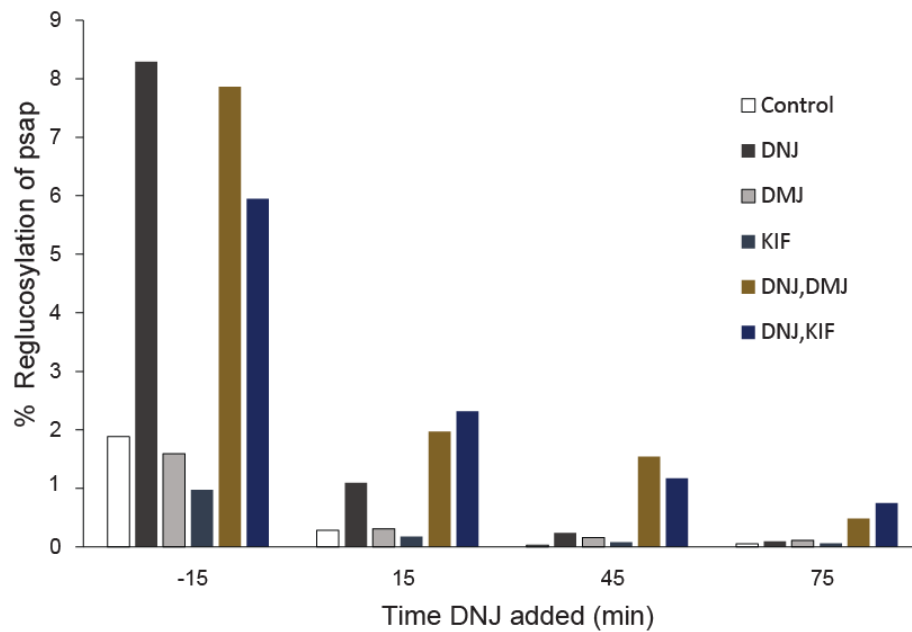


Figure 3.5 Inhibiting mannose trimming leads to the accumulation of prosaposin in the monoglucosylated state

A) MI8-5 CHO cells were radiolabeled for 30 min. Cells were pre-treated for 2 hrs before the pulse with 1 mM DMJ and 0.1 mM KIF, as well as during the pulse and the chase, and 0.5 mM DNJ was added 15 min before the end of the pulse where indicated. DNJ was also added at the indicated time of the chase, followed by 15 min incubation. Prosaposin was isolated with prosaposin antisera from 10% of the total lysate and an equal fraction was treated with EndoH where indicated. Monoglucosylated prosaposin was isolated by GST-CRT pull-down from 70% of the total lysate followed by immunoprecipitation with prosaposin antisera. All samples were resolved on a 9% SDS-PAGE reducing gel. B) The quantification of the percentage of reglucosylation of prosaposin was performed by dividing the lanes 49 to 72 by the corresponding EndoH sensitive band in the lanes 1 to 48, which was multiplied by 7 to account for differences of sample used. Reglucosylation was calculated as a percentage of the EndoH-sensitive prosaposin since this represents ER localized protein compared to EndoH-resistant protein localized to the Golgi and lysosome. The bar graphs are representative of one experiment. Yet, similar trends were observed when the experiment was repeated.

## CHAPTER 4

### THE ROLE OF FREE CYSTEINES IN SUBSTRATE RECOGNITION BY UGT1

#### Introduction

The environment of the ER creates favorable conditions for the oxidation of the cysteines (Cys) of maturing proteins (Oka and Bulleid, 2013; Hagiwara and Nagata, 2012). As a result, these Cys form either intra or inter molecular disulfides. The presence of unpaired or mispaired cysteines on maturing substrates in the ER could be an indication of an aberration in their folding (Nishikawa et al., 2005; Hebert et al., 1996). Thus, unpaired or mispaired cysteines of immature and misfolded substrates can be recognized by quality control machinery proteins, such as oxidoreductases, to be retained in the ER (Isidoro et al., 1996; Anelli et al., 2003). While UGT1 binds to non-native glycoproteins with exposed hydrophobic residues (Sousa and Parodi, 1995; Caramelo et al., 2003; Sousa et al., 1992), the possibility that it can select for substrates with unpaired or mispaired cysteines has not been addressed previously in the literature. Moreover, the previous test tube assays that looked at recognition specificities of UGT1 did not recapitulate the redox conditions of the ER, which could affect the activity of UGT1. Some lines of evidence indicate that UGT1 may directly or indirectly select for substrates depending on their cysteine redox status. UGT1 is associated with an oxidoreductase, Sep15, (Labunskyy et al., 2007; Labunskyy et al., 2009) which could bind these substrates via disulfide bridging, and thus assist in their recruitment to UGT1. Moreover, the cysteine rich prosaposin, which contains 16 disulfides, has been shown to be the endogenous substrate that is most predominantly recognized by UGT1 (Pearse and



Hebert, 2010), suggesting UGT1 might favor cysteine rich proteins. Finally, the recent identification in the sensor region of UGT1 of the thioredoxin-like domains (Zhu et al., 2014), which are characteristic of oxidoreductases, points to a redox mechanism of substrate recognition by UGT1.

UGT1 has ten cysteine residues, and it is not known whether or not they could form intramolecular disulfides. One potential mechanism of substrate recognition by UGT1 could involve free cysteines in UGT1 that perform a nucleophilic attack on aberrant disulfides on the substrates. Alternatively, free cysteines on immature or defective substrates could be involved in a nucleophilic attack on disulfides on UGT1. A third possibility could involve the abovementioned oxidoreductase Sep15 to bridge the substrates via disulfide bond formation.

