THE IDENTIFICATION AND CHARACTERIZATION OF A GROUP OF ER TPR-CONTAINING ADAPTER PROTEINS

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The identification and characterization of a group of ER TPR-containing adapter proteins

A Dissertation Presented

By

JOHAN C. SUNRYD

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

DOCTOR OF PHILOSOPHY

September 2014

Molecular and Cellular Biology
The identification and characterization of a group of ER TPR-containing adapter proteins

A Dissertation Presented
By
JOHAN C. SUNRYD

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DEDICATION

To my amazing wife Brianna and our wonderful daughter Isadora.
ACKNOWLEDGEMENTS

The first person I must thank is Dr. Daniel Hebert for all of his advice, support and patience over the years. To my great fortune, Dan accepted me as his student despite all of my eccentricities. My success as a graduate student is in large part due to Dan's ability to direct my creative energy into a productive, analytical and systematic force. Dan has trained me how to design experiments, analyze and evaluate results, create new hypothesis and to test them experimentally. Though I could cover page upon page with all that I have learned from Dan, the one quote that always rings in the back of my mind is "look at your data with your mind, not your heart". I would like to thank my thesis committee members: Drs. Lila Gierasch, Danny Schnell, and Scott Garman for their input and guidance in developing my work. In addition, I would like to thank Dr. Rafael Fissore for his help during the later part of my thesis.

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ABSTRACT
THE IDENTIFICATION AND CHARACTERIZATION OF A GROUP OF ER TPR-
CONTAINING ADAPTER PROTEINS

September 2014
JOHAN C. SUNRYD, B.S., MICHIGAN STATE UNIVERSITY
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Directed by: Professor Daniel Hebert

The endoplasmic reticulum (ER) is an organelle responsible for a variety of functions in all eukaryotic cells. Some of these functions are localized to specific regions in the ER, such as the ribosome-studded rough ER sheets or the ribosome-free smooth ER sheets. The smooth ER sheets have the ability to form ER tubules, which extend throughout the cell and make contact with other organelles. In order to accommodate these localized functional regions, a certain degree of heterogeneity and compartmentalization into sub-domains exists within the ER. Since the ER membrane and lumen are contiguous, the compartmentalization of the ER into sub-domain cannot exclusively be created by membrane barriers. Adapter proteins nucleate the formation of protein complexes to create sub-domains in the ER. Tetratricopeptide repeats (TPR) containing proteins are known to organize protein complexes involved in a wide range of cellular processes. While the human genome is predicted to encode ~ 180 TPR proteins, only two have previously been shown to localize to the ER. We hypothesized that there are additional TPR-containing adapter proteins that contribute to the organization or compartmentalization of ER processes. To this end, we screened an in silico library of putative TPR containing proteins from the Regan laboratory (Yale University) to identify proteins that potentially possess an N-terminal ER targeting signal sequence. This screen, combined with additional in silico approaches, identified nine putative ER proteins that contained between three to ten TPR motifs. While some of the identified proteins are soluble ER proteins (i.e. TTC13 and TTC17), others were found to
reside in the ER membrane with their TPR domains facing the ER lumen (TMTC1-4). TMTC1 and TMTC2 were found to interact with the ER calcium pump SERCA2B, and TMTC2 with calnexin. Additionally, live cell calcium measurements showed a role for TMTC1 and TMTC2 in calcium homeostasis. Overall, this strategy was successful in identifying novel ER proteins with TPR motifs, and this approach can be applied to identify proteins with specific motifs in the ER or other organelles.
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CHAPTER 1

THE COMPARTMENTALIZATION AND ORGANIZATION OF THE ENDOPLASMIC RETICULUM

Introduction

The endoplasmic reticulum (ER) is the gateway to the secretory pathway, and approximately one third of the human genome encodes proteins targeted to the ER. A number of cellular functions such as secretory protein folding, degradation of terminally misfolded secretory proteins, trafficking of secretory proteins, calcium homeostasis, lipid synthesis and xenobiotic metabolism are all dependent upon the ER and ER-resident proteins (Figure 1.1A) (Leitman et al., 2013; Lynes and Simmen, 2011). Early electron microscopy studies highlight morphological differences between regions of the ER categorizing these regions as rough and smooth ER sheets. The rough ER sheets are studded with ribosomes while the smooth ER sheets lack ribosomes (Figure 1.1B) (Palade and Siekevitz, 1956). These ER sheets are connected by helicoidal turns, and the low curvature of these turns allows docking of ribosomes (Terasaki et al., 2013). The smooth ER contains proteins that enhance membrane curvature that form tubules; which have the ability to fuse and separate (Voeltz et al., 2006). Additionally, the ER is highly dynamic and capable of changing its morphology from sheets to tubules, and vise versa, as the need arises (Friedman and Voeltz, 2011). The ER sheets and tubules extend throughout the cell.

The dispersal of the ER allows for discrete contact points to form between the ER and other organelles such as the mitochondria, plasma membrane, pre-peroxisomal compartments and the cytoskeleton. The ER membrane is also contiguous with the nuclear envelope. Interactions between the ER and other organelles and the cytoskeleton influence its shape, size and movement in the cell (Voeltz et al., 2006). In eukaryotes, the movement of the ER is guided along microtubules by motor proteins (Nikonov et al., 2007), and although the actin cytoskeleton is
important for yeast and plant ER, it is not for mammalian ER (Liebe and Menzel, 1995; Prinz et al., 2000). Collectively, these data demonstrate a high level of organization within the ER and that the shape and movement of the ER are highly dynamic.

Although the luminal space of the ER exists as an unbroken unit without interruptions, it nevertheless appears to possess some heterogeneity in protein composition among the different regions. The clearest example of this is in the rough ER, which is enriched with translating ribosomes. More recent work has demonstrated an enrichment of certain factors at contact sites between the ER and various organelles (Lynes and Simmen, 2011). Further experimental support for the compartmentalization of the ER was shown by accumulation of both soluble and membrane proteins required for disposal of misfolded secretory proteins in a specific region of the ER (Lederkremer, 2009). Proteomic studies further support the heterogeneity of the ER as most ER proteins have a preferential localization in rough versus smooth ER (Gilchrist et al., 2006). On the whole, a growing body of work suggests that the diverse functions of the ER are localized to specific sub-domains within this continuous lumen.

**Rough ER sheets**

The ribosome studded rough ER sheets, which are essential for co-translational insertion of secretory proteins into the ER, were the first example of ER sub-domains (Figure 1.2) (Schnell and Hebert, 2003). At the rough ER, proteins with a N-terminal signal sequence are inserted co-translationally as the ribosome docks onto the SEC61 translocation complex located in the ER membrane. This large multi-protein complex facilitates the insertion of the nascent protein and releases transmembrane domains into the ER membrane as they are synthesized. Alternatively, a secretory protein can be targeted post-translationally to the ER by means of an internal transmembrane domain (Johnson et al., 2013). Once the nascent protein reaches the ER lumen, a plethora of covalent modifiers, chaperones and foldases aid to enhance the folding efficiency of the client protein (Tamura et al., 2010).
Secretory proteins commonly acquire disulfides, which are formed in the oxidizing environment of the ER with the aid of ER localized oxidoreductase or protein disulfide isomerase enzymes. Additionally, most secretory proteins obtain covalent asparagine (N) linked glycan during their ER residency. Modification of these sugar structures by ER localized enzymes allow for recruitment of ER resident lectin chaperones to aid glycoprotein folding (Hebert et al., 2005). Protein maturation is further augmented by a number of chaperones that recognize misfolded proteins independently of N-linked glycans (Pearse and Hebert, 2010). The collective aim of these covalent modifications and chaperones in the ER is to guide the folding of the client proteins to a functional state. Once deemed properly folded by the ER quality control machinery, a protein can be targeted for trafficking to other parts of the secretory pathway.

**Smooth ER sheets and tubules**

Proteins selected for secretion move from the rough ER to the ER exist sites (ERES), which are located in the smooth ER (Figure 1.2). The ERES are characterized by the presence of SEC16A and SEC16B, which recruit components of COPII vesicles to the cytoplasmic side of the ER membrane to form vesicles (Watson et al., 2006). These vesicles buds off from the ER membrane and head to the ER-Golgi intermediate compartment and Golgi for further processing and sorting (Bhattacharyya and Glick, 2007). The process of selection for secretion through ERES is poorly understood. Some membrane proteins are selected for secretion by diacidic motifs in their cytoplasmic C-terminal tails, which recruits components of the COPII vesicles for proper secretion (Nishimura and Balch, 1997; Nishimura et al., 1999). Additionally, it is possible that changes in local lipid or cholesterol composition of the ER membrane could play a role. ER resident membrane proteins have slightly shorter transmembrane domains than other membrane resident proteins, which is attributed to the difference in lipid composition of the respective membranes. Additionally, fusing a 17 or a 22 amino acids transmembrane domain to ER targeted fluorescent proteins resulted in either ER retention or ERES dependent secretion, respectively.
(Ronchi et al., 2008). Thus, lipid composition, length of transmembrane domains as well as ER resident and cytoplasmic proteins aid in proper trafficking of proteins through the ERES. Thus, the ERES are critical in shuttling proteins deemed as properly folded out of the ER toward their intended destination.

As protein folding and maturation is an error-prone process, a fraction of secretory proteins inevitably misfold and must be rapidly degraded to prevent toxic build up (Smith et al., 2011). These misfolded secretory proteins are recognized in the ER, by a process named ER-associated degradation (ERAD). The ERAD substrate is recognized by ERAD receptors and targeted to a retrotranslocation complex in the ER membrane, retrotranslocated, ubiquitinated in the cytoplasm, extracted from the ER membrane and ultimately degraded by the 26S proteasome. A number of ERAD factors are localized to a sub-domain of the ER called the ER quality control compartment (ERQC) (Figure 1.2) (Avezov et al., 2008; Kondratyev et al., 2007). It has been proposed that adapter proteins, which nucleate multiprotein complexes required for the disposal of aberrant secretory proteins, maintain the ERQC. SEL1L is an ER type I membrane adapter protein that interacts with multiple ER luminal and membrane proteins required for ERAD, and could aid in the maintenance of the ERQC (Christianson et al., 2008; Mueller et al., 2006). The protein complex nucleated by SEL1L is believed to contain, among other factors, an unidentified pore that forms a channel through the ER membrane and the E3 ligase, HRD1, that ubiquitinates misfolded proteins as they emerge into the cytoplasm (Hosokawa et al., 2008; Mueller et al., 2008). Once ubiquitinated, the cytoplasmic ATPases associated with diverse cellular activities (AAA ATPase) p97, provides the mechanical force to extract misfolded proteins from the ER membrane and prepare them for proteasomal degradation in the cytoplasm (Stolz et al., 2011; Ye et al., 2001).

While the SEL1L-HRD1 complex is the best-studied E3 ligase complex in the ER membrane, it is important to note that the ER contains approximately 40 E3 ligases, some of
which are completely uncharacterized (Claessen et al., 2012; Neutzner et al., 2011). Thus, it is possible that there are unknown paralogues of SEL1L that aid in the organization of proteins involved ERAD and ERQC.

Despite the efforts by quality control machinery and the rapid disposal of the failures by ERAD, misfolded proteins can nevertheless accumulate in the ER and activate the unfolded protein response (UPR) (Figure 1.2) (Hetz, 2012; Yoshida et al., 2001). The UPR is a diverse ER rescue mechanism that has three distinct branches in mammals, mediated by ATF6, IRE1α and PERK. These proteins initiate various signaling pathways that aim to repress general protein translation, increase translation of chaperones, enhance degradation of misfolded proteins, activate autophagy and expand the size of the ER (Hetz, 2012; Ng et al., 2000). While all three branches of the UPR are activated by severe ER stress, differences in responses to the three branches to some forms of ER stress have been observed (DuRose et al., 2006). Although originally considered to be activated solely by the accumulation of unfolded proteins, the UPR has also been implicated in glucose metabolism, innate immunity and cell differentiation (Hotamisligil, 2010; Iwakoshi et al., 2003; Martinon and Glimcher, 2011). Notably in B-cells, the UPR is activated prior to immunoglobulin synthesis, indicating that UPR can be activated before ER stress occurs (Cenci and Sitia, 2007; Hu et al., 2009). Thus, the overarching goal of the UPR is to overcome ER stress and restore ER homeostasis. If homeostasis cannot be re-established and the stress signal is prolonged, the UPR signal will eventually become proapoptotic and the cell will be cleared by apoptosis (Tabas and Ron, 2011). Taken together, the UPR is a broad umbrella signal transduction pathway that uses a multi tool approach to maintain ER homeostasis and to ensure ER fitness.

The ER interfaces with multiple organelles

While the ER consists of an uninterrupted membrane and lumen, the ER appears to possess a higher level of compartmentalization, which becomes evident when looking at the
discrete contact points between the ER and other organelles (Figure 1.1C) (Leitman et al., 2013). These contact points can be considered sub-domains of the ER, enriched in factors that are needed for specific functions. Perhaps the clearest ER-organelle interface sub-domain is the nuclear envelope, which is a continuation of the ER membrane that wraps around the nucleus. Unique to the nuclear envelope are the nesprin family of proteins, which provide a link between the lamina and the cytoskeleton by bridging the inner and outer membrane of the nuclear envelope, thus influencing shape and positioning of the nucleus in the cell (Mellad et al., 2011). Additionally, the mammalian nuclear envelope completely disintegrates and regenerates before and after cell division, further highlighting the dynamic nature of the ER. During nuclear envelope breakdown, the ER was found to lose interactions with microtubule plus-end binding proteins and enhanced binding to microtubule minus-end binding proteins (Schlaitz et al., 2013; Smyth et al., 2012). This would cause the ER to migrate towards the spindle poles and has been proposed to prevent interference during chromosomal segregation. Thus, the dynamic nature of the nuclear envelope aid in cell division and maintain interactions with the cytoskeleton.

The ER also makes extensive contact with the mitochondria at sites that are called mitochondria-associated membranes (MAM), which are important for lipid transfer, calcium signaling and apoptosis (Figure 1.1C) (Hayashi et al., 2009; Rusinol et al., 1994). The ER and mitochondria come in close proximity, 10-50 nm, which could allow the transfer of lipids to occur between the two membranes, in a vesicle independent manner (Csordas et al., 2006). The ER is primarily responsible for the synthesis of phospholipids and cholesterol (Figure 1.2) (van Meer et al., 2008). Although the ER is the site of cholesterol synthesis, it contains a low level of cholesterol. While not all ER localized lipid synthesis enzymes are found in the MAM, some are enriched at these locations. Lipids synthesized in the ER are dispersed throughout endomembranes by membrane vesicles and lipid transport proteins (van Meer et al., 2008). Enrichment of lipid synthesis enzymes and lipid transfer at the MAM is needed since
mitochondria are not part of the endocytic pathway, thus mitochondria would require a vesicle independent system to acquire the appropriate lipids. Additionally, the ER is unique in its lipid composition in that it is the only organelle that has a completely symmetrical distribution of lipids, unlike the plasma membrane and the endomembranes that have a preference for certain lipids between the different leaflets (Devaux and Morris, 2004). Thus the ER serves as a platform to create much of the lipids necessary for maintaining the endomembrane systems.