We first set out to investigate the potential role of free cysteines in substrate recognition by UGT1. Antithrombin III (ATIII) was chosen as a model substrate, since it is a secreted soluble glycoprotein and was shown to be efficiently recognized by UGT1 in our preliminary assays (30% reglucosylation). ATIII pertains to the family of serine protease inhibitors (Serpins) and inhibits the activity of thrombin and other serine protease coagulation factors (Gettins, 2002). Thus, it plays a critical role in preventing blood clotting, and several of its inactive mutants have been associated with thrombosis (Perry and Carrell, 1996). ATIII has four glycosylation sites and six cysteines, forming three disulfides. Two nested disulfides are positioned at the N-terminal end of the protein and one disulfide at the C-terminal end (Perry and Carrell, 1996) (Figure 4.1).

Our lab has extensively studied the maturation of ATIII and its cysteine mutants in WT CHO cells. It was observed that ATIII with all its cysteines mutated to alanines (ATIII Cys-less), was secreted as efficiently as wild type. Despite being efficiently secreted, the ATIII Cys-less was incapable of binding and inhibiting thrombin (unpublished data), indicating that ATIII Cys-less failed to fold to the active form. These observations hinted that the absence of the cysteines from ATIII prevented its identification as misfolded by the ER quality control machinery, thus resulting in its release from the ER. This finding is surprising as it contradicts what is widely known in the literature about the stringency of the ER quality control in retaining defective substrates in the ER. Being a bona fide substrate for UGT1, we hypothesized that the lack of cysteines from ATIII Cys-less prevented its recognition by UGT1, leading to its erroneous escape out of the ER in-lieu of its retention.

Moreover, deletion of the most C-terminal disulfide of ATIII (ATIII C247-430A) prevented the formation of the two N-terminal disulfides, suggesting the four cysteines of ATIII at the N-terminus remained unpaired. Interestingly, ATIII lacking the C-terminal disulfide was almost fully retained in the ER (unpublished data). We hypothesized that this was due to the recognition of the unpaired cysteines by the folding sensor domain of UGT1, which would retain the substrate in the ER. Thus, using ATIII as a model substrate allows us to examine whether free cysteines are used by UGT1 as “handles” to retain immature proteins in the ER.

## **Results**

To investigate if UGT1 uses cysteines as “handles” to retain substrates in the ER, we compared reglucosylation levels of ATIII WT to Cys-less. ATIII WT secretes with a half-life of 54 min, and is detected in the media 30 min after synthesis (unpublished data). A fraction of ATIII can reach its native state in 30 min, although non-native intermediates were observed at that time point, indicating ATIII is not fully folded yet (unpublished data). We performed our cell based reglucosylation assay in MI8-5 CHO cells as described in previous chapters. Since we were initially interested in comparing the overall levels of reglucosylation rather than the timing of reglucosylation cycles, MI8-5 CHO cells were pre-incubated with 0.5 mM DNJ for 30 min before the pulse, and the same concentration of DNJ was maintained during the pulse and the chase. The cells were transfected with ATIII WT or Cys-less containing a myc-tag at the C-terminus; cells were radiolabeled for 30 min and chased for the indicated times. ATIII was isolated from the cell lysate or the media by immunoprecipitation with myc antiserum. Monoglucosylated ATIII resulting from reglucosylation by UGT1 was isolated by a GST-CRT pull down, which was followed by immunoprecipitation with myc antibody.

Both ATIII WT and Cys-less were secreted efficiently (Figure 4.2, lanes 9-12 and 33-36). Quantification of secretion levels illustrated that ATIII Cys-less secretes equally or better than ATIII WT, which indicates a lack of retention of ATIII Cys-less in the ER (Figure 4.2, Panel B, left). These results agree with previous observations in WT CHO cells (unpublished data). In the presence of DNJ, there was a noticeable reduction in secretion of both ATIII WT and Cys-less compared to non-treated cells (Figure 4.2, Compare lanes 21-24 to 9-12 and lanes 45-48 to 33-36). This is consistent with our

previous findings that trapping a substrate in the monoglucosylated state reduces its secretion (Chapter 2). Thus, we concluded that reglucosylation by UGT1 of ATIII WT, as well as ATIII Cys-less, delays their exit from the ER.

To evaluate the reglucosylation activity of UGT1, monoglucosylated substrates were isolated with a GST-CRT pull-down followed by immunoprecipitation. Interestingly, even in the absence of DNJ, monoglucosylated ATIII WT and Cys-less could be isolated after up to 4 hrs of chase (Figure 4.2, lanes 5-8 and 29-32). As expected, these levels increased in the presence of DNJ, since the substrates were maintained in the monoglucosylated form (Figure 4.2, Compare lanes 17-20 to 5-8 and lanes 41-44 to 29-32). When comparing the level of reglucosylation, lower amounts of monoglucosylated ATIII Cys-less were noted compared to ATIII WT, particularly at 10 and 60 min chase (Figure 4.2, Panel B, right). This could imply that the absence of cysteines in ATIII results in less efficient recognition by UGT1 at earlier time points. We observed a general trend of lower reglucosylation of ATIII Cys-less relative to ATIII WT when the experiment was repeated, but more data are needed to apply statistical analysis.

We then used the ER retained ATIII C247-430A to compare its reglucosylation and maturation to ATIII Cys-less in MI8-5 CHO cells. ATIII C247-430A lacks the C-terminal disulfide. Deletion of the ATIII C-terminal disulfide prevents the formation of the two N-terminal disulfides, presumably by leaving the protein with four unpaired cysteines at the N-terminus. Since ATIII C247-430A is almost fully retained in the ER, we predicted that UGT1 is the reason for its retention, and that the reglucosylation level of ATIII C247-430A would be higher than that of ATIII Cys-less.