The MAM also contains factors important for calcium homeostasis, which help maintain a three to four order of magnitude concentration gradient in the ER relative to the cytoplasm (Brini and Carafoli, 2009; Lewis, 2007). Members of the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) class of proteins primarily maintain this gradient. These ER membrane pumps are responsible for sequestering calcium from the cytoplasm and into the ER lumen. Humans possess three different SERCA proteins named SERCA1, SERCA2 and SERCA3, which have two, three and six splice variants, respectively (Brini and Carafoli, 2009). SERC2B is ubiquitously expressed and is the primary ER calcium pump in non-muscle tissue, thus SERCA2B is the Sisyphus of the ER, forever tasked with the laborious task of pumping calcium from the cytoplasm into the ER lumen against the concentration gradient.

Calcium can be released from the ER into the cytoplasm by stimulation of inositol triphosphate (IP3) receptors by various signaling pathways (Patterson et al., 2004; Zalk et al., 2007). While IP3 receptors are found throughout the smooth ER, their localization at the MAM ensures efficient transfer of calcium from the ER into the mitochondria. Extra calcium in the mitochondria initially enhances ATP production; however, prolonged calcium influx into the cytoplasm or mitochondria will activate apoptotic signals (Hajnoczky et al., 2006; Hayashi et al., 2009). Thus, the calcium flux must be promptly stopped upon activation to prevent toxic build up of cytoplasmic calcium. While SERCA2B is the main pump responsible for sequestering calcium into the ER, calcium can also be sequestered into the Golgi or mitochondria by Secretory
Pathway Ca2+-ATPase (SPCA) proteins or by the mitochondrial calcium uniporter (MCU), respectively (Brini and Carafoli, 2009; Hayashi et al., 2009). Excess calcium can also be cleared from the cytoplasm by expulsion into the extracellular space by Na⁺-Ca²⁺ exchangers located at the plasma membrane. Thus, the eukaryotic cell has adopted multiple ways of avoiding chronic elevation of cytoplasmic calcium levels.

Calcium homeostasis is regulated by posttranslational events such as oxidation of disulfide bonds in either SERCA2B or the IP3 receptor by ER oxidoreductases (Higo et al., 2005; Li and Camacho, 2004). The formation of these disulfides is believed to lock the IP3 receptor and SERCA2B in a confirmation that inhibits release or sequestering of calcium, respectively. The activity of SERCA2B is also influenced by interactions with the lectin chaperone calnexin and the phosphorylation or palmitoylation status of calnexin (Lynes et al., 2013; Roderick et al., 2000). Finally and perhaps more drastically, the IP3 receptor can be targeted for ERAD shortly after stimulation to prevent excess release of ER calcium (Lu et al., 2011). These data demonstrate the multiple levels of calcium regulation, including compartmentalization, palmitoylation, phosphorylation, oxidation and protein degradation that a eukaryotic cell employs to modulate calcium homeostasis.

The ER also comes in close contact (10-25 nm) with the plasma membrane in regions called the plasma membrane-associated membranes (PAM) (Figure 1.1C). Similar to the MAM, PAM is important for sterol transfer, lipid synthesis and calcium signaling (Pichler et al., 2001; Yeromin et al., 2006). When ER calcium levels become low, a calcium-sensitive ER membrane protein oligomerizes and moves toward the plasma membrane where it stimulates influx of extracellular calcium into the cytoplasm through a plasma membrane calcium channel (Roos et al., 2005). The elevated cytoplasmic calcium is then quickly sequestered by SERCA2B into the ER, thus extracellular calcium can be quickly targeted to the ER with minimal dwell time in the cytoplasm (Lewis, 2007). Additionally, work conducted in yeast indicates that endocytosis only
occurs in regions of the plasma membrane devoid of PAM, since PAM could interfere with retrograde trafficking (Stradalova et al., 2012).

Parts of the smooth ER membrane are used to form peroxisomes at specific ER sub-domains named pre-peroxisomal compartments (Figure 1.1C) (Tabak et al., 2013). Peroxisomes are disperse eukaryotic organelles with multiple metabolic functions, primarily beta-oxidation of long chain fatty acids. Though the exact molecular events of peroxisomal formation are unclear, it is known that multiple peroxisome proteins are originally targeted to the ER, and after some unknown cue, vesicles loaded with peroxisomal proteins bud off from the smooth ER to form mature peroxisomes (van der Zand et al., 2010; Yonekawa et al., 2011). In summary, a growing body of work implies that the ER is compartmentalized into multiple sub-domains, each dedicated to unique functions.

**Tetratricopeptide repeats, a resourceful adapter motif.**

A multitude of cellular functions rely on adapter proteins to mediate multi-protein complexes and aid in compartmentalization. By utilizing adapter proteins, the cell can increase the local concentration of specific factors without having to increase the total amount of the protein (Good et al., 2011). One such example is the ERQC, which is organized by SEL1L; the ER membrane adapter protein that nucleates a protein complex important for disposal of misfolded secretory proteins. Specifically, SEL1L utilizes ER luminal tetratricopeptide repeats (TPR) motifs to mediate the multi-protein ERQC complex consisting of both ER luminal and membrane proteins.

TPR motifs consist of 34 amino acids that fold into two anti-parallel α helices with a short loop connecting the two helices (Figure 1.3A) (D’Andrea and Regan, 2003; Zeytuni and Zarivach, 2012). The consensus sequence for a TPR motif is degenerate. While no amino acid is absolutely conserved, there tends to be a propensity for some amino acids at certain positions.
forming a delicate jigsaw puzzle wherein opposing amino acid side chains have evolved to complement each other (Main et al., 2003). Likely due to its small size, a single TPR motif is insufficient to form a functional unit, hence TPR domains are always found in a sequential organization to form functional clusters of individual TPR motifs. A minimum of three sequential TPR motifs form a functional cluster, but as many as sixteen have been predicted in an open reading frame in archaea (D'Andrea and Regan, 2003). Clusters of three TPR motifs are also the most common organization. Additionally, each cluster of TPR domains is followed by a C-terminal capping helix needed for structural support or stability (Figure 1.3B). The sequential organization of TPR motifs creates a concave groove for the ligand and a convex backside needed for structural integrity (Figure 1.3C). While clusters of three to four TPR motifs tend to bind to a well-defined ligand, proteins with five or more sequential TPR motifs appear to be more promiscuous in their binding partners, with no clear consensus sequence among its substrates (Gatto et al., 2000; Lazarus et al., 2011; Scheufler et al., 2000). Thus, despite being a relatively simple motif, the number and organization of the TPR motifs can greatly influence scaffolding abilities and give them the ability to bind to a wide range of ligands.

TPR domains exhibit a high degree of variability in their sequence and bioinformatic results have shown that certain positions are more variable than others, the so called hypervariable positions (Magliery and Regan, 2005). Interestingly, these hypervariable positions coincide with the amino acids that are important for recognizing the ligand in the structures of various TPR domains co-crystalized with their ligands (Gatto et al., 2000; Scheufler et al., 2000). Furthermore, mutating these hypervariable positions in a cluster of TPR motifs affects the binding specificity without affecting protein folding (Jackrel et al., 2009). This demonstrates that TPR motifs are highly moldable scaffolds, which can accommodate a wide range of ligands.

The best-studied TPR domain containing protein is Hop (Hsp70-Hsp90 Organizing Protein), which possesses nine TPR domains in three separate clusters of three TPR motifs.
(Figure 1.4 A) (Smith et al., 1993). The N-terminal TPR cluster of Hop recognizes the C-terminus of Hsp70 while the middle TPR cluster associates with the C-terminus of Hsp90, and the last cluster does not appear to bind a distinct ligand (Figure 1.4A). By bringing Hsp70 and Hsp90 together in a complex, Hop facilitates the transfer of substrates from Hsp70 to Hsp90 (Chen and Smith, 1998a; Johnson et al., 1998). Both the N-terminal and middle clusters of TPR motifs recognize the C-terminal cytoplasmic EEVD tail of Hsp70 and Hsp90, respectively, implying that the EEVD sequence cannot be the sole determinant for selectivity (Brinker et al., 2002; Scheufler et al., 2000). The interactions between the clusters of TPR motifs in Hop and their respective ligands are mediated by both the side chain and the peptide backbone of the EEVD ligand. A synthetic heptamer peptide corresponding to the C-terminus of Hsp90 showed a clear preferential binding to the central TPR cluster of Hop as compared to the TPR cluster that is designated to interact with Hsp70 (Scheufler et al., 2000). Thus, the TPR domains of Hop can support proper chaperone interactions to create a multi-protein complex to assist with protein folding. While some TPR containing proteins like Hop have well-defined ligands for their TPR domains, others appear more promiscuous in selecting binding partners.

O-linked β-N-acetylglucosamine transferase (OGT) possesses thirteen predicted TPR motifs ordered in a sequential manner and utilizes TPR motifs to recognize substrates for attachment of N-acetyl glucosamine to the side chains of serines or threonines (Figure 1.4 B) (Iyer and Hart, 2003; Kreppel and Hart, 1999). Addition of N-acetyl glucosamine to client proteins has been shown to be important for the nuclear import of proteins as well as sensing cellular glucose levels (Hart et al., 2007). Of the thirteen predicted TPR motifs in OGT, eleven were solved in a crystal structure in the presence of a peptide ligand; however, the ligand crystallized with the catalytic domain instead of the TPR motifs (Jinek et al., 2004; Lazarus et al., 2011). While no consensus sequence among the ligands has been observed, it has been speculated that the TPR domains keep the substrate in a unfolded conformation in order to present the substrate to the
catalytic domain of the OGT. Hop and OGT highlight a trend among adapter proteins with TPR domains, wherein stretches of three to five sequential TPR motifs tend to recognize a shorter and more defined ligands whereas a higher number of sequential TPR motifs is less specific in their binding.

The difference in ligand preference between shorter and longer clusters of TPR motifs is exhibited in Tom70, which uses a cluster of three TPR domains to interact with the C-terminal EEVD tail sequence of Hsp70 and a cluster of seven TPR motifs interacts with proteins targeted for mitochondrial import (Figure 1.4 C) (Wu and Sha, 2006; Young et al., 2003). Similarly to OGT, no clear mode of interaction has been described between a client protein and the large TPR cluster. Yet another similarity between Tom70 and OGT is that both appear to form homodimers using interactions with the convex backside of the their TPR domains (Jinek et al., 2004; Wu and Sha, 2006; Young et al., 2003). Similarly, Toc64 and Pex5 recognize the C-terminal EEVD of Hsp90 or the C-terminal SKL tag used for peroxisomal import (Gatto et al., 2000; Qbadou et al., 2006). Thus TPR domains are versatile adapters that can be modulated to interact with a specific ligand or a broad range of substrates.

TPR motifs appear to be used by proteins involved in post-translational translocation into mitochondria (tom70), chloroplast (Toc64) and peroxisomes (Pex5) (Chan et al., 2006; Gatto et al., 2000; Qbadou et al., 2006). Additionally, *Saccharomyces cerevisiae* contains the TPR protein Sec72p, which aids in post-translational translocation of proteins into the ER (Feldheim and Schekman, 1994; Jermy et al., 2006). The observation that TPR domains are found at sites of posttranslational protein translocation across cellular membranes could reflect the fact that translocation events are complicated processes and multiple proteins are needed to ensure the fidelity of this process and that substrates generally need to be unstructured to be translocated. TPR domains are excellent candidates for nucleating the necessary factors of protein translocation into a functional complex, and maintain the substrate in a translocation competent state.
To date only a few TPR domain-containing proteins have been localized to the ER, these are Sec72p, ERdj6 and SEL1L. Sec72p is an ER membrane proteins with three predicted cytoplasmic TPR motifs, which are believed aid in the posttranslational translocation of proteins targeted to the ER (Plath et al., 1998). While conflicting data have been published on the topology and function of ERdj6, recent work indicates that ERdj6 is an ER membrane protein with nine TPR motifs and a J-domain facing the ER lumen (Oyadomari et al., 2006; Rutkowski et al., 2007). ERdj6 has been shown to bind misfolded through the TPR domain whereas the J-domain was important for recruiting BiP (Petrova et al., 2008). SEL1L is a type I ER membrane protein that possesses eleven TPR domains in three separate clusters of four, five and two TPR motifs, respectively (Figure 1.4 D). Degradation of a variety of misfolded proteins depended on SEL1L (Mueller et al., 2006). SEL1L is an important converging point in ERAD, as it facilitate formation of a multi-protein membrane complex and mediates interactions with proteins in the ER lumen (Christianson et al., 2008; Cormier et al., 2009; Hosokawa et al., 2008; Iida et al., 2011; Mueller et al., 2008). Interestingly, SEL1L retained its interaction with luminal binding partners when the transmembrane domain was removed, implying that a luminal TPR domain containing portion of SEL1L is sufficient to mediate protein-protein interactions with the soluble quality control receptors, EDEM1, OS-9 and XTP3-B (Christianson et al., 2008). All together, the degenerate nature of TPR motifs can be utilized to nucleate a host of protein complexes for various functions across multiple organelles, although only a few have been localized to the ER.

**A bioinformatics screen for novel TPR containing ER adapter proteins**

The ER appears to have a high-level of organization, and ER-resident adapter proteins contribute to this organization by nucleating large multi-protein complexes. Mammalian cells have ~180 predicted TPR domain containing proteins, and one-third of the proteome is targeted to the ER (Letunic et al., 2012). Therefore, we hypothesized that there might be novel uncharacterized ER adapter proteins that utilize TPR motifs to aid in the compartmentalization
and organization of ER processes. To explore this possibility further, we acquired a partial *in silico* TPR protein library from Dr. L. Regan, (Magliery and Regan, 2005) (Figure 1.5). This partial TPR protein library contains ~ 300 proteins from a variety of organisms and was created at random to perform bioinformatic studies on TPR domain proteins. The amino acid sequence of proteins within the library was subjected to a signal sequence prediction algorithm to predict whether any of these proteins were likely to be targeted to the ER via an N-terminal signal sequence (Bendtsen et al., 2004). It should be noted that proteins that are targeted to the ER independent of a N-terminal signal sequence would be missed using this approach (Johnson et al., 2013).

NASP (Nuclear autoantigenic sperm protein) and TMTC1 (Transmembrane and TPR repeat-containing protein 1) were two original hits discovered with this search (Figure 1.6). While NASP has been linked to histone assembly in the nucleus, TMTC1 was completely uncharacterized (Batova and O’Rand, 1996; Finn et al., 2012). The original two hits were further expanded using BLAST searches to identify additional uncharacterized TPR containing proteins with a predicted N-terminal ER targeting sequence. This approach identified seven additional putative secretory TPR proteins named TMTC2, TMTC3, TMTC4, TTC9B, TTC13, TTC17, and TTC35. This *in silico* screen scheme expanded our results to nine putative secretory adapter proteins that contain TPR domains (Figure 1.6).