The reglucosylation assay with the ATIII C247-430A construct was carried out as described above (Figure 4.3). Consistent with previously observed results in WT CHO, ATIII C247-430A was secreted at a lower level than ATIII Cys-less in untreated cells (Figure 4.3, Compare lanes 9-12 to 33-36, and Panel B, left). Monoglucosylated ATIII Cys-less and ATIII C247-430A were detected even in the absence of DNJ, and their amounts increased in the presence of DNJ as would be expected (Figure 4.3, Compare lanes 17-20 to 5-8 and 41-44 to 29-32). Higher levels of monoglucosylated ATIII C247-430A compared to ATIII Cys-less were observed at 10 min and 240 min, although the trend was not consistent for the other time points (Figure 4.3, Panel B, right). The elevated levels of reglucosylated ATIII C247-430A could indicate that UGT1 might favor the ATIII C247-430A mutant due to the presence of unpaired cysteines. However, since this trend was not the same at all the time points, it is difficult to make a definitive conclusion. Moreover, the higher reglucosylation of ATIII C247-430A is not sufficient to be solely responsible for the retention of this mutant in the ER.

The approach used so far compares the reglucosylation levels after one round of reglucosylation. This approach does not give information about the timing of reglucosylation, nor its persistence. Finding if reglucosylation cycles are persistent could provide an explanation of the retention of substrates in the ER. Thus, instead of adding DNJ throughout the pulse and the chase, DNJ was added in a shorter time window before lysing the cells. A window of 30 min rather than the previously used 15 min was chosen to ensure that sufficient ATIII was trapped in the reglucosylated form (See Chapter 3, Figure 3.2 for experimental design). The time point -20 min indicates DNJ was added 20 min before the start of the chase, and the cells were chased for 10 min in the presence of

DNJ (Figure 4.4). The rest of the time points refer to the time DNJ was added where indicated after the start of chase. The persistence of reglucosylation as well as secretion efficiency of ATIII C247-430A was compared to that of ATIII WT in MI8-5 CHO cells. In the absence of any drug treatment, ATIII WT was secreted significantly better than ATIII C247-430A (Figure 4.4, compare lanes 9-12 to 33-36, Panel B, left). The addition of DNJ showed that reglucosylation of ATIII C247-430A persisted, particularly when DNJ was added 90 min after the chase (Figure 4.4, Compare lane 44 to 20). Quantification of reglucosylated ATIII showed higher levels of reglucosylation of ATIII C247-430A by comparison to ATIII WT when DNJ was added after 30 and 90 min of the start of the chase. In summary, it appears that ATIII C247-430A is more persistently reglucosylated than ATIII WT, which suggests that UGT1 persistently reglucosylates ER retained substrates with unpaired cysteines.

### **Discussion**

The folding sensor domain of UGT1 has been well known to recognize exposed hydrophobic regions on immature substrates. Another hallmark of non-native glycoproteins is the presence of unpaired or mispaired cysteines. Here, we addressed the possibility that UGT1 can recognize proteins containing unpaired cysteines using ATIII as a model substrate.

We first analyzed whether the presence of cysteines in ATIII is important to its recognition by UGT1 by comparing reglucosylation of ATIII WT to Cys-less. We found that reglucosylation of the misfolded ATIII Cys-less was marginally lower than that of ATIII WT. This does not necessarily indicate that cysteines are not playing a role in

substrate recognition by UGT1, but the preference of UGT1 for misfolded substrates that we observed previously (Chapter 2) could be compensating for their absence, explaining why no major differences were observed.

Chaperones in the ER, such as BiP, can act as a backup for the UGT1/CNX cycle, to retain misfolded proteins in the ER (Pieren et al., 2005). However, it appears that the retention of misfolded ATIII Cys-less by BiP has failed, suggesting that ATIII is not a BiP substrate. Whether ATIII is a BiP substrate or not should be the subject of further investigation.

To directly address if unpaired cysteines contribute to substrate recognition by UGT1, we compared the levels of reglucosylation of ATIII Cys-less to C247-430A. ATIII C247-430A was proposed to carry four free cysteines at its N-terminus. This was supported by the observation that, on non-reducing gels, it runs to the same position as reduced ATIII WT (unpublished data). Further studies are needed to confirm that the four cysteines at the N-terminus of ATIII C247-430A are unpaired, which can be done by comparing gel mobility shifts of ATIII C247-430A that is modified or not with maleimide functionalized PEG (Polyethylene glycol) groups that react with free thiols. The presence of these four unpaired cysteines did not seem to cause much of an increase in ATIII C247-430A reglucosylation by comparison to ATIII Cys-less. Yet, it seems to support more persistent reglucosylation. That the level of persistent reglucosylation observed is sufficient to explain the ER retention of ATIII C247-430A is still an open question and these preliminary results require further confirmation.

Future work should focus on determining if having a single free cysteine in ATIII would result in an increase in reglucosylation relative to ATIII Cys-less. We examined this possibility in our preliminary studies, but our results were not conclusive due to the fact that ATIII mutants with single Cys at certain positions formed dimers, which would present double the number of glycans to be possibly modified by UGT1 compared to the monomer. Reglucosylation of the monomer must be assessed with non-reducing samples obtained from the cell-based reglucosylation assay, where only the bands corresponding to the monomer are quantified.

According to a recent study, the crystal structure of a portion of the folding sensor domain of UGT1 revealed a domain with a thioredoxin-like fold and two additional domains with a thioredoxin-like fold were predicted (Zhu et al., 2014) (Figure 1.5, Chapter 1). Thioredoxin-like domains are found in oxidoreductases and are characterized by a CXXC motif that catalyzes oxidation or isomerization of disulfides (Oka and Bulleid, 2013; Rutkevich and Williams, 2011). However, UGT1's two most N-terminal thioredoxin-like domains lack cysteines, and the third thioredoxin-like domain contains a single cysteine, suggesting that these domains are unlikely to have an oxidoreductase activity. However, a redox mechanism cannot be excluded yet. Future work should determine the functions of these domains.