Multiple TPR containing adapter proteins are anchored in a membrane, and membrane insertion can greatly enhance the ability of adapter proteins to nucleate protein complexes by restricting their diffusion to two dimensions. Thus, all nine proteins identified in our screen were also analyzed using the ΔG prediction software, which predicts the free energy of inserting a hydrophobic domain into a membrane (Hessa et al., 2007). While TMTC1-4 were predicted to have various numbers of transmembrane domains, TTC9B, TTC13, TTC17, TTC35 and NASP were all predicted to be soluble proteins. Taken together, we have discovered nine putative
adapter proteins that possess multiple TPR motifs and appear to be targeted to the ER by N-terminal signal sequences. The biochemical and cell biological characterization of these nine proteins will allow us to determine if they are targeted to the ER, reside in the ER and act as adapter proteins to nucleate large protein complex formation that are used to organize functional complexes.
Figure 1.1 Organization of the endoplasmic reticulum.

(A) Cos7 cells were stained for ERp57 as a marker for the endoplasmic reticulum (ER). The staining highlights how the ER surrounds the nuclear envelope and extends throughout the cell. (B) The ER can be divided into three major domains termed the nuclear envelope, sheets and tubules. (C) ER is in close contact with other organelles through specialized sub-domains of the ER such ER exist sites (ERES), mitochondria associated membranes (MAM), plasma membrane-associated membranes (PAM) and the pre-peroxisomal compartments.
The first identified sub-domain of the ER was the rough ER (RER), which is enriched in factors needed for co-translational translocation into the ER and early protein folding and modification events. The ER is also the site of synthesis for the bulk of the phospholipids and cholesterol and hosts many enzymes important for lipid synthesis (blue), and flippases (green and gray) in the ER membrane. The majority of the intracellular calcium is stored in the ER, where SERCA2B (green) is the primary pump to sequester calcium into the ER and inositol trisphosphate receptor (pink and purple) stimulation causes release of calcium from the ER. Properly folded secretory proteins are targeted to the ER exit sites (ERES), where cargo is packaged into COPII covered vesicles (blue ovals) for sorting to the ER-Golgi-intermediate compartment then the Golgi. Due to the inherent difficulties of protein folding, a portion of secretory proteins fail to reach their native state and must be degraded to prevent toxic build up of misfolded proteins. The ER quality control compartment (ERQC) utilizes a membrane embedded adapter protein (SEL1L, red) to nucleate a multi protein complex that can efficiently dispose of misfolded proteins. The overall health of the ER is monitored by the unfolded protein response (UPR), which senses
accumulation of misfolded proteins or other stress signals and launches a stress response to restore ER homeostasis.
Figure 1.3 Tetratricopeptide repeats as adapter motifs.

(A) Tetratricopeptide repeats (TPR) are structural motifs that consist of 34 amino acids and fold into two helices (PDB# 1ELR). Sequential TPR motifs create a functional domain. The crystal structure represents one of the TPR motifs of Hsp70/Hsp90-organizing protein (Hop) (Scheufler et al., 2000). (B) The different TPR motifs of Hop are color coded blue, purple and cyan, with
ligand binding residues colored red. The capping helix is pale blue and the Hsp90 ligand is designated in green. (C) Structure as in B tilted 90 degrees towards the reader.
Figure 1.4 Various TPR domain adapter proteins found in eukaryotic cells.

(A) Hop is an adapter protein that mediates the interaction between Hsp70 and Hsp90, thereby facilitating the transfer of substrates from one chaperone to the other (Chen and Smith, 1998b; Johnson et al., 1998). (B) O-linked β-N-acetylglucosamine transferase uses thirteen sequential TPR domains to prepare client proteins for O-linked glycosylation (Iyer and Hart, 2003; Kreppel and Hart, 1999). (C) TOM70 is a TPR domain containing protein that utilizes one cluster of TPR domains for Hsp70 docking and a second larger cluster for client proteins to be translocated (Wu and Sha, 2006; Young et al., 2003). (D) SEL1L utilizes both asparagine (N)-linked glycans and TPR domains to nucleate a multi-protein complex needed for endoplasmic reticulum-associated degradation (ERAD) in the ERQC sub-domain (Avezov et al., 2008; Christianson et al., 2008; Cormier et al., 2009; Hosokawa et al., 2008).
An in silico library of known TPR proteins was a generous gift from Dr. Lynne Regan at Yale University (Magliery and Regan, 2005). The library of TPR proteins was analyzed with SignalP 3.0, a software that predicts N-terminal signal sequences (Bendtsen et al., 2004). The original hits were further expanded by BLAST searches and other in silico methods to generate a larger list of proteins containing TPR domains and predicted ER targeting sequences (Altschul et al., 1990). Finally, identified proteins were analyzed with ΔG predictor, a software that predicts transmembrane domains (Hessa et al., 2011).
Figure 1.6 Putative ER adapter proteins.

Our screen for novel secretory adapter proteins identified nine putative TPR-containing proteins that were potentially targeted to the ER by an N-terminal signal sequence (black squares). The names, position of tetratricopeptide repeat (TPR) (red rectangles), hydrophobic domains (green squares), asparagine (N)-linked glycans (black branched structures), proline-rich domains (blue squares) and histone binding domains (brown rectangles) are designated.
CHAPTER II

TMTC1 AND TMTC2 ARE REGULATORS OF CALCIUM HOMEOSTASIS.

Abstract

The endoplasmic reticulum (ER) is organized in part by adapter proteins that nucleate the formation of large protein complexes. Tetratricopeptide repeats (TPR) are well-studied protein structural motifs that support intermolecular protein-protein interactions. TMTC1 and TMTC2 were identified by an in silico search as TPR containing proteins possessing N-terminal ER targeting signal sequences and multiple hydrophobic segments, suggestive of polytopic membrane proteins that are targeted to the secretory pathway. A variety of cell biological and biochemical assays were employed to demonstrate that TMTC1 and TMTC2 are both ER resident integral membrane proteins with multiple clusters of TPR domains orientated within the ER lumen. Proteomic analysis followed by co-immunoprecipitation verification found that both proteins associated with the ER calcium uptake pump SERCA2B, while TMTC2 also bound to the carbohydrate-binding chaperone calnexin. Live cell calcium measurements revealed that overexpression of either TMTC1 or TMTC2 caused a reduction of calcium released from the ER following stimulation, whereas the knockdown of TMTC1 or TMTC2 increased stimulated calcium released. Together, these results implicate TMTC1 and TMTC2 as ER proteins involved in calcium homeostasis.

Introduction

The endoplasmic reticulum (ER) is an organelle comprised of a continuous membrane-envelope and lumen that is compartmentalized into numerous functional regions (Voeltz et al., 2002). The organization of the ER is directed in part by extrinsic factors that organize the rough ER (ribosomes) and the contiguous nuclear envelope (lamina and lamina receptors), as well as through interactions with the cytoskeleton (microtubules), organelles (Golgi and mitochondria) or
the plasma membrane (Levine and Rabouille, 2005). ER resident adapter proteins that nucleate the formation of large protein complexes also support the compartmentalization of the ER (Carvalho et al., 2006; Christianson et al., 2008; Mueller et al., 2008). The organization of the ER contributes to its ability to efficiently perform functions in the maturation, quality control and trafficking of secretory pathway cargo, calcium regulation and lipid synthesis (English et al., 2009; Hebert and Molinari, 2007; Lynes et al., 2013). These activities contribute to the maintenance of general cellular homeostasis; however, many questions still remain as to how the organization of the ER is maintained.

Tetratricopeptide repeats (TPR) are protein structural motifs that support protein-protein interactions. A single TPR domain consists of a degenerate 34 amino acid sequence that is comprised of two anti-parallel helices (D'Andrea and Regan, 2003; Das et al., 1998). A minimum of three consecutive TPR domains is required to form a functional unit, and this is the most common number of TPR found in a cluster (D'Andrea and Regan, 2003). Clusters comprised of up to sixteen sequential TPR have been observed. Proteins with three TPR domains in a cluster favor the recognition of short and defined sequences, whereas proteins with long stretches of consecutive TPR domains tend to be more promiscuous in their selectivity. For example, the Hsp70-Hsp90 Organizing Protein (HOP) uses two separate clusters of three consecutive TPR domains to bind the EEVD sequence located at the C-termini of Hsp70 and Hsp90, facilitating the hand-off of a substrate from one chaperone to another (Chang et al., 1997; Scheufler et al., 2000). In contrast, the O-linked N-acetylglucosamine transferase possesses twelve consecutive TPR domains that are proposed to bind and prepare substrates for modification (Lazarus et al., 2011). Tom70 displays a combination of TPR clusters as it uses a cluster of three TPR domains to recognize the C-terminus of Hsp70 for substrate delivery and a second cluster of seven TPR domains to potentially prepare substrates for mitochondrial import (Wu and Sha, 2006). These
diverse cellular functions are coordinated by TPR domains and their ability to bind to a range of ligands.

SEL1L and p58IPK (also called ERdj6) are the only ER TPR containing proteins that have been characterized in mammalian cells. SEL1L nucleates a large ER membrane complex involved in ER-associated degradation (ERAD) (Mueller et al., 2008). p58IPK is an ER co-chaperone that interacts with BiP to facilitate protein folding (Petrova et al., 2008; Rutkowski et al., 2007). Given the extensive utilization of TPR domains in metazoans to support protein-protein interactions, the ER is expected to contain additional TPR domain proteins that contribute to complex formation and ER organization.

An in silico approach was used to expand our understanding of ER adapters that participate in the organization of the ER using TPR domains. TMTC1 and TMTC2 were identified as TPR containing proteins that possess potential N-terminal ER targeting sequences and multimembrane spanning regions. They were both found to reside in the ER membrane with their TPR domains orientated within the ER lumen. A role for TMTC1 and TMTC2 as adapter proteins was supported by their appearance in large molecular weight complexes. A shotgun proteomics approach was employed that identified SERCA2B as a potential binding partner for TMTC1 and TMTC2, as well as calnexin for TMTC2. Manipulation of the expression levels of TMTC1 and TMTC2 combined with live cell calcium measurements demonstrated that TMTC1 and TMTC2 influenced calcium sequestering in the ER. Collectively, these findings showed that TMTC1 and TMTC2 are two novel TPR containing ER adapters involved in calcium homeostasis.
**Results**

**TMTC1 and TMTC2 are ER resident proteins.**

As adapter proteins commonly use clusters of TPR domains to modulate protein-protein interactions, we hypothesized that the ER might contain TPR domain-containing proteins, in addition to SEL1L and p58IPK that contribute to the organization of the ER. Databases (SMART7 ([Letunic et al., 2012](http://www.smart7.org)) and a Regan lab TPR protein library (Yale University) ([Magliery and Regan, 2005](http://www.ncbi.nlm.nih.gov/protein/NP_689801.1)) were queried with SignalP 3.0 to identify TPR proteins that were potentially targeted to the secretory pathway ([Bendtsen et al., 2004](http://www.ncbi.nlm.nih.gov/protein/NP_787057.2)). TMTC1 (Transmembrane and TPR repeat-containing protein 1 (NCBI Accession # NP_787057.2) ([http://www.ncbi.nlm.nih.gov/protein/NP_787057.2](http://www.ncbi.nlm.nih.gov/protein/NP_787057.2)) and TMTC2 (NCBI Accession # NP_689801.1 ([http://www.ncbi.nlm.nih.gov/protein/NP_689801.1](http://www.ncbi.nlm.nih.gov/protein/NP_689801.1)) were identified as TPR-containing proteins with potential N-terminal signal sequences. These proteins were initially identified, but not characterized, in a large human sequencing study that identified opening reading frames from human cells ([Gerhard et al., 2004](http://www.ncbi.nlm.nih.gov/protein/NP_001180380.1); [Ota et al., 2004](http://www.ncbi.nlm.nih.gov/protein/NP_787057.2)). *In silico* analysis of TMTC1 and TMTC2 indicated that each protein contained a potential N-terminal signal sequence and ten C-terminal TPR domains (Figure 2.1A).

Two TMTC1 isoforms are reported in the NCBI Protein Database with NCB Accession # NP_001180380.1 ([http://www.ncbi.nlm.nih.gov/protein/NP_001180380.1](http://www.ncbi.nlm.nih.gov/protein/NP_001180380.1)) and NP_787057.2 ([http://www.ncbi.nlm.nih.gov/protein/NP_787057.2](http://www.ncbi.nlm.nih.gov/protein/NP_787057.2)), for isoform 1 and 2 respectively ([Sayers et al., 2009](http://www.ncbi.nlm.nih.gov/protein/NP_001180380.1)). TMTC1 splice variants present in HEK293T cells were determined by PCR using isoform specific primers on a cDNA library generated from total mRNA. A PCR product was observed using the primers directed against regions of the TMTC1 isoform 2, while a product was not generated for isoform 1 (Figure 2.1B and C). qRT-PCR using a variety of primer sets failed to amplify a PCR product that would correlate with TMTC1 isoform 1. As only the TMTC1 isoform 2 was expressed in HEK293T cells, this isoform will be referred to as TMTC1.
TMTC1 and TMTC2 cDNAs were subcloned into mammalian expression vectors encoding a C-terminal myc-tag, and their cellular localization was determined by confocal immunofluorescence microscopy. COS7 cells were transfected with either TMTC1-myc or TMTC2-myc, and staining was compared against an ER (ERp57) or Golgi (GM130) marker (Figure 2.2A). Both TMTC1 and TMTC2 colocalized with ERp57, while colocalization was not observed with GM130, suggesting that TMTC1 and TMTC2 are both ER resident proteins.

Secretory proteins are commonly modified in the ER with N-linked glycans at the consensus site Asn-X-Ser/Thr. TMTC1 and TMTC2 both possess a single N-linked glycosylation consensus site (Figure 2.1A), therefore a glycosylation assay was used to further analyze ER targeting and localization. As the molecular weight of an N-linked glycan is ~2.5 kD, the removal of N-linked glycans by glycosidase treatment results in a corresponding increase in mobility for the deglycosylated protein. Endoglycosidase H (Endo H) trims the high mannose glycans encountered in the ER while Peptide-N-Glycosidase F (PNGase F) removes complex glycans acquired in the Golgi in addition to high mannose glycans.

HEK293T cells transfected with TMTC1 or TMTC2 containing a C-terminal S-tag were pulsed with [35S]-Met/Cys for 60 min. Cell lysate and media fractions were affinity precipitated with S-protein agarose beads followed by glycosidase treatment. Shifts upon glycosidase treatment were not observed for either TMTC1 or TMTC2 indicating that neither protein appeared to be glycosylated (Figure 2.2B). As not all glycosylation sites are efficiently recognized and modified, two N-linked glycosylation sites were introduced to the C-terminal portion of TMTC1 (TMTC1\textsuperscript{N626, 633}) and TMTC2 (TMTC2\textsuperscript{N602, 818})(Figure 2.1A, see asterisks). These constructs migrated slower than the wild type constructs, and their treatment with PNGase F produced faster migrating proteins indicating that TMTC1\textsuperscript{N626, 633} and TMTC2\textsuperscript{N602, 818} were both modified with N-linked glycans (Figure 2.2C). A similar increase in mobility was observed upon Endo H treatment indicating that the carbohydrates were high mannose glycoforms. Therefore,
the cellular distribution for TMTC1 and TMTC2 and the glycosylation profiles for the glycan addition mutants are consistent with TMTC1 and TMTC2 residing in the ER.

**TMTC1 and TMTC2 are upregulated by oxidative stress.**

Proteins that reside in the secretory pathway are frequently transcriptionally upregulated by stress as their functions can help to alleviate stress (Walter and Ron, 2011). To determine whether the TMTC1 and TMTC2 genes are transcriptionally regulated by ER stress, HEK293T cells were exposed to different ER stress conditions. Cells were subjected to oxidative stress (dithiotreitol, DTT), calcium depletion (thapsigargin), N-glycan synthesis inhibition (tunicamycin) or amino acid starvation (-Cys/Met). RNA was harvested from cells followed by reverse transcription to generate cDNA and changes in gene expression were measured by qRT-PCR. TMTC1 and TMTC2 gene expression was increased with DTT treatment by 3.9 and 2.3 fold, respectively (Figure 2.3). However, thapsigargin, tunicamycin or amino acid starvation did not produce a significant increase in gene expression of TMTC1 or TMTC2, although the transcription of BiP was significantly stimulated by DTT, thapsigargin and tunicamycin. Thus, only oxidative stress increased the transcription of TMTC1 and TMTC2.

**TMTC1 and TMTC2 are ER membrane proteins with luminal orientated TPR domains.**

Analysis of the TMTC1 and TMTC2 protein sequences with ΔG prediction demonstrated that TMTC1 and TMTC2 contained 9 and 10 hydrophobic segments, respectively, that could potentially serve as transmembrane domains to create polytopic membrane proteins (Figure 2.1A) (Hessa et al., 2007). Alkaline extraction of membrane fractions was performed to separate membrane and soluble forms of proteins following centrifugation to determine if TMTC1 and TMTC2 are integral membrane proteins (Giorda et al., 2012; Mostov et al., 1981).
HEK293T cells were transfected with either TMTC1 or TMTC2, and proteins were radiolabeled with $[^{35}\text{S}]-\text{Met/Cys}$ for 60 min. Cells were homogenized in isotonic buffer and fractions were separated by centrifugations. TMTC1 and TMTC2 were found in the total membrane and nuclear fractions (Figure 2.4A). The nuclear localization of TMTC1 and TMTC2 is likely explained by the contiguous nature of the ER and nuclear membrane prohibiting their separation as the ER proteins calnexin and calreticulin were also in the nuclear fractions. Alkaline extraction of the total membrane fractions followed by centrifugation found TMTC1 and TMTC2 exclusively in the membrane pellet. This profile was observed for the ER membrane protein calnexin and not its soluble parologue calreticulin, which largely accumulated in the supernatant. Therefore, both TMTC1 and TMTC2 are integral membrane proteins.

Since TMTC1 and TMTC2 are membrane proteins, a trypsin protection assay was employed to determine if their C-terminal TPR domains are positioned in the ER lumen or the cytoplasm. HEK293T cells were transfected with either TMTC1 or TMTC2 constructs containing C-terminal S-tags and pulsed with $[^{35}\text{S}]-\text{Met/Cys}$ for 60 min followed by homogenization and isolation of ER-enriched microsomes. Isolated microsomes were resuspended in an isotonic buffer, and aliquots were treated with Triton-X 100 and trypsin as indicated. Affinity purification using S-agarose beads allowed the isolation of proteins containing a protected C-terminus. Trypsin treatment produced discrete TMTC1 and TMTC2 fragments of 62.5 and 51.6 kD, respectively (Figure 2.4B, compare lanes 5 to 6 and 9 to 10). As the TPR domains and S-tags are both at the C-termini, this demonstrated that the TPR domains of TMTC1 and TMTC2 were positioned in the ER lumen. Combined with the modification of the glycosylation sites added to the TPR rich regions (Figure 2.2C), these results demonstrated that the TPR domains for both of the membrane proteins TMTC1 and TMTC2 were facing the ER lumen.
**TMTC1 and TMTC2 associate with SERCA2B.**

To identify binding partners of TMTC1 and TMTC2, a shotgun liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) approach was used. Cells expressing either TMTC1 or TMTC2 with a C-terminal S-tag were homogenized and ER-derived microsome fractions were isolated. Proteins associated with TMTC1 and TMTC2 were isolated using the S-protein agarose beads and resolved by SDS-PAGE. The gels were subjected to either silver staining or in-gel trypsin digestion followed by LC-MS/MS analysis (Figure 2.5A). The proteins associated with the S-tagged proteins obtained from the LC-MS/MS analysis were scored according to the number of unique peptides identified from the corresponding protein (Table 2.1). The top four associated proteins for TMTC1 and TMTC2 were identical: SERCA2B, calnexin, BiP and the glucosidase II α subunit.

To verify the interactions identified using the mass spectrometry approach, HEK293T cells were transfected with the C-terminal S-tagged TMTC1 or TMTC2, followed by affinity purification and immunoblotting against endogenous associated proteins. Both TMTC1 and TMTC2 readily interacted with SERCA2B, a polytopic ER calcium pump responsible for calcium uptake from the cytoplasm into the ER (Figure 2.5B). TMTC2, but not TMTC1, interacted with calnexin. Calnexin is a carbohydrate-binding chaperone of the ER membrane that associates with maturing glycoproteins that possess monoglucosylated glycans (Pearse and Hebert, 2010). The TMTC2-calnexin interaction appeared to be specific since TMTC2 did not interact with calreticulin, the soluble paralog of calnexin, or the calnexin-associated oxidoreductase ERp57 (Figure 2.5B). The interaction between calnexin and TMTC2 was reduced though not abolished by glucosidase inhibition with N-butyl-deoxynojirimycin even though TMTC2 was not glycosylated (Figure 2.5C). These results suggested that an additional glycosylated component might be involved in TMTC2 binding to calnexin. Despite being identified by the mass
spectrometry results (Table 2.1), no interaction between TMTC1 and TMTC2 with either BiP or the glucosidase II α subunit was observed (Figure 2.5B).

To determine the approximate size of the TMTC1 and TMTC2 complexes, velocity centrifugation was performed. HEK293T cells were transfected with either S-tagged TMTC1 or TMTC2. Cells were lysed in Triton-X 100, and cell lysates were layered on a 10-40% linear sucrose gradient followed by centrifugation. TMTC1 and TMTC2 were exclusively found in the larger fractions indicating that both proteins resided in large protein complexes (Figure 2.5D, fractions 7-10). The SERCA2B sedimentation profile overlapped with the TMTC1 and TMTC2 profiles, though SERCA2B was also found in lighter fractions absent of TMTC1 and TMTC2 (Figure 2.5D, fractions 5 and 6). While calnexin was predominantly localized to smaller size fractions (Figure 2.5D, fractions 1-4), a portion was also observed in the TMTC1 and TMTC2 fractions. Collectively, these results indicate that TMTC1 and TMTC2 are found in large protein complexes that include SERCA2B, as well as calnexin for TMTC2.

To determine if the interactions with TMTC1 and TMTC2 involved their TPR domains, the C-terminal TPR rich portion of TMTC1 (TMC1\textsuperscript{TPR}) and TMTC2 (TMTC2\textsuperscript{TPR}) were targeted to the ER in HEK293T cells using the signal sequence of BiP and a C-terminal S-tag followed by an ER KDEL retention sequence to support ER residency. TMTC1\textsuperscript{TPR} and TMTC2\textsuperscript{TPR} were both efficiently targeted to and retained in the ER as observed by confocal immunofluorescence microscopy (Figure 2.6A) and the absence of the appearance of the constructs in the cell media (data not shown). TMTC1\textsuperscript{TPR} and TMTC2\textsuperscript{TPR} maintained their interaction with SERC2B, suggesting that these interactions were mediated through the TPR domains of TMTC1 or TMTC2. In contrast, TMTC2\textsuperscript{TPR} did not associate with calnexin, implying that this interaction required the transmembrane domain of TMTC2 (Figure 2.6B). Thus, the TPR domains of TMTC1 and TMTC2 were utilized to mediate the interactions with SERCA2B.
TMTC1 and TMTC2 alter cytoplasmic calcium levels.

As TMTC1 and TMTC2 both interact with SERCA2B, live cell calcium measurements were used to elucidate the impact of TMTC1 and TMTC2 on calcium regulation. Initially, cytoplasmic calcium levels were monitored after the overexpression of TMTC1 or TMTC2. Green fluorescent protein (GFP) was fused to the C-termini of TMTC1 (TMTC1\textsubscript{GFP}) and TMTC2 (TMTC2\textsubscript{GFP}) to mark efficiently transfected cells for analysis (Figure 2.7A). Cells were incubated with Fura-2 AM, a membrane permeable fluorophore that is rendered membrane impermeable by cytoplasmic esterases. The treatment of cells with ATP and carbachol releases intracellular calcium stores, as carbachol is a non-cleavable acetylcholine analog that stimulates muscarinic receptors at the plasma membrane resulting in the downstream release of calcium from the ER via the inositol trisphosphate receptor (Luo et al., 2001; Wu et al., 2002).

Cell expressing TMTC1\textsubscript{GFP} or TMTC2\textsubscript{GFP} and treated with ATP and carbachol showed calcium responses that reached peaked levels earlier than non-transfected cells (Figure 2.7B). Nevertheless, a 33.8%, 45.2% and 30.4% decrease in calcium release was observed in cells expressing either TMTC1\textsubscript{GFP}, TMTC2\textsubscript{GFP} or both compared to non-transfected cells, respectively. These results suggest that TMTC1 and TMTC2 affect calcium homeostasis, possibly by modifying the calcium sequestering capacity of SERCA2B to decrease the duration of the calcium signal. No synergy between TMTC1 and TMTC2 was observed when both proteins were expressed together.

Intracellular calcium release can also be stimulated by addition of calcium ionophores such as ionomycin, which promote release mostly from intracellular stores when the exposure is performed in the absence of extracellular calcium. Therefore, cells expressing TMTC1\textsubscript{GFP} or TMTC2\textsubscript{GFP}, bathed in calcium free media and exposed to ionomycin had a 20.2% and a 27.4% reduction in calcium release, respectively (Figure 2.7C-D). This implies that intracellular calcium stores were reduced by overexpression of TMTC1\textsubscript{GFP} or TMTC2\textsubscript{GFP}.
Next, the effect of TMTC1 or TMTC2 knockdown on cytoplasmic calcium levels was investigated. TMTC1 or TMTC2 was knocked down by transfecting HEK293T cells with a polycistronic plasmid that expressed an shRNA directed towards transcripts for either TMTC1 or TMTC2, as well as a cytoplasmic localized GFP to mark transfected cells. To verify knockdown efficiencies, RNA was harvested from HEK293T cells 24 hr post-transfection, and changes in gene expression were measured by qRT-PCR (Figure 2.8A). Fluorescence-activated cell sorting of GFP expressing cells was used to measure transfection efficiency of the different shRNA constructs. TMTC1 shRNA1 and TMTC2 shRNA3 achieved the maximum knockdown of 55% and 48%, respectively, in a cell mixture of transfected and non-transfected cells. These shRNA constructs were used for further analysis of effects on calcium measurements. Since cytoplasmic GFP was also expressed from the shRNA encoding plasmid, transfected and non-transfected cells were readily distinguishable by microscopy. Knockdown of either TMTC1, TMTC2 or both resulted in a 33.1%, 64.1% or 71.0% increase in carbachol stimulated ER calcium release, respectively (Figure 2.8B). While peak calcium levels were not greatly affected by knockdown of either molecule, calcium release was enhanced, especially after TMTC2 knockdown, which prolonged the response suggesting that it promoted greater calcium influx. Collectively, these results indicate that overexpression of TMTC1 or TMTC2 decreases stimulated calcium release, whereas their knockdown, especially for TMTC2 or both TMTC1 and TMTC2 combined enhanced calcium increases into the cytoplasm supportive of a role for TMTC1 and TMTC2 in calcium regulation.

**Discussion**

We identified and characterized two novel TPR-rich ER proteins, TMTC1 and TMTC2. They share a 54% similarity and 27% identity in the amino acid sequence, suggestive of a common evolutionary origin (Sievers et al., 2011). Basic Local Alignment Search Tool (BLAST) analysis indicated that TMTC1 and TMTC2 homologues are conserved in the chordata phylum.
and are absent in lower eukaryotes such as yeast as is their most prominent associated protein SERCA2B (Brini and Carafoli, 2009). Published microarray data shows that TMTC1 and TMTC2 are transcribed in a wide range of human tissues (Wu et al., 2013). TMTC1 and TMTC2 are divided into two regions that appear to create two functionally distinct domains, an N-terminal hydrophobic region and a C-terminal domain that contains multiple TPR clusters (Figure 2.1A).

Alkaline extraction of total membrane preparations demonstrated that TMTC1 and TMTC2 are integral membrane proteins. ΔG analysis predicts that the N-termini of TMTC1 and TMTC2 have ten and eleven potential transmembrane domains, respectively. A negative ΔG associated with a high probability of membrane integration was calculated for five (TMTC1) and three (TMTC2) of these hydrophobic segments. Hydrophobic domains with a positive ΔG frequently insert into membranes upon stabilization by efficiently inserted transmembrane domains (Hessa et al., 2007). Therefore, TMTC1 and TMTC2 appear to be polytopic membrane proteins with their hydrophobic N-termini providing a long stretch of multiple transmembrane segments. The presence of a number of hydrophilic and charged residues within some of the hydrophobic domains of TMTC1 and TMTC2 imply that these residues could be used for intramembrane interactions, such as the TMTC2-calnexin interaction.