The selenocysteine containing protein Sep15 is known to bind to UGT1 and has been proposed to act as an oxidoreductase in the ER (Korotkov et al., 2001; Labunskyy et al., 2005). That Sep15 can modulate UGT1 activity has not been tested. It is possible that Sep15 can first recognize non-native substrates with unpaired cysteines, and then present them to UGT1 for reglucosylation.



Taken together, the apparent contribution of free Cys to substrate retention in the ER implied from our preliminary data, and the association of UGT1 with Sep15 strongly point to a redox mechanism that could define the function of UGT1. Future work could uncover a previously unpredicted mode of action of UGT1 in ER quality control.

### **Materials and Methods**

Refer to the Materials and Methods section in Chapter 2

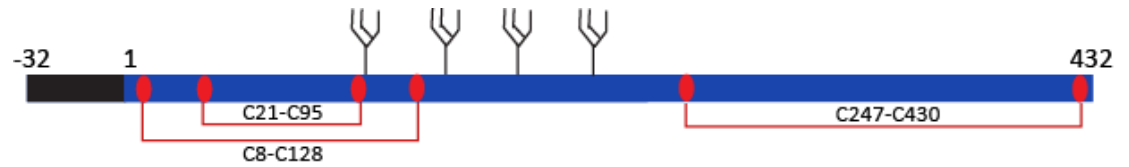
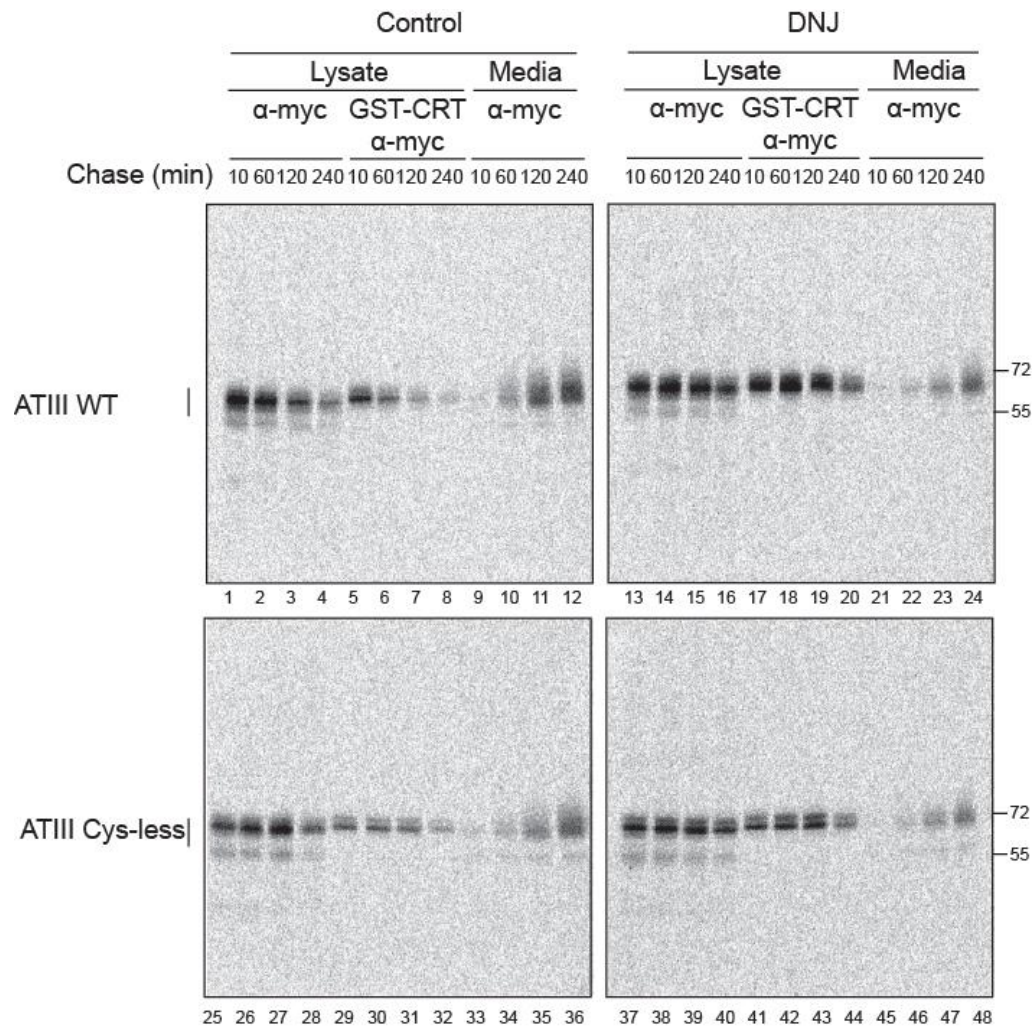


Figure 4.1 Schematic of antithrombin (ATIII)

ATIII is 464 amino acids in length. The signal sequence (in black) covers the first 32 amino acids and is cleaved upon translocation of the protein into the ER. It contains four glycosylation sites (branched structures) and six cysteines (red ovals). The six cysteines form three disulfides, two nested disulfides at the N-terminal end of the protein, and one disulfide at the C-terminal end of the protein.