The single natural N-glycosylation site present in the N-terminal hydrophobic portion of either TMTC1 or TMTC2 was not modified as probed by a glycosidase mobility shift assay. Lack of apparent glycosylation could be due to positioning of the consensus sites in the cytoplasm or being too close to the membrane for recognition by the oligosaccharyltransferase (Nilsson and von Heijne, 1993). Alternatively, the shift caused by glycosidase treatment and the removal of a single glycan might be too slight to be visualized for these large hydrophobic proteins. However, ER targeting was verified by placing efficiently modified glycosylation sites in the C-terminal TPR-rich regions of both TMTC1 and TMTC2 and by confocal immunofluorescence microscopy.
The glycosylation of sites added to the TPR regions of TMTC1 and TMTC2, combined
with the trypsin protection of the C-terminal domains from isolated membranes, placed the TPR
domains within the ER lumen for both TMTC1 and TMTC2. The trypsin protected fragments of
63 kD (TMTC1) and 52 kD (TMTC2) corresponded to the complete C-terminal TPR domains
(Figure 2.1A). UniProt predicted that TMTC1 has a total of ten TPR domains organized into
clusters of seven and three, while TMTC2 has ten total TPR domains found in clusters of four and
six. In contrast, analysis of TMTC1 by TPR domain prediction software TPRpred did not
recognize the second and sixth TPR domains of TMTC1 as bona fide TPR domains. The space
between the seventh and eighth TPR domains in TMTC1, which was not identified by UniProt,
was predicted to be an additional TPR domain by TPRpred, UniProt and TPRpred predictions are
in strong agreement for TMTC2. The only discrepancy between the analyses was that TPRpred
did not recognize the first TPR motif of TMTC2. The differences in organization of the TPR
domains of TMTC1 and TMTC2 could have implications for their functions.

TPR domains are found in proteins across species and organelles, and participate in a
plethora of activities including protein folding, post-translational modification, translocation and
signal transduction. Clusters of TPR domains form discrete domains that nucleate protein-protein
interactions (D'Andrea and Regan, 2003; Scheufler et al., 2000; Zeytuni and Zarivach, 2012).
Sucrose sedimentation analysis suggested that both TMTC1 and TMTC2 resided in large
molecular weight complexes. SERCA2B was the most prominent associated proteins for both
TMTC1 and TMTC2, and this interaction was mediated through the TPR domains as the
association persisted with a soluble construct comprised solely of the TPR-rich C-terminal region.
The dependency of the TPR domains of TMTC1/TMTC2 for the interactions with SERCA2B
was unexpected given the hydrophobic nature of TMTC1/TMTC2 and SERCA2B, and the small
portion of SERCA2B that is exposed to the ER lumen. It cannot be ruled out at this time that the
TMTC1 or TMTC2 interaction with SERCA2B is mediated through an additional protein in the large complex.

The carbohydrate-binding ER chaperone, calnexin, was also found to interact with the non-glycosylated TMTC2 but not TMTC1. Binding was reduced by 50% in the presence of glucosidase inhibition. Calnexin did not interact with the soluble TPR domain construct of TMTC2. The interaction could be mediated through the transmembrane segments of SERCA2B and calnexin (a type I membrane protein) as calnexin has been proposed to monitor proteins within the ER bilayer (Swanton et al., 2003). Alternatively, an additional glycosylated substrate such as SERCA2B may be involved in the interaction between TMTC2 and calnexin, as calnexin is known to associate with SERCA2B (Roderick et al., 2000).

TMTC1 and TMTC2 overexpression reduced the amount of calcium released from the ER after stimulation with carbachol and ATP. This reduction might be caused by a decrease in total amount of calcium stored in the ER, implying a defect in calcium uptake and/or storage. This is consistent with the observed higher baseline calcium values, especially in cells overexpressing TMTC2. Elevated baselines and reduced calcium release was also seen in studies using ionomycin implying that overexpression of TMTC1 or TMTC2 disrupts calcium homeostasis in these cells. It is unclear how these proteins might interfere with calcium ER levels, although SERCA2B is likely a target, given the evidence of direct interaction between these two molecules.

In contrast, knockdown of TMTC1 and TMTC2 caused an increase in the calcium response to the same agonists. While the amplitude of the peak response was not higher, it was longer, especially after TMTC2 knockdown. An interpretation of these results is that the knockdown of TMTC2 promotes greater calcium influx, possibly caused by an initial larger calcium release and robust activation of the stored calcium entry mechanism. The other
interpretation of the results, that knockdown of TMTC1 or TMTC2 reduces the capacity for SERCA2B to sequester cytoplasmic calcium is not supported by the comparable amplitude of the calcium rise induced by the agonists. The observation that no additive effect was observed with either co-expression or co-knockdown of both TMTC1 and TMTC2 implies that TMTC1 and TMTC2 do not act synergistically. It is worth noting that in both overexpression and knockdown studies, TMTC2 gave a stronger response then TMTC1, which may be attributed to the interaction between TMTC2 and calnexin. Future studies should address the role of calnexin on the function of TMTC2.

The interaction between calnexin and SERCA2B can be modified by reversible post-translational phosphorylation or palmitoylation of calnexin (Lynes et al., 2013; Roderick et al., 2000). Phosphorylation of the C-terminal cytoplasmic tail of calnexin is believed to reduce sequestering by SERCA2B, whereas palmitoylation of calnexin is proposed to increase SERCA2B activity. Since knockdown of TMTC2 increased calcium release, it is possible that this is accompanied by changes in post-translational modifications of calnexin. Furthermore, the decreased interaction between TMTC2 and calnexin caused by inhibition of glucosidase could be due to changes in post-translational modifications on either SERCA2B or calnexin (Figure 2.5C). Collectively, these modifications allow for a dynamic control of calcium sequestering adjustable to the current needs of the cell.

An RNAi based screen for proteins that effect protein trafficking found that knockdown of TMTC1 caused a reduction in the level of a viral glycoprotein localized to the cell surface and that the Golgi appeared dispersed (Simpson et al., 2012). In our study, knockdown of TMTC1 or TMTC2 in HeLa cells did not cause a significant defect in either ER or Golgi morphology. This discrepancy could be caused by differences in the HeLa strains employed (Landry et al., 2013).
The identification of TMTC1 and TMTC2 as ER membrane adapters and their interactions with SERCA2B highlights the exquisite organization and functional regulation of the ER and its components to carry out specific functions. The discovery of two additional regulators of intracellular calcium further highlights how central calcium signaling is to many aspects of cell biology and how carefully the signal is controlled and regulated. While both TMTC1 and TMTC2 appeared to disrupt calcium sequestering, only TMTC2 appeared to do so in cohort with calnexin. The precise mechanism for how TMTC1 and TMTC2 regulate SERCA2B activity is unclear, future studies will be aimed to further investigate how TMTC1 and TMTC2 influence calcium homeostasis.

**Materials and methods**

**Reagents and plasmids**

Dulbecco’s modified Eagle medium (DMEM), calcium free DMEM, fetal calf serum (FCS), penicillin, streptomycin, and Zysorbin were purchased from Invitrogen. 1-deoxynojirimycin (DNJ) and Easy-Tag \[^{35}\text{S}\]-Cys/Met were purchased from Toronto Research Chemicals and PerkinElmer, respectively. S-protein agarose beads and S-tag antibody were purchased from EMD Millipore. EndoH, PNGase F, AMV first strand synthesis kit, and all cloning reagents were purchased from New England Biolabs. FastStart Sybr Green qPCR mix was purchased from Roche, and all primers were acquired from IDT DNA. Anti-mouse-HRP IgG, anti-rabbit-HRP IgG, and protein A Sepharose CL-4B were purchased from GE Healthcare. Antibodies directed towards the following antigens were also purchased: SERCA2 (Cell Signaling); myc (Cell Signaling); calnexin (Enzo Life Sciences); calreticulin (Thermo scientific); and GM130 (BD Biosciences). TMTC1 and TMTC2 cDNA were purchased (Open Biosystems), and cloned into a pcDNA3.1 A-, a plasmid harboring either a C-terminal S- or myc- tag, using standard molecular biology techniques. TMTC1 and TMTC2 were tagged with GFP by inserting the GFP cDNA between the coding region and the C-terminal S-tag. These constructs were
termed TMTC1GFP and TMTC2GFP. The pcDNA3.1 A- S-tag backbone was modified to possess a N-terminal BiP signal sequence prior to the multiple cloning site and a KDEL sequence after the C-terminal S-tag. The TPR domains of TMTC1 and TMTC2 were cloned into this plasmid to create the TMTC1TPR and the TMTC2TPR constructs. shRNA plasmids were purchased from Qiagen. All other chemicals were obtained from Sigma-Aldrich.

**In silico analysis of TMTC1 and TMTC2**

The primary amino acids sequence of TMTC1 and TMTC2 were analyzed by UniProtKB and TPRpred to identify the number and position of putative TPR domains (Karpenahalli et al., 2007; UniProt, 2013). Hydrophobic domains were identified by the ΔG software, which predicts transmembrane domains (Hessa et al., 2007). Homologues of TMTC1 and TMTC2 were identified by Basic Local Alignment Search Tool (BLAST) searches (Altschul et al., 1990). The similarity and identify of TMTC1 and TMTC2 were determined using Clustal Omega software (Sievers et al., 2011).

**Tissue culture**

Human embryonic kidney (HEK) 293T or COS7 cells were grown in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin, and incubated at 37 °C in 5% CO2. Cells were transfected with polyethylenimine (PEI) and the appropriate plasmids for 16 hr.

**Microscopy**

Cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature followed by permeabilization with methanol for 10 min at -20 °C. Slides were stained with the indicated primary antibody followed by staining with appropriate Alexa Fluor 488 or 594 secondary antibodies in immunostaining buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 2 mM MgCl2, 1 mM EGTA, and 2% bovine serum albumin). Slides were rinsed and
mounted onto cover slips with VectaShield (Vector Laboratories). Images were obtained with a
Flouview 1000 MPE, IX81 motorized inverted research microscope (Olympus Inc.) equipped
with a Hamamatsu C8484-05G camera. All images were acquired with a Plan Apo N 60x 1.42NA
lens and processed by using the FV10-ASW and the Adobe Photoshop software.

Radiolabeling, affinity purification and glycosylation assay

Radiolabeling of proteins with $[^{35}\text{S}]$-Cys/Met was performed as previously described
(Svedine et al., 2004). Cells were lysed in MNT buffer (0.5% Triton X-100, 20 mM MES, 100
mM NaCl, 20 mM Tris-HCl [pH 7.5]). All steps were conducted at 4°C. The post-nuclear
supernatant (PNS) was isolated by centrifugation followed by pre-clearing with Zysorbin for 1 hr.
Cleared supernatant was incubated with S-protein agarose beads overnight and subsequently
washed twice with MNT buffer. After the final MNT wash, glycosylation assays were performed
by adding appropriate buffers and either mock, Endo H or PNGase F enzymes according to the
manufacturer’s protocol. Finally, reducing sample buffer was added to all samples, and they were
analyzed by SDS-PAGE.

Alkaline extraction

Alkaline extraction was performed as previously described (Tamura et al., 2011). Briefly,
radiolabeled cells were resuspended in ice-cold homogenization buffer (20 mM HEPES, pH 7.5,
5 mM KCl, 120 mM NaCl, 1 mM EDTA, and 0.3 M sucrose) and passed through a 22-gauge
needle 20 times. All subsequent steps were conducted at 4°C. The homogenate was centrifuged
at 1,000 g for 10 min to pellet the nuclear fraction. The remaining PNS was centrifuged at 45,000
rpm in Beckman rotor (TLA 120.2) for 10 min to separate the cytosol (supernatant) from the
cellular membranes (pellet). The cellular membrane fraction was resuspended in homogenization
buffer, and a portion of the resuspended membranes was incubated with 0.1M Na$_2$CO$_3$ (pH 11.5)
for 30 min on ice. The alkaline extracted portion was centrifuged at 65,000 rpm for 20 min
through a sucrose cushion (50 mM triethanolamine, 0.3 M sucrose, pH 7.5) to separate soluble
proteins from membrane proteins in the supernatant and pellet, respectively. The pH was adjusted in the alkaline extracted sample with 1 M Tris-HCl (pH 7.5). An excess of MNT was added to all fractions, and immunoprecipitation or affinity precipitation was performed with protein-A sepharose and appropriate antisera or with S-protein agarose, respectively.

**Trypsin protection**

Radiolabeled cells were homogenized, and the microsomes were purified as described above. Microsomes were resuspended in homogenization buffer, and 10 µg trypsin and/or Triton X-100 was added to a final concentration of 0.1%. After incubation for 15 min at 27 °C, the reaction was quenched with 5 µg soybean trypsin inhibitor. Samples were then resuspended in MNT, and affinity purification was performed as described above.

**qRT-PCR**

HEK293T cells were treated with either 5 mM dithiothreitol, 0.5 µM thapsigargin, 5 µg/ml tunicamycin or starved for Cys and Met for 8 hr prior to RNA isolation with RNeasy Mini Kit (Qiagen). One µg of purified RNA was reverse transcribed into cDNA using the AMV Reverse Transcriptase kit (New England Biolabs). Quantitative real time polymerase chain reactions (qRT-PCR) were performed in 20 µL reactions using FastStart universal SYBR Green master (Rox) kit (Roche Diagnostics Corp.) on a Mx3000P real-time PCR machine (Agilent Technologies Inc) according to manufacturer’s instructions. Changes in mRNA levels were calculated using the change in cycle threshold value method with β-actin as the reference gene (Pfaffl, 2001). Statistical analysis of the data was calculated using GraphPad Prism 4.0 (GraphPad Software), significance between treatment groups was determined using one-way ANOVA followed by Tukey multiple comparison tests.

The following primers were used: Beta actin (5’ GCACTCTTCCAGCCTTCC 3’, 5’ TGTCCACGTCACACTTCATG 3’), BiP (5’ GCTGGTTCTATTGCGGCTTTCTC 3’, 5’
Polycistronic plasmids that express a cytoplasmic GFP and a predesigned shRNA were purchased from Qiagen. HEK293T cells were transfected with the shRNA for 24 hr prior to RNA purification and qRT-PCR as described above. To determine transfection efficiency, HEK293T cells were transfected for 24 hr followed by trypsinization and resuspension in DMEM. Resuspended cells were loaded onto a Guava Easy Flow Cytometer (Millipore). Transfected versus non-transfected cells were distinguished based on fluorescence from the cytoplasmic GFP. A total of 5,000 events were counted per measurement.

**In gel digestion and LC-MS/MS**

Transfected cells were homogenized and microsomes were purified as described above. Microsomes were resuspended in mass spectrometry buffer (50 mM Hepes pH 7.4, 300 mM NaCl, 25 mM EDTA, 1% Triton X-100) followed by affinity purification with S-protein agarose beads. Beads were washed twice in MS buffer and twice with 50 mM ammonium bicarbonate followed by resuspension in reducing sample buffer. Samples were loaded onto a SDS-PAGE and allowed to run approximately 1 cm into a gel before trypsin digestion. Tryptic peptides were lyophilized, resuspended and loaded onto a custom column that eluted into a Proxeon Easy nanoLC system directly coupled to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific). The Orbitrap was set to achieve full scans from 350-2000 m/z with a resolution of 6000. The LTQ ion trap instrument performed ten scans. Data was analyzed with either Mascot Distiller (Matrixsciences, Ltd.) or Extract_MSN (Thermo Scientific). The Mascot Search engine (Matrixsciences, Ltd.) and SwissProt database were used for protein identification. Trypsin
digestion and subsequent steps were performed by the Proteomics and Mass Spectrometry Facility, University of Massachusetts Medical School for mass spectrometric analysis.

**Immunoblotting and affinity purification**

Transfected cells were suspended in homogenization buffer. Part of the homogenate was centrifuged at 45,000 rpm for 10 min to purify microsomes; the pellet was resuspended in reducing sample buffer and was considered the total membrane fraction. An excess of MNT buffer was added to an equal portion of homogenate and preclearing was performed with control agarose beads, followed by affinity purification with S-protein agarose beads overnight. Beads were washed twice in MNT buffer. Reducing sample buffer was added, and samples were loaded onto a SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane and immunoblotted with the appropriate antisera. Blots were developed and TIFF files were acquired by a G:Box (New England BioGroup), densitometric quantification of western blots were performed using MultiGauge software (Fuji film). The TMTC2 and calnexin interaction was calculated by dividing the amount of calnexin in the S-tag AP with the amount of calnexin in the total membranes fraction, with or without DNJ. The interaction with TMTC2 and calnexin in absence of DNJ was then set to 100%. Error bars represent the standard deviation for three independent experiments.

**Sucrose gradient centrifugation**

Transfected cells were lysed in MNT, and the post-nuclear supernatant was layered on top of a continuous 10-40% sucrose gradient with a 60% cushion in MNT buffer. The gradients were centrifuged for 16 hr at 4 °C with a Beckman SW41 at 145,000g. Standards used to determine sedimentation velocities were bovine serum albumin (4.6S, 66 kD), beta-amylase (8.9S, 200 kD) and bovine thyroglobulin (19S, 669 kD). After centrifugation, 9% of total gradient volume was collected per fraction, and proteins were isolated with trichloroacetic acid precipitation. Samples were resuspended in reducing sample buffer and analyzed by
immunoblotting with the appropriate antisera. After the last fraction was collected, the pellet was processed by adding sample buffer directly to the ultracentrifugation tube.

**Live cell calcium measurement**

HEK293T cells were plated on a glass bottom 3.5 cm dish and transfected the following day. Cells were loaded with 2.5 μM Fura2-AM (Molecular Probes, Eugene, OR, USA) and 2.5 μM Pluronic F-127 for 45 min 16 hr post-transfection. Plates were washed with fresh media and incubated for 45 min to allow hydrolysis of the Fura2-AM ester. Plates were then rinsed with fresh media and placed on an inverted microscope with a 20× objective (Nikon Corp., Tokyo, Japan). Fluorescence was measured every 20 sec, and the excitation wavelength was switched between 340 and 380 nm by a filter wheel. After stable baseline fluorescence had been established, carbachol and ATP (final concentration 100 μM for each) were added to media to release intracellular calcium. Light emitted by Fura2 was collected by a cool SNAP ES digital camera, and all obtained data was processed using SimplePCI software. Statistical analysis and area under curve calculations were completed using GraphPad Prism 4.0 software. The area under the curve was calculated by subtracting the baseline for each of the cells prior to the addition of carbachol. Once individual areas under the curve were obtained, the measurements were averaged for all cells in that treatment. The average calculated for the mock was set to 1.00 and the values of the treated cells were calculated relative to this number. Statistical significance was calculated using an unpaired t test. Cells analyzed in all Fura2 experiments were from at least five different plates collected on three separate days. Experiments with ionomycin were carried out in the same manner with the exception of calcium free media being used during imaging.
<table>
<thead>
<tr>
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<td>TMTC1</td>
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<tr>
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<tr>
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Table 2.1 Putative binding partners of TMTC1 and TMTC2

Putative binding partners of TMTC1 and TMTC2 as determined by LC-MS/MS. Proteins are ranked by number of unique peptides identified that originates from the corresponding proteins compared to mock transfected cells.
Figure 2.1 Organization and transcription of TMTC1 and TMTC2 isoforms.

(A) The organization of TMTC1 (TMTC1 isoform 2) and TMTC2 with signal sequences (black boxes), hydrophobic domains (green boxes) and TPR domains (red boxes) as designated. Endogenous and introduced N-linked glycosylation sites are indicated by black branched structures and asterisks, respectively. (B) There are two possible isoforms of TMTC1 designated as TMTC1.1 (TMTC1 isoform 1 (NCB Accession # NP_001180380.1 (http://www.ncbi.nlm.nih.gov/protein/NP_001180380.1)) and TMTC1.2 (TMTC1 isoform 2 (NCB Accession # NP_787057.2 (http://www.ncbi.nlm.nih.gov/protein/NP_787057.2))). The open reading frame is indicated in black while the different 5’-untranslocated regions are designated by brown (TMTC1.1) and green (TMTC1.2) boxes. Different 5’ primers (P1-P4) were designed to amplify specific isoforms using the same 3’ primer (P5). (C) RNA was reverse
transcribed to cDNA followed by PCR with TMTC1 isoform specific primers. P1 and P2 are specific for TMTC1.1 (lanes 1 and 2) while P4 is specific for TMTC1.2 (lane 4). P3 is directed towards both isoforms (lane 3). PCR products were resolved by agarose gel electrophoresis with the nucleotide base pair makers (kbp) indicated to the right.
Figure 2. TMTC1 and TMTC2 are ER localized.

(A) Cellular localization of TMTC1 and TMTC2 was investigated by confocal microscopy. COS7 cells were transfected with TMTC1 or TMTC2 cDNA. Fixed cells were stained with myc, ERp57 (ER) or GM130 (Golgi) antisera. Nuclei were visualized by DAPI staining (blue). Scale bars correspond to 10 µm. (B) HEK293T cells were transfected with S-tagged TMTC1 or TMTC2 as indicated and radiolabeled for 1 hr with [35S]-Cys/Met. Cells and media were collected. TMTC1 and TMTC2 from the media and lysed cells were affinity purified using S-protein agarose. Samples were then subjected to a glycosylation assay with either Endo H (lanes 2, 5, 8 and 11) or PNGase F (lanes 3, 6, 9 and 12) digestion as indicated. Reducing sample buffer was added, and the samples were analyzed by 7.5% SDS-PAGE. (C) Two N-linked glycosylation sites
were introduced into TMTC1 (TMTC1\textsuperscript{N626, 633}) and TMTC2 (TMTC2\textsuperscript{N602, 818}). Added glycan positions are indicated by the asterisks in Figure 1A. Samples were treated and digested as in B. Molecular weight markers are designated in kD to the right for all SDS-PAGE panels.
Figure 2.3 TMTC1 and TMTC2 are upregulated by oxidative stress.

HEK293T cells were treated with regular growth media or media containing 5 mM DTT, 1 μM thapsigargin, 5 μg/mL tunicamycin or media lacking Cys and Met (-Cys/Met) for 8 hr prior to RNA purification. RNA was reverse transcribed to cDNA followed by qRT-PCR with appropriate primers, and changes in gene expression were calculated using beta actin as a reference. Statistical significance between treatment groups was determined using one-way ANOVA followed by Tukey multiple comparison tests, ** and *** indicates a P-value of less than 0.01 and 0.001, respectively. Error bars represent standard deviation from at least three independent experiments.
Figure 2.4 TMTC1 and TMTC2 are ER membrane proteins with their TPR domains facing the ER lumen.

(A) HEK293T cells expressing S-tagged TMTC1 or TMTC2 were radiolabeled for 1 hr followed by homogenization, fractionation and alkaline extraction. The fractions collected were whole cell lysate (WCL), nucleus (N), cytosol (C), total membrane (TM), as well as supernatant (S) and pellet (P) fractions upon alkaline extraction of the TM. Samples were resolved by reducing 7.5% SDS-PAGE. (B) TMTC1-S-tag or TMTC2-S-tag was expressed in HEK293T cells. After radiolabeling for 1 h, cells were homogenized, and microsomes were purified by ultracentrifugation then resuspended in homogenization buffer. Aliquots of the ER microsomes were incubated for 10 min at 27 °C without (lanes 1, 5 and 9) or with 0.1% Triton X-100 and 10 µg trypsin (lanes 4, 8 and 12) as indicated. TMTC1 or TMTC2 were affinity purified with S-protein agarose beads. Samples were resolved on a reducing 9% SDS-PAGE.
Figure 2.5 TMTC1 and TMTC2 interact with SERCA2B.

(A) HEK293T cells were transfected as indicated and homogenized prior to purification of microsomes by ultracentrifugation. TMTC1 or TMTC2 was isolated with its associated factors from microsomes in MS buffer using S-protein agarose affinity precipitation. A portion of the samples was loaded onto a SDS-PAGE and silver stained to verify enrichment of putative binding partners. A separate portion of the affinity-purified sample was run on a short SDS-PAGE before excision of the sample and in gel trypsin digestion. Tryptic peptides were injected into a LC-MS/MS instrument followed by peptide identification. (B) HEK293T cells expressing TMTC1 or TMTC2 were harvested in homogenization buffer. A portion of the cell homogenate was subjected to ultracentrifugation and resuspended in reducing sample buffer. This was considered the total membrane fraction. An excess of MNT was added to an equal amount of cell homogenate and subjected to S-protein agarose affinity precipitation. Proteins were detected by immunoblotting with appropriate antisera directed against the S-tag epitope, SERCA2B, calnexin (CNX), BiP, glucosidase II subunit alpha (Gls II α), calreticulin (CRT) and ERp57. (C) HEK293T cells were treated with N-butyl deoxynojirimycin (DNJ) for 4 hr prior to homogenization where indicated, and samples were processed as in B. Quantification of the TMTC2 and calnexin interaction was calculated as described above, error bars represent standard deviation of three independent experiments. Statistical significance between treatment groups was determined by an unpaired t test, measurements designated (*) have a P value of 0.0233. (D) HEK293T cells were transfected as indicated above. Cells were lysed in MNT, and samples were layered on top of a continuous 10-40 % sucrose gradient in MNT buffer prior to ultracentrifugation. Fractions were collected from the top of the gradient, and proteins were precipitated with trichloroacetic acid. Immunoblotting was then performed with appropriate antisera as described above. Sizing of detected complexes was estimated by comparison to the following standards designated at the bottom of the immunoblots; bovine serum albumin (4.6S, 66 kDa); beta-amylase (8.9S, 200 kDa); and bovine thyroglobulin (19S, 669 kDa).
Figure 2.6 The TPR domains of TMTC1 and TMTC2 are sufficient to interact with SERCA2B.

(A) COS7 cells were transfected with TMTC1$^{TPR}$ and TMTC2$^{TPR}$, and cells were treated and stained as described in Figure 1B. (B) HEK293T cells were transfected with either TMTC1$^{TPR}$ or TMTC2$^{TPR}$, harvested and homogenized. Total membrane fractionation and affinity precipitation were performed as described in Figure 5B. Proteins were detected with appropriate antisera against the S-tag, SERCA2B, calnexin (CNX), BiP, glucosidase II subunit alpha (Gls II α), calreticulin (CRT).
Figure 2.7 Overexpression of TMTC1 and TMTC2 decreased stimulated calcium release.

(A) COS7 cells were transfected with TMTC1\textsubscript{GFP} or TMTC2\textsubscript{GFP}, treated, stained and analyzed as in Figure 2.1B. (B) HEK293T cells transfected with TMTC1\textsubscript{GFP}, TMTC2\textsubscript{GFP} or cotransfected with both were incubated with Fura2-AM followed by incubations in fresh media. Changes in cytoplasmic calcium were determined by measuring the ratio of fluorescence emission after excitation at 340 and 380 nm. Basal calcium levels were recorded to create a stable baseline before calcium release was stimulated with the addition of 100 \(\mu\)M ATP and 100 \(\mu\)M carbachol to the media (see bar). A total of 386, 91, 103 and 106 cells were measured for non-transfected control, TMTC1\textsubscript{GFP}, TMTC2\textsubscript{GFP} and cotransfection of TMTC1\textsubscript{GFP} with TMTC2\textsubscript{GFP}, respectively. Error bars represent standard error of the mean. The area under the curve is relative to the total amount of calcium released from the ER upon stimulation (bar graph). Statistical significance between non-transfected cells and either TMTC1\textsubscript{GFP}, TMTC2\textsubscript{GFP}, or the TMTC1\textsubscript{GFP} TMTC2\textsubscript{GFP} cotransfection was calculated by using an unpaired t-test. Measurements designated (***) had a \(P\) value of <0.0001. Error bars represent standard error of the mean. (C) HEK293 cells were transfected with TMTC1\textsubscript{GFP}, and live cell calcium measurements were performed as in B. Basal calcium levels were recorded before 2 \(\mu\)M ionomycin (see bar) was added to media to release intracellular calcium. All recordings were performed in calcium free media to prevent interference from extracellular calcium. A total of 130 and 78 cells were analyzed for non-transfected control and TMTC1\textsubscript{GFP}, respectively. The area under the curve is relative to the total amount of calcium released from the ER upon stimulation (bar graph). Statistical significance between non-transfected cells and TMTC1\textsubscript{GFP} was calculated using an unpaired t-test, measurements designated (**) have a \(P\) value of 0.0022. Error bars represent standard error of the mean. (D) HEK293 cells were transfected with TMTC2\textsubscript{GFP}, and live cell calcium measurements were performed as in C. A total of 140 and 100 cells were analyzed for non-transfected control and TMTC2\textsubscript{GFP}, respectively. Statistical significance between non-transfected cells and TMTC2\textsubscript{GFP} was calculated by using an unpaired t-test. Measurements designated (**) have a \(P\) value of > 0.0001. Error bars represent standard error of the mean.
Figure 2.8 Knockdown of TMTC1 and TMTC2 increased stimulated calcium release.