A)



B)

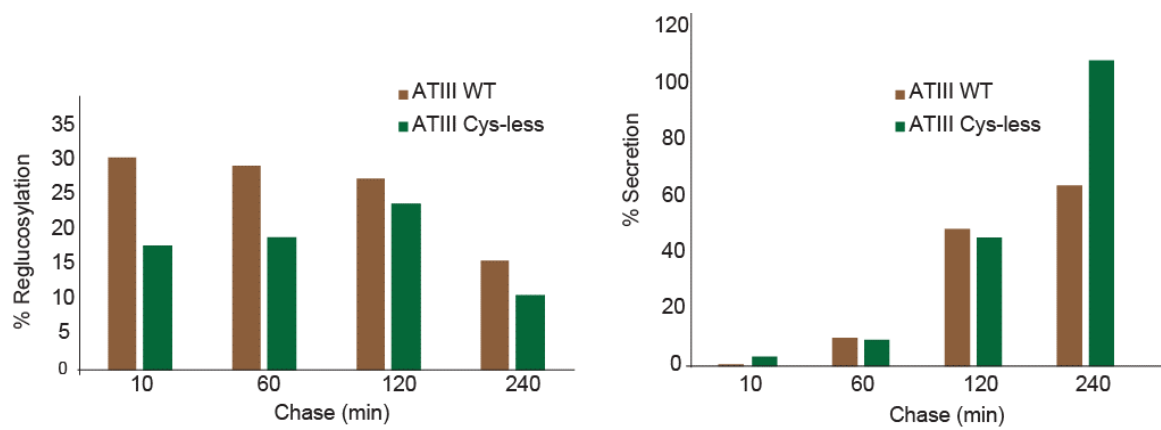
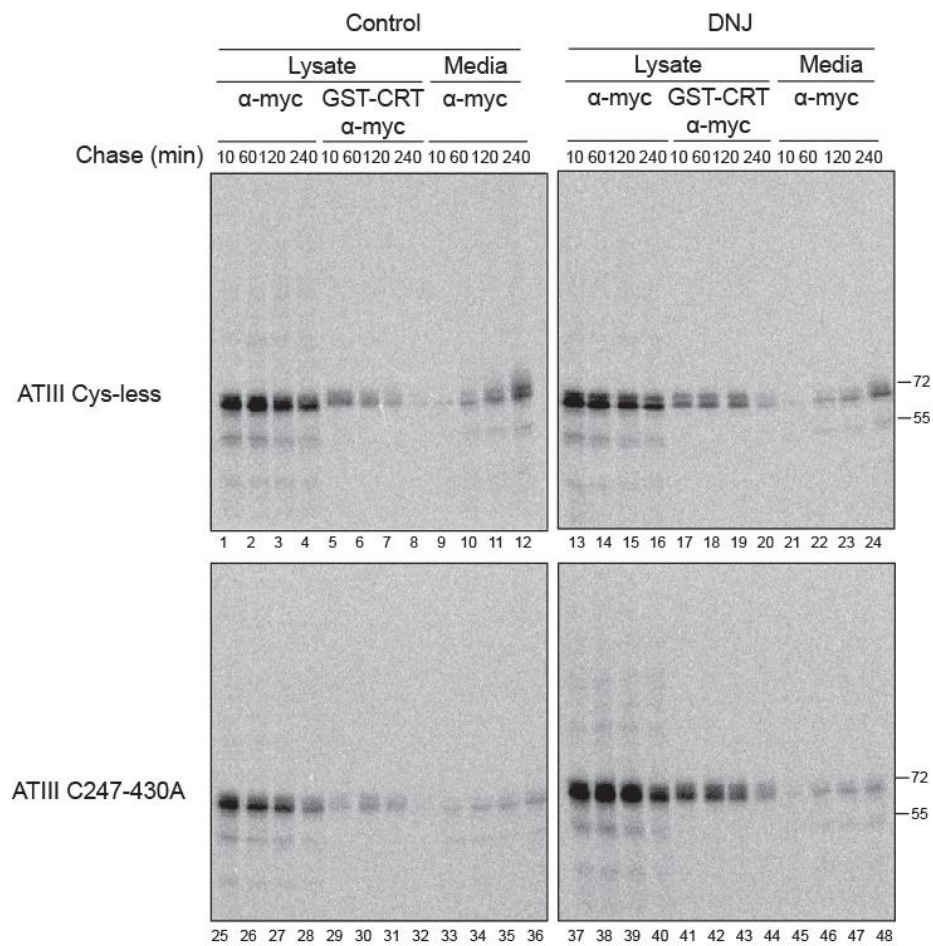


Figure 4.2 Comparison of reglucosylation and secretion of ATIII WT to Cys-less

A) MI8-5 CHO cells were transiently transfected with ATIII WT or Cys-less, radiolabeled for 30 min and chased for the indicated times. 0.5 mM DNJ was added throughout the pulse and chase where indicated. ATIII was isolated with myc antibody from 20 % of the cell lysate or the media. Monoglucosylated ATIII was isolated by GST-CRT pull down followed by immunoprecipitation with myc antibody from 70% of the cell lysate. All samples were resolved on an 8% reducing SDS-PAGE. B) Quantification of reglucosylation and secretion levels of ATIII WT and Cys-less. The percentage of reglucosylation of ATIII WT was obtained by dividing lanes 17-20 by the corresponding lanes 13-16, which were multiplied by 7.5 to account for differences in sample used. Similarly, the percentage of reglucosylation of ATIII Cys-less was obtained by dividing lanes 41-44 by the corresponding lanes 37-40, multiplied by 7.5. The secretion percentage of ATIII WT was obtained by dividing lanes 9-12 by lane 1, and the secretion percentage of ATIII Cys-less by dividing lanes 33-36 by lane 25. The bar graphs are representative of one experiment.