(A) HEK293T cells were transfected with polycistronic plasmids that expressed shRNA (directed towards TMTC1 or TMTC2 transcripts) and cytoplasmic GFP. RNA was purified from HEK293T cells, and the RNA was reverse transcribed to cDNA followed by qRT-PCR. Changes in gene expression were measured using beta actin as a reference gene. Fold reduction in TMTC1
or TMTC2 mRNA abundance was compared to mock transfected cells. Four different shRNA and a scrambled negative control were tested for knockdown of TMCT1 and TMTC2. Error bars indicated standard deviation from three independent experiments. HEK293T cells transfected with the different shRNA constructs and GFP were also analyzed and scored as non-transfected or transfected based on GFP fluorescence using flow cytometry. A total of 5,000 cells were counted per experiment and standard deviations were calculated from at least two separate experiments. (B) HEK293T cells were transfected with either TMTC1 shRNA1, TMTC2 shRNA3 or cotransfection with TMTC1 shRNA1 and TMTC2 shRNA3. Live cell calcium measurements were performed as in Figure 7B. Once a stable baseline was obtained, 100 µM ATP and 100 µM carbachol was added to the media (see bar). A total of 297, 137, 115 and 208 cells were measured for non-transfected control, TMTC1 shRNA1, TMTC2 shRNA3 and the cotransfection of TMTC1 and TMTC2 shRNAs, respectively. Error bars represent standard error of the mean. The area under the curve is relative to the total amount of calcium released from the ER upon stimulation. Statistical significance between non-transfected cells and either TMTC1 shRNA1 or TMTC2 shRNA3 was calculated by using an unpaired t test. Measurements designated (****) have a P value of <0.0001. Error bars represent standard error of the mean.
CHAPTER III

TMTC3, TMTC4, TTC13 AND TTC17 ARE ER TPR CONTAINING PROTEINS

Abstract

Although the endoplasmic reticulum (ER) consists of a contiguous membrane and lumen, evidence of compartmentalization into sub-domains within the organelle has emerged. The ER is believed to be dependent on adapter proteins, which can aid in ER compartmentalization by nucleating the formation of protein complexes. Tetratricopeptide repeats (TPR) are adapter motifs that are known to organize proteins into complexes in a wide range of cellular processes. While a large number of TPR containing proteins have been predicted to exist in the human genome, only a few have been localized to the ER. To this end, a bioinformatics screen was conducted to identify novel ER TPR containing proteins as described in Chapter I. Subsequent biochemical and cell biological assays identified TTC13 and TTC17 as ER luminal proteins, whereas TMTC3 and TMTC4 were found to be ER membrane proteins with their TPR domains facing the ER lumen. A shotgun proteomics approach was employed to identify novel binding partners of TTC13, TTC17, TMTC3 and TMTC4. An interaction with TMTC4 and the lectin chaperone calnexin was verified by co-affinity precipitation, though the significance of this interaction is currently unknown. Collectively, this study provided a useful method for identifying novel ER TPR proteins and the approach could be employed to study another protein fold in the ER.

Introduction

The endoplasmic reticulum (ER) extends throughout the cell body and is responsible for a plethora of functions in the ER such as protein folding, trafficking, degradation, lipid synthesis, calcium homeostasis and xenobiotic degradation (Lynes and Simmen, 2011). The ER can be classified as rough ER sheets where ribosomes dock on the ER membrane or smooth ER sheets for regions of the ER membrane lacking ribosomes (Levine and Rabouille, 2005; Voeltz et al.,
2002). Furthermore, the ER can also be shaped into tubules that extend outward and make contacts with other organelles. The shape, size and intracellular distribution of the ER is under constant modification by external cues such as contacts with other organelles and the cytoskeleton. Despite being a continuous organelle, the ER appears to be organized into sub-domains (Lynes and Simmen, 2011). It has been postulated that this organization is maintained by adapter proteins that organize multi-protein complexes to assist in the various functions of the ER in the different sub-domains.

Tetratricopeptide repeats (TPR) are a common motif found in various adapter proteins (D’Andrea and Regan, 2003; Zeytuni and Zarivach, 2012). They are universally found in all organisms and multiple and diverse pathways. These motifs consist of 34 amino acids that fold into two anti-parallel alpha helices. While there is no consensus sequence of the motifs, there tends to be a preference for certain amino acids at defined positions. A search of databases predicts that the human genome encodes for ~170 proteins with TPR motifs (Letunic et al., 2012; UniProt, 2013). Out of these, only ERdj6 and SEL1L have previously been localized to the ER. Since one third of the human genome is predicted to be targeted to the secretory pathway, we hypothesized that there might be additional currently ER resident TPR proteins that contribute to ER organization.

To further our understanding of TPR proteins in the ER and their role in organization and compartmentalization of the ER, a screen was conducted as described in Chapter II. Briefly, an in silico library was acquired from L Regan’s lab (Yale University) (Magliery and Regan, 2005), and the library was expanded by utilizing BLAST database (Altschul et al., 1990). This list generated nine hits, and of these, TMTC1 and TMTC2 were extensively characterized in Chapter II. However, this bioinformatics screen also resulted in the identification of TMTC3, TMTC4, TTC9B, TTC13, TTC17, TTC35 and NASP. This chapter will focus on the characterization of these additional seven proteins. By employing various biochemical and cell biological assays, we
found that TMTC3, TMTC4, TTC13 and TTC17 were targeted to the secretory pathway. While TMTC3 and TMTC4 were found to be integral membrane proteins with their TPR domains facing the ER lumen, TTC13 and TTC17 were found to be ER luminal proteins. Shotgun proteomics implied that TMTC4 interacts with fatty acid synthase, calnexin and p97 (a cytoplasmic AAA ATPase). Co-affinity precipitation studies verified the TMTC4 and calnexin interaction but the remaining interaction remain to be validated.

**Results**

The bioinformatics screen conducted in Chapter II resulted in nine hits (Figure 1.1), while TMTC1 and TMTC2 were characterized in the previous chapter. TMTC3, TMTC4, TTC9B, TTC13, TTC17, TTC35 and Nuclear autoantigenic sperm protein (NASP) were also predicted to be ER targeted (Figure 3.1). NASP was previously shown to be important for histone assembly in the nucleus, and TTC35 had been found to be a cytoplasmic protein localized to the cytoplasmic face of the ER membrane (Batova and O’Rand, 1996; Christianson et al., 2012). Analysis by the ΔG algorithm predicted that TMTC3 and TMTC4 were membrane inserted, whereas TTC9B, TTC13, TTC17, TTC35 and NASP appear to be soluble proteins (Hessa et al., 2011). To test these predictions and to query the cellular localization of TMTC3, TMTC4, TTC9B, TTC13, TTC17, TTC35 and NASP, RNA was harvested from HEK293T cells and converted into cDNA. This library of cDNA was then used as a PCR template to amplify specific open reading frames, which were subcloned into a mammalian expression vector with a C-terminal S-tag.

The cellular localization of TMTC3, TMTC4, TTC9B, TTC13, TTC17, TTC35 and NASP was investigated with confocal microscopy by transfecting Cos7 cells with corresponding a C-terminal S-tag constructs. Cells were co-stained with either an ER (ERp57) or a Golgi (GM130) marker, and the nucleus was visualized by DAPI staining. TMTC3, TMTC4, TTC13 and TTC17 all showed a clear colocalization with the ER marker but not with the Golgi marker.
(Figure 3.2). Conversely, TTC9B and TTC35 appeared cytoplasmic and NASP was primarily found in the nucleolus. The subcellular localization for NASP and TTC35 agrees with previously published data (Christianson et al., 2012; Richardson et al., 2000). TMTC3, TMTC4, TTC13 and TTC17 were identified as ER localized TPR containing proteins by confocal microscopy.

**TMTC3 and TTC17 are upregulated by DTT**

Proteins that reside in the secretory pathway are frequently transcriptionally induced by stress as their functions can help to alleviate the stress (Walter and Ron, 2011). To this end, we measured the changes in transcription during oxidative stress (dithiothreitol, DTT), SERCA2B inhibition (thapsigargin), inhibition of N-linked glycan synthesis (tunicamycin) or starvation of cysteine and methionine for 8 hr. TMTC3, TMTC4, TTC9B, TTC13 and TTC17 had a 2.19, 1.64, 2.77, 2.09 and a 3.10-fold transcriptional activation upon DTT treatment, respectively (Figure 3.3B). Additionally, administration of thapsigargin caused a 2.14-fold increase in TTC13 transcription, whereas tunicamycin caused a 1.99 fold transcriptional increase of TTC17.

Addition of DTT, thapsigargin or tunicamycin caused a 26.1, 12.3 and 11.9 fold induction of BiP transcript levels, which agrees with published data (Hetz, 2012). Additionally, a 0.53 and 0.59 fold reduction of NASP transcript levels were observed upon tunicamycin treatment or starvation of cysteine and methionine. For most genes tested, DTT caused the strongest response in transcription levels, which could imply that these genes are specifically upregulated by oxidative stress. Alternatively, the upregulation of these proteins by thapsigargin or tunicamycin could occur on a different time frame than induction by DTT. The observation that TTC9B transcription is upregulated by oxidative stress is interesting since this protein is not targeted to the secretory pathway, thus TTC9B might be important for ER stress signaling in the cytoplasm.
TMTC3, TMTC4 and TTC17 have high mannose N-linked glycans, indicative of ER resident proteins

N-linked glycans are large hydrophilic sugar structures that are added co- or post-translationally to most secretory proteins upon ER targeting (Hebert et al., 2005). N-linked glycans are added to proteins with the consensus sequence N-X-S/T, where X is any amino acid except proline. However, recent studies have highlighted that there is a propensity for certain amino acids before and after the consensus sequence, which can affect how well a glycosylation site is recognized (Zielinska et al., 2012; Zielinska et al., 2010). To corroborate the ER localization implied by the confocal microscopy data, a glycosylation assay was employed. This assay depends on the observation that addition of a N-linked glycan to a protein increases its molecular weight by approximately 2.5 kD, which can be visualized by a slower migration by SDS-PAGE. Furthermore, these N-linked glycans can be removed by either Endoglycosidase H (Endo H) and/or Peptide N-Glycosidase F (PNGase F), enzymes that trim carbohydrates from polypeptides. Endo H can only trim the high mannose glycans encountered in the ER whereas PNGase F can also trim the complex glycans acquired in the Golgi. A gel shift upon PNGase F treatment implies that a protein was N-linked glycosylated, and a gel shift by Endo H treatment implies that the protein had high mannose glycan indicative of ER residency.

HEK293T cells were transfected with TMTC3, TMTC4, TTC13 or TTC17, and proteins were radiolabeled for 1 hr with [35S]-Cys/Met followed by cell lysis and affinity purification with S-protein agarose beads. While TMTC3, TMTC4 and TTC17 appeared to be glycosylated, TTC13 did not (Figure 3.4). TMTC3 has five consensus sites for N-linked glycans, though not all appeared to be recognized as judged by the relatively small shift on the glycosylation assay. The first two glycosylation sites are positioned in the hydrophobic N-terminus of TMTC3 and might be either too close to the ER membrane to be recognized by oligosaccharyl transferase (OST). Alternatively, the glycosylation sequences could face the cytoplasm (Nilsson and von Heijne,
1993). Furthermore, of the three glycosylation sequences in the TPR domains, only the third glycan was predicted to be recognized by the NetNGly algorithm (http://www.cbs.dtu.dk/services/NetNGlyc/), which predicts likelihood of a consensus site being modified. TMTC4, compared to TMTC3, gave a larger shift on a SDS-PAGE and was likely to possess multiple glycans, although only the first glycan was predicted to be recognized by the NetNGly algorithm. The relatively large shift for TTC17 implied that many of the twelve potential glycosylation sites were modified. Interestingly, NetNGly predicted that only seven would be efficiently recognized. The glycans encountered on TMTC3, TMTC4 and TTC17 were all EndoH sensitive, which suggested that these proteins are ER resident proteins. TTC13 has only a single potential glycosylation site, which did not appear to be recognized. While the NetNGly algorithm predicted that TTC13 is glycosolated, the size of TTC13 could make it difficult to detect a 2.5 kD shift in mobility on a SDS-PAGE.

To test if any of the proteins were secreted, the media fraction was collected after 1 hr of radiolabeling with $[^35\text{S}]-\text{Cys/Met}$, followed by S-protein agarose affinity purification and the glycosylation assay. Neither TMTC3, TMTC4, TTC13 nor TTC17 were secreted into the media, which is consistent with an ER localization (Figure 3.4A). Additionally, the glycosylation data for TMTC3, TMTC4 and TTC17 agrees with the microscopy data, further indicating that these proteins are ER resident proteins. While no glycosylation was observed for TTC13, the observation that no protein was detected in the media and the microscopy supports ER residency.

**TMTC3 and TMTC4 are ER membrane proteins, whereas TTC13 and TTC17 ER luminal**

Analysis of the TMTC3, TMTC4, TTC13 and TTC17 protein sequences with $\Delta G$ prediction algorithm suggested that TMTC3 and TMTC4 contained eight and eleven hydrophobic domains, respectively. In contrast, TTC13 and TTC17 were not predicted to have hydrophobic domains (Figure 3.1). Since these hydrophobic domains could serve as transmembrane domains, an alkaline extraction assay was employed to determine if TMTC3, TMTC4, TTC9B, TTC13,
TTC17 and/or NASP were integral membrane proteins. HEK293T cells were transfected with a C-terminally S-tagged TMTC3, TMTC4, TTC9B TTC13, TTC17 and NASP and radiolabeled for 1 hr with $[^{35}\text{S}]$-Cys/Met followed by lysis in an isotonic buffer and separation using a series of centrifugation steps.

Both TMTC3 and TMTC4 were found in the nuclear, total membrane and the pellet fractions, which is the same distribution as encountered for calnexin, an ER membrane anchored lectin chaperone. The occurrence of TMTC3 and TMTC4 in the nucleolus is likely due to the fact that the ER membrane is continuous with the nuclear envelope. TTC13 and TTC17 were encountered in the nuclear, total membranes and primarily in the soluble fraction with some protein in the pellet fraction. This enrichment of TTC13 and TTC17 in the soluble fraction implies that these proteins are luminal proteins, as observed for calreticulin, the soluble paralogue of calnexin. The presence of TTC13, TTC17 and calreticulin in the pellet fraction is likely an artifact of the alkaline extraction. In this protocol, proteins are incubated with 0.1 M sodium carbonate (pH 11.5) for 30 min on ice. During this treatment, a portion of the proteins might aggregate due to the harsh buffer conditions, and these aggregated proteins would then be pelleted along side membrane proteins during the last centrifugation step. In accordance with the microscopy data, TTC9B localized primarily to the nuclear and the cytoplasm fractions whereas NASP localized primarily to the cytosolic and nuclear fraction. Collectively these data indicate that TMTC3 and TMTC4 are integral membrane ER proteins, whereas TTC13 and TTC17 are soluble ER proteins.

The TPR domains of TMTC3, TMTC4, TTC13 and TTC17 are in the ER lumen

To further support the ER localization of TTC13 and TTC17 and to address the topology of the TPR domains of TMTC3 and TMTC4, a trypsin protection assay was employed. HEK293T cells were transfected with TMTC3, TMTC4, TTC13 or TTC17 harboring a C-terminal S-tag and radiolabeled for 1 hr with $[^{35}\text{S}]$-Cys/Met. Cells were lysed in an isotonic buffer and microsomes
were purified by ultracentrifugation followed by re-suspension in homogenization buffer and incubation with trypsin and Triton X-100 as indicated. After trypsin digestion, trypsin was inhibited by an excess of soybean trypsin inhibitor, and affinity purification was performed using the C-terminal S-tag. If the S-tagged, and TPR rich, C-terminus was localized to the ER lumen, it would be protected from trypsin. Conversely, if the C-terminal S-tag was facing the cytoplasm, it would be readily available to trypsin. After trypsin inhibition, affinity purification using S-agarose beads allowed for the isolation of proteins containing a protected C-terminus.

A smaller, protected fragment of TMTC3 was observed on a SDS-PAGE after the trypsin assay. However only a portion of TMTC3 was digested by trypsin, implying that the TPR domains of TMTC3 are facing the ER lumen and that the cytoplasmic portion of TMTC3 is partially sensitive to trypsin digestion (Figure 3.5). TMTC4 remained completely protected upon trypsin treatment, implying that the TPR domains are facing the ER lumen. Both TTC13 and TTC17 were completely protected from trypsin digestion, suggesting that they are soluble proteins located to the ER lumen. Collectively, these data imply that the TPR domains of TMTC3, TMTC4, TTC13 and TTC17 are all facing the ER lumen.