A)



B)

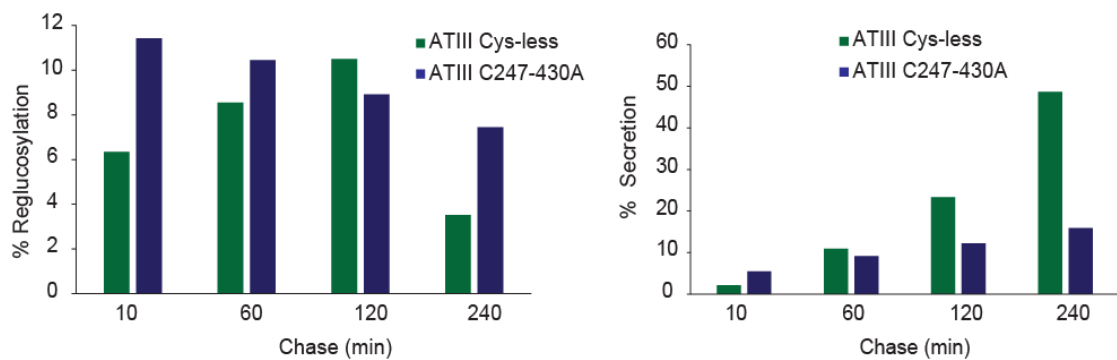
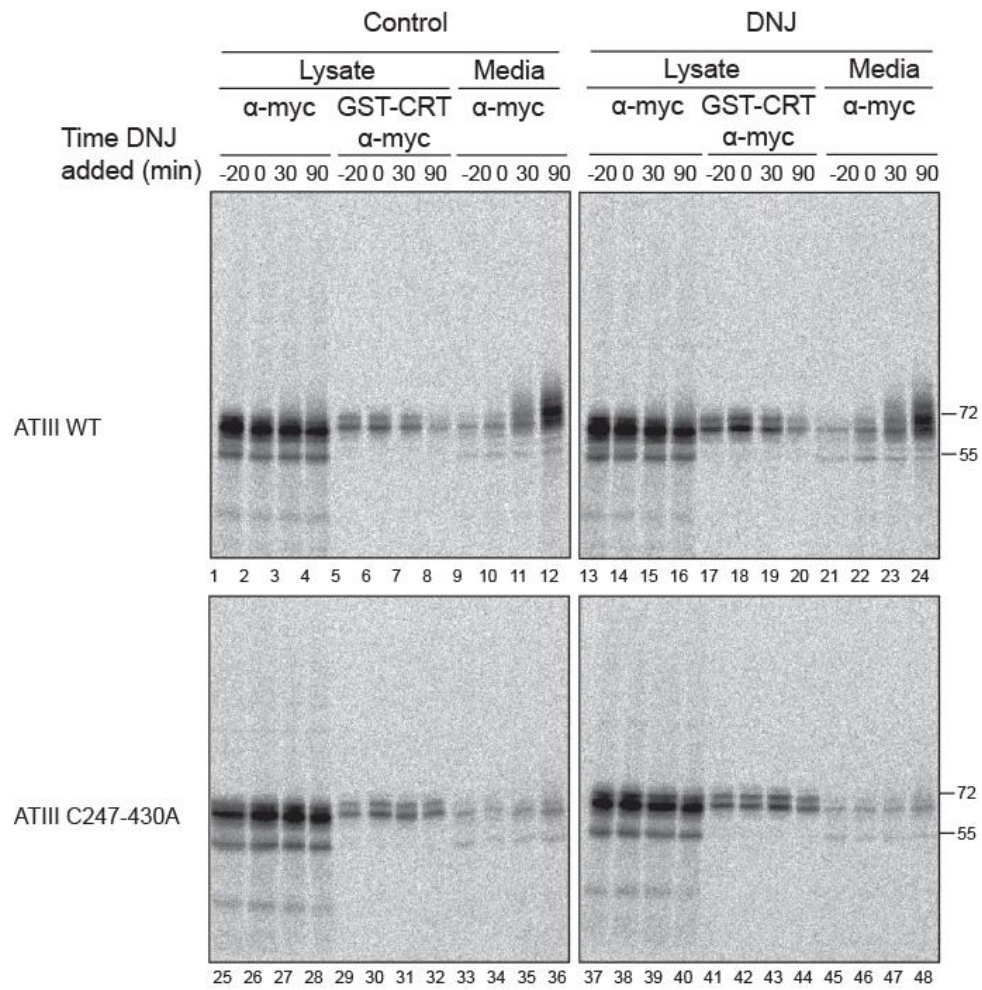


Figure 4.3 Comparison of reglucosylation and secretion of ATIII Cys-less to C247-430A.

A) MI8-5 CHO cells were transiently transfected with ATIII Cys-less and C247-430A, radiolabeled for 30 min and chased for the indicated times. 0.5 mM DNJ was added to the pulse and chase media where indicated. ATIII was isolated with myc antibody from 20 % of the cell lysate or the media. Monoglucosylated ATIII was isolated by GST-CRT pull down followed by immunoprecipitation with myc antibody from 70% of the cell lysate. All samples were resolved on an 8% SDS-PAGE reducing gel. B) Quantification of reglucosylation and secretion levels of ATIII Cys-less and C247-430A. The percentage of reglucosylation of ATIII Cys-less was obtained by dividing lanes 17-20 by the corresponding lanes 13-16, which were multiplied by 7.5 to account for differences in sample used. Similarly, the percentage of reglucosylation of ATIII C247-430A was obtained by dividing lanes 41-44 by the corresponding lanes 37-40, multiplied by 7.5. The secretion percentage of ATIII Cys-less was obtained by dividing the quants from lanes 9-12 by lane 1, and the secretion percentage of ATIII C247-430A by dividing lanes 33-36 by lane 25. The bar graphs are representative of one experiment.

A)



B)

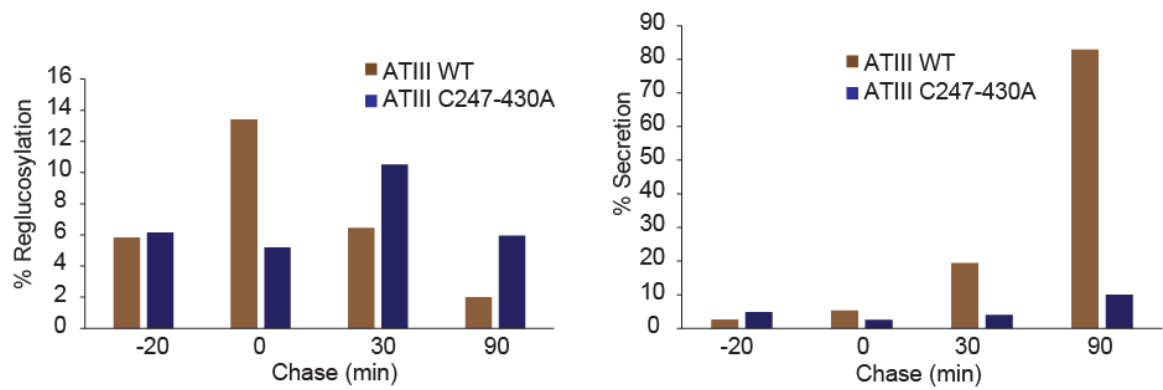


Figure 4.4 Comparison of the persistence of reglucosylation, and secretion levels of ATIII WT to C247-430A

A) MI8-5 CHO cells were transiently transfected with ATIII WT and C247-430A, radiolabeled for 30 min and chased for the indicated times. 0.5 mM DNJ was added to the cells at the indicated time relative to the start of the chase. ATIII was isolated with myc antibody from 20 % of the cell lysate or the media. Monoglucosylated ATIII was isolated by a GST-CRT pull down followed by immunoprecipitation with myc antibody from 70% of the cell lysate. All samples were resolved on an 8% reducing SDS-PAGE. B) Quantification of reglucosylation and secretion levels of ATIII WT and C247-430A. The percentage of reglucosylation of ATIII WT was obtained by dividing lanes 17-20 by the corresponding lanes 13-16, which were multiplied by 7.5 to account for differences in sample used. Similarly, the percentage of reglucosylation of ATIII C247-430A was obtained by dividing lanes 41-44 by the corresponding lanes 37-40, multiplied by 7.5. The secretion percentage of ATIII WT was obtained by dividing lanes 9-12 by lane 1, and the secretion percentage of ATIII C247-430A by dividing the quants from lanes 33-36 by lane 25. The bar graphs are representative of one experiment.



## CHAPTER 5

### CONCLUSIONS AND FUTURE DIRECTIONS

A great load of secretory and membrane proteins is constantly trafficking through the ER. To cope with this heavy flow, the eukaryotic ER has evolved an intricate network of factors that reduce folding errors or dispose of the failures. In this highly crowded environment, UGT1 stands at the intersection between the folding and the degradation routes. Thus, determining if UGT1 can direct proteins to these different routes is of paramount importance in expanding our understanding of the ER quality control.

The function of UGT1 in the ER has been extrapolated from past work that studied the properties of UGT1 in cell free assays. While these approaches were necessary and indispensable for propelling our understanding of the role of UGT1, there has been a lack of cellular assays that addressed its function, particularly in mammalian cells. Thus, we adopted an assay that allowed us to directly assess the reglucosylation activity of UGT1, and its impact on substrate maturation in intact cells. In this thesis, we uncovered fundamental details of the mechanism of UGT1 in the surveillance of glycoproteins. Moreover, our findings opened the door into new avenues of investigation, which could grant a better understanding of protein maturation and trafficking.

#### **The properties of substrates recognized by UGT1**

We found that UGT1 recognized both substrates that fold successfully in the ER, such as prosaposin or wild type  $\alpha 1$ -antitrypsin and substrates deemed misfolded, such as the ERAD clients NHK and A1ATZ. This suggests a less discriminatory role of UGT1

than previously proposed in the literature and that UGT1 can recognize both proteins capable of attaining their native fold and those that are deemed misfolded, although UGT1 seemed to favor misfolded substrates. Furthermore, the orphan subunit of the T-cell receptor complex TCR $\alpha$ , which is an ERAD substrate, and disease mutants of tyrosinase and  $\alpha$ -N-acetylgalactosaminidase were also recognized by UGT1 (Chapter 2, Figure 2.1). While we have used several substrates with different biophysical properties and topologies, testing additional model substrates would broaden the scope of this conclusion, particularly by comparing the reglucosylation of bona fide ERAD substrates to their wild type counterparts.

To further define the extent to which the requirement for recognition by UGT1 correlates with the folding efficiency of the substrate, future work should focus on comparing reglucosylation of fast folding to slow folding substrates. Fast folding substrates are expected to be less efficiently recognized by UGT1 because they stay in the form of incompletely folded intermediates and reside in the ER for a brief time, while the opposite applies for slow folding substrates. RNase and CFTR consist of well-studied model substrates for fast and slow folding proteins in the ER, respectively (Ward and Kopito, 1994; Geiger et al., 2011). We have attempted to use RNase, but we have faced the problem of severe hypoglycosylation. Thus, further optimization or different substrates are needed to perform this study.

Although the literature provided us with a number of good model substrates that helped us to expand our understanding of the properties of UGT1, this does not necessarily make them ideal substrates. In addition to the folding status of the substrate, features of the substrate like size, topology (membrane or soluble), number of glycans,

number of cysteines or even the overall hydrophobicity could determine whether or not a substrate will be recognized or even be favored to be modified by UGT1. Although all the wild type glycoproteins that we have tested so far were reglucosylated by UGT1, there have been differences in the reglucosylation efficiency, with prosaposin and ATIII seemingly being the most efficiently recognized. The identification of endogenous substrates of UGT1, and classification of these substrates based on their biochemical properties, could uncover features favored by UGT1. A proteomics approach with our GST-CRT pull down assay might provide clues for trends in substrates selection of UGT1. Yet, instead of the MI8-5 CHO cell line, ideally, this experiment should be done in an ALG6 deficient human cell line that transfers glucose deficient N-linked glycans to substrates (Shrimal and Gilmore, 2015). Taken together, a proteomic analysis would help to further clarify the basis of substrate selection by UGT1, and could uncover an even larger role of UGT1 in protein homeostasis in the ER.

### **The implications of the localization of UGT1 for the persistence of reglucosylation cycles**

We found that UGT1 transiently reglucosylated wild type substrates, while it reglucosylated misfolded substrates more persistently (Chapter 2, Figure 2.5.). The latter finding is in agreement with the previously determined localization of UGT1 beyond the rough ER, which positions UGT1 to reglucosylate substrates at later time points. However, it is contradictory with the observation that reglucosylation can be seen at early time points, suggesting UGT1 is also active early in the maturation pathway. The data in the literature are not clear about the location of UGT1 within the ER, as it is suggested to be more concentrated in either the smooth ER, ER exit sites or the pre-Golgi intermediate

compartment (Zuber et al., 2001; Gilchrist et al., 2006). Separation of the rough and smooth ER fractions from MI8-5 CHO (Gilchrist et al., 2006), followed by isolating monoglucosylated substrates like NHK or WT A1AT by the GST-CRT pull-down from each fraction could help in determining whether reglucosylation can begin in the rough and persist in the smooth ER. Moreover, super resolution microscopy techniques can help to further clarify the detailed location of UGT1 in the ER, and determine if it co-localizes with the elements of the reglucosylation cycle.

### **Defining the players that terminate reglucosylation cycles**

We have determined that mannose trimming of glycoproteins contributes to their removal from reglucosylation cycles (Chapter 3, Figure 3.3 and 3.5). Several questions arise from this observation. What is the identity of the ER mannosidases responsible for mannose trimming? Are all these mannosidases required? Which branches of the glycan are trimmed to reduce or stop reglucosylation?

While the identity of the mannosidases responsible for terminating reglucosylation cycles is not known, several mannosidases have been identified in the ER such as ER mannosidase I and the mannosidase-like proteins EDEM1-3. Whether or not one of these mannosidases could be responsible for removing substrates from reglucosylation cycles can be determined either by knocking down or overexpressing each of them individually in MI8-5 CHO then performing the reglucosylation assay to look at persistence of substrates in the reglucosylation cycles. Since these mannosidases could act as a back-up for each other, it might be necessary to knock-down or overexpress a combination of multiple proteins.

Defining which branch of the glycan is trimmed to reduce or arrest reglucosylation might be more challenging. Mass spectrometry can be used to determine the composition of the glycan(s) of different substrates upon over-expression or knockdown of mannosidases, though this is far from trivial. Alternatively, our cell based reglucosylation assay can be performed, but with cell lines that transfer glycans with reduced mannose composition and lack glucose residues such as the MadIA214 CHO cells, which only transfers Man5 GlcNAc2, a glycan missing the branches B and C (Ermonval et al., 2000; Ermonval et al., 2001). If these glycans get reglucosylated efficiently in the cell based reglucosylation assay, this would indicate that the B and the C branches do not influence glycan recognition by UGT1. If a reduction in reglucosylation occurs upon overexpression of mannosidases in these cells, it would point to a trimming of the A branch. This approach can provide direct evidence, not only to which branch in the glycan is responsible for reducing or stopping substrate recognition by UGT1, but also to the identity of the mannosidase that performs the trimming.

### **Free thiols potentially represent a new mechanism of selection of immature substrates by UGT1**

Using ATIII as a model substrate, we have accumulated some preliminary evidence suggesting that free thiols on an incompletely folded or misfolded substrate contribute to its recognition by UGT1 and its subsequent ER retention by the lectin chaperones. Another possibility is that UGT1 could have unpaired cysteines that mediate the recognition of substrates with aberrant disulfides. Modification with PEG groups functionalized with thiol reactive moieties such as PEG-maleimide would confirm that UGT1 has free cysteines if a mobility shift is observed on an SDS-PAGE. In addition,

non-reducing gels would demonstrate whether or not UGT1 and the substrate form covalent intermolecular disulfides if a complex is detected on the gel.

Alternatively, the interacting partner of UGT1, Sep15, which is hypothesized to be an oxidoreductase, could recognize substrates with unpaired or mispaired cysteines, and subsequently present them for reglucosylation by UGT1 (Labunskyy et al., 2005; Labunskyy et al., 2007). By performing our cell-based reglucosylation assay in MI8-5 CHO with over-expression or knockdown of Sep15, we can define if Sep15 plays a role in recognizing substrates and passing them over to UGT1.

Finally, it would be of interest in future experiments to investigate the function of the thioredoxin-like domains of UGT1. These domains do not contain any cysteines except for one in the third domain thus, it is unlikely that they have a catalytic function in oxidation or isomerization of disulfide bonds (Zhu et al., 2014). Yet, they could still be responsible for substrate recognition by UGT1. They can be expressed individually in mammalian cells to investigate if they still maintain interaction with UGT1 substrates. Moreover, although the crystal structure of the third domain has been solved, that of the first two domains is just predicted (Zhu et al., 2014). In addition, the solved crystal structure of the third domain is from the *C. thermophilum*, which shares only 35% homology with UGT1 (Zhu et al., 2014). Thus, the observed thioredoxin-like fold in *C. thermophilum* UGT1 may not certainly be shared by its human homolog, making it essential to solve the crystal structure of human UGT1.

## **Summary**

To conclude, we have determined that reglucosylation of wild type substrates by UGT1 results in delay in their trafficking and in their prolonged binding to lectin chaperones, while reglucosylation of misfolded substrates does not affect their degradation by ERAD. In addition, misfolded substrates are more persistently reglucosylated than wild type substrates and seem to be favored by UGT1. Moreover, mannose trimming contributes to ending reglucosylation cycles. Future work should focus on determining the mannosidases responsible for this trimming, and the branches of the glycans that are trimmed. Lastly, preliminary data and evidence from the literature indicates that free Cys could be hallmarks that UGT1 uses to select immature glycoproteins for reglucosylation. Dissecting the details of the involvement of free Cys in recognition by UGT1 could uncover a new mechanism of UGT1 in protein quality control that potentially involves other players in the ER.

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