**LC-MS MS to identify binding partners of TMTC1 and TMTC2**

Since TMTC3, TMTC4, TTC13 and TTC17 all are ER proteins with their TPR domains facing the ER lumen, the next step was to identify potential binding partners of these putative ER adapters. HEK293T cells were transfected with TMTC3, TMTC4, TTC13 or TTC17 harboring a C-terminal S-tag, cells were lysed in an isotonic buffer, and ER microsomes were purified by ultracentrifugation. The microsomes were then resuspended in affinity purification buffer, and affinity purification was performed with S-protein agarose beads. A portion of the affinity-purified protein was then run on a SDS-PAGE followed by silver staining to verify enrichment of putative binding partners compared to mock transfected cells (Figure 3.6A).
While TTC13 and TTC17 expression was high and the proteins were clearly visible on a silver stained SDS-PAGE, no clear enrichment of binding partners was observed when the affinity purification was performed in 1% Triton X-100, a relatively harsh detergent. The experiment was repeated, but the AP was performed in 1% CHAPS, despite CHAPS being a milder detergent, no clear enrichment of putative binding partners were observed upon silver staining of a SDS-PAGE. To circumvent this problem, we plan to repeat the AP with either a gentler detergent such as digitonin or a chemical crosslinker to covalently trap the complex. Dithiobis(succinimidyl propionate) (DPS) is a lysine specific crosslinker with a 12Å length, which has the advantage of having an internal disulfide that can reduced by DTT. Thus the crosslinker can be released on a reducing SDS-PAGE and enrichment of putative binding partners can be visualized by silver staining.

TMTC3 had a relatively low expression in the large scale AP, and only a weak enrichment was observed in Triton X-100 lysis (Figure 3.6). Thus the TMTC3 AP might need to be repeated with either a gentler detergent or a covalent crosslinker. The identification of putative binding partners of TMTC3, TTC13 and TTC17 is an active area of research in our laboratory. Conversely, expression and purification of TMTC4 in a 1% Triton X-100 buffer resulted in enrichment of a number of bands on a silver stained gel that could represent putative binding partners of TMTC4. To identify the proteins, a portion of the large AP was loaded onto a SDS-PAGE and the dye front was allowed to run – 1 cm into the resolving gel. The gel segment above the dye front was then excised and processed for in gel trypsin digestion, followed by analysis by liquid chromatography–mass spectrometry/mass spectrometry (LC-MC/MS) to identify the tryptic peptides. This LC-MS/MS indentified a large number of putative binding partners of TMTC4 (Table 3.1). Potential binding partners were scored based on number of unique peptides that were identified from the corresponding protein. The top hits included BiP (the ER Hsp70),
p97 (a cytoplasmic AAA ATPase associated with diverse cellular activities, also called VCP),
calnexin (a membrane bound lectin chaperone) and fatty acid synthase.

**TMTC4 interacts with calnexin**

To verify the binding partners of TMTC4 as implied by the LC-MS/MS, HEK293T cells
were transfected with TMTC4 harboring a C-terminal S-tag and lysed in an isotonic buffer. A
portion of the lysate was subjected to affinity purification with S-tag agarose beads while another
fraction was subjected to ultracentrifugation to obtain the total membranes fraction.
Immunoblotting against the S-tag or endogenous putative binding partners indicated that TMTC4
interacted with calnexin but not with BiP or calreticulin (Figure 3.6). Since TMTC4 has multiple
N-linked glycans, the interaction between TMTC4 and calnexin could be because calnexin is a
lectin chaperone that is required for proper folding of TMTC4. Alternatively, TMTC4 could form
a complex with protein(s) that have N-linked glycans, and the TMTC4 calnexin binding could be
mediated through a ternary complex. No interaction between TMTC4 and BiP was observed,
likely reflecting that BiP has a propensity to nonspecifically bind agarose and sepharose beads
and frequently appears as a false positive in LC-MS/MS analysis of ER proteins. We are actively
pursuing the validity of the interactions between p97, clathrin heavy chain 1 and fatty acid
synthase with TMTC4.

**Discussion**

The ER sheets and tubules form a continuous network that extends throughout the cell
(Voeltz et al., 2002). Despite the continuous nature of the ER, a higher level of organization and
 compartmen talization into sub-domains has been observed (Lynes and Simmen, 2011). It has
been proposed that this organization is in part created by adapter proteins that nucleate multi
protein complexes. This study explored the possibility that there might be additional adapter
proteins in the ER, some of which rely upon TPR domains. TMTC3, TMTC4, TTC13 and TTC17
were localized to the ER as demonstrated by microscopy, cell fractionation, and glycosylation assays.

TMTC3, TMTC4, TTC9B, TTC13 and TTC17 were transcriptionally upregulated by oxidative stress (Figure 3.3A). Additionally, TTC13 and TTC17 were transcriptionally upregulated by inhibition of SERCA2B or of glycan synthesis, respectively. While the statistical analysis implies that the observed transcriptional induction was statistically significant, the actually fold induction was relatively low. This could indicate that TMTC3, TMTC4, TTC9B, TTC13 and TTC17 only play a minor or secondary role in restoring ER homeostasis after stress. Additionally, during the course of this study a manuscript was published that implied that silencing of TMTC3 sensitized HeLa cells to ER stress, however TMTC3 was not upregulated by ER stress in our study (Racape et al., 2011). The transcriptional upregulation of TTC9B is interesting since this suggests that TTC9B somehow is linked to the ER stress signaling in the cytoplasm. Why oxidative ER stress commonly resulted in a larger response then other treatment is not clear. It could suggest that these proteins are sensitive to oxidative damage and their misfolding causes transcriptional upregulation as a compensatory mechanism. Alternatively, the different stress conditions tested could be active on different time scales and the 8 hr treatment used in this study failed to account for this. However, this is unlikely since a significant induction of BiP was observed in DTT, thapsigargin and tunicamycin treatment, implying a robust activation of the unfolded protein response.

The number of sequential TPR motifs in a cluster of TPR motifs can give clues as to the nature of the ligand. Smaller clusters tend to interact with more specific ligands whereas larger clusters tend to be more promiscuous in their binding partner (D'Andrea and Regan, 2003; Zeytuni and Zarivach, 2012). In the case of Hop, Pex5 and TOM70, clusters of 3 TPR motifs recognize a peptide of three or four amino acids located at the extreme C-termini of their substrate (Chen and Smith, 1998a; Gatto et al., 2000; Young et al., 2003). Analogous to this,
several ER proteins, including BiP and GRP94, possess C-terminal KDEL sequences, which are utilized for ER retention. Since TTC17 has two separate clusters of three TPR domains, it is conceivable that these domains are designed to interact with a well-defined ER resident ligand, such as the KDEL. To address this, the different clusters of TPR domains as well as the wild type TTC17 could be expressed in HEK293T cells and binding to BiP and GRP94 could be addressed by co-affinity purification. Furthermore, expressing individual clusters of TPR motifs from TTC17 might enhance our chances of identifying specific binding partners of TTC17 in the LC-MS/MS experiments as these individual domains could act like dominant negatives. The same approach of only expressing the clusters of TPR motifs from TMTC3 and TTC13 could enhance our detection of putative binding partners by LC-MS/MS.

While the sequential nature of the TPR domains are important, the exact number of TPR motifs in a protein can be hard to predict due to discrepancies between available databases and algorithms. While UniprotKB predicts that TTC9B has three TPR domains whereas TPRpred, another algorithm that predicts TPR domains, predicts that a gap between the first and second TPR domains of TTC9B is also a TPR domain (Figure 3.1) (Karpenahalli et al., 2007; UniProt, 2013). Similarly, TTC17 has two clusters of three TPR domains that likely form two individual binding modules while the first orphan TPR motif is most likely not functional, it might not be a bona fide TPR motif. Additionally, TTC17 is also a large protein consisting of 1141 amino acids and only 238 of these are accounted for in the seven TPR motifs. Thus it is likely that the segments of TTC17 that are not TPR motifs would fold into another specific fold. To this end, the primary amino acid sequence of TTC17 was analyzed by a variety of algorithms that predicts secondary structure and protein domains. However, analysis by UniprotKB, SuperFam and the BioSmart 7 algorithms did not detected any other domains in TTC17 (Karpenahalli et al., 2007; Letunic et al., 2012; UniProt, 2013).
In addition to their TPR domains, TMTC3 and TMTC4 have eight and nine hydrophobic domains, respectively, some of which are used as transmembrane domains (Figure 3.1) (Hessa et al., 2011). Due to the number of the transmembrane domains and the presence of charged and hydrophilic residues in some of these putative transmembrane domains, TMTC3 and/or TMTC4 could form a pore in the ER membrane. Additionally, proteins with TPR domains are frequently found in protein complexes designed for post-translational translocation, such as Tom70 (mitochondria), Toc64 (chloroplast), PEX5 (peroxisome) and Sec72p (yeast ER) (Feldheim and Schekman, 1994; Gatto et al., 2000; Qbadou et al., 2006; Young et al., 2003). In this light, the interaction between TMTC4 and p97 is of great interest since p97 is a cytoplasmic AAA ATPase associated with pulling misfolded secretory proteins out of the ER membrane and into the cytoplasm for ubiquitination and degradation by the 26S proteasome (Smith et al., 2011). If an interaction between TMTC4 and p97 can be verified, it could imply that TMTC4 is important to form a pore for misfolded proteins in the ER membrane. While a number of proteins have been proposed to function as the conduit for misfolded proteins to pass through the ER membrane and into the cytoplasm, the matter is still controversial (Carvalho et al., 2010; Lilley and Ploegh, 2004; Plemper et al., 1997; Ploegh, 2007; Ye et al., 2004). Since posttranslational translocation is a complicated processes, nucleation of a multi-protein complex is likely required, thus TMTC4 is an ideal candidate to form such a pore with its multiple hydrophobic domains and TPR motifs. While this is a highly attractive hypothesis, it is possible that TMTC4 readily misfolds and becomes targeted for degradation and subsequent extraction by p97. Nevertheless, we have characterized four novel ER adapters, TMTC3, TMTC4, TTC13 and the TTC17, which might aid in the compartmentalization of the ER into subdomains.

**Materials and methods**

**Reagents and plasmids**
Dulbecco’s modified Eagle medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, and Zysorbin were purchased from Invitrogen. 1-deoxynojirimycin (DNJ) and Easy-Tag \textsuperscript{35}S-Cys/Met were purchased from Toronto Research Chemicals and PerkinElmer, respectively. S-protein agarose beads and S-tag antibody were purchased from EMD Millipore. EndoH, PNGase F, AMV first strand synthesis kit, and all cloning reagents were purchased from New England Biolabs. FastStart Sybr Green qPCR mix was purchased from Roche, and all primers were acquired from IDT DNA. Anti-mouse-HRP IgG, anti-rabbit-HRP IgG, and protein A Sepharose CL-4B were purchased from GE Healthcare. Antibodies directed towards the following antigens were also purchased as follows: myc (Cell Signaling); calnexin (Enzo Life Sciences); calreticulin (Thermo scientific); and GM130 (BD Biosciences). TMTC3, TMTC4, TTC9B, TTC13, TTC17, TTC35 and NASP were open reading frames were acquired by harvesting total RNA from HEK293T cells followed by reverse transcriptase to form cDNA. The cDNA was then PCR amplified and cloned into a pcDNA3.1 A-, a plasmid harboring either a C-terminal S- or myc- tag, using standard molecular biology techniques. The individual clusters of TPR domains of TMTC3, TMTC4, TTC13 and TTC17 were cloned into a pcDNA3.1 A- S-tag backbone, which was modified to possess a N terminal BiP signal sequence prior to the multiple cloning site and a KDEL sequence after the C-terminal S-tag. All other chemicals were obtained from Sigma-Aldrich.

**Tissue culture**

Human embryonic kidney (HEK) 293T or Cos7 cells were grown in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin, and incubated at 37 °C in 5% CO\textsubscript{2}. Cells were transfected with polyethylenimine (PEI) and the appropriate plasmids for 16 hr.
Confocal microscopy

Cos7 cells were transfected with TMTC3, TMTC4, TTC9B TTC13, TTC17, TTC35 and NASP harboring a C-terminal S-tag as indicated. Cells were fixed in 4% paraformaldehyde in PBS for 10 min followed by permeabilization for 10 min in methanol at -20 °C. Slides were rehydrated in immunostaining buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 2 mM MgCl₂, 1 mM EGTA, and 2% bovine serum albumin) and stained with ERp57, GM130 or S-tag antisera in immunostaining buffer. Slides were rinsed and mounted onto cover slips with VectaShield (Vector Laboratories), which contained DAPI for staining of the nucleus. Images were obtained with a Flouview 1000 MPE, IX81 motorized inverted research microscope (Olympus Inc.) equipped with a Hamamatsu C8484-05G camera. All images were acquired with a Plan Apo N 60x 1.42NA lens and processed by using the FV10-ASW and the Adobe Photoshop software.

qRT-PCR

HEK293T cells were treated with 5 mM dithiothreitol, 0.5 µM thapsigargin, 5 µg/ml tunicamycin or starved for Cys and Met for 8 hs prior to RNA isolation with RNeasy Mini Kit (Qiagen). One µg of purified RNA was reverse transcribed into cDNA using the AMV Reverse Transcriptase kit (New England Biolabs). Quantitative real time polymerase chain reactions (qRT-PCR) were performed in 20 µL reactions using FastStart universal SYBR Green master (Rox) kit (Roche Diagnostics Corp.) on a Mx3000P real-time PCR machine (Agilent Technologies Inc) according to manufacturer’s instructions. Changes in mRNA levels were calculated using the change in cycle threshold value method with β-actin as the reference gene (Pfaffl, 2001). Statistical analysis of the data was calculated using GraphPad Prism 4.0 (GraphPad Software). The following primers were used: Beta actin (5’ GCACCTCTCCAGCCCTTCC 3’, 5’ TGTCCACGTCACACTTCATG 3’), BiP (5’ GCTGTGTCTATAGGCCTTCTC 3’, 5’ TGTCTCCTTCACAGCATCG 3’), XBP1 (5’ GCCCTGGTTGCTGAAGAG 3’, 5’ GTCAATACCGCCAGAATCC 3’), TMTC3 (5’ TTTTCTAAGCCATCCCCCTG 3’, 5’
Glycosylation assay

HEK293T cells were transfected as indicated for 16 hr followed by radiolabeling of proteins with $[^{35}S]$-Cys/Met was performed for 1 hr. Cells were lysed in MNT buffer (0.5% Triton X-100, 20 mM MES, 100 mM NaCl, 20 mM Tris-HCl [pH 7.5]). All steps were conducted at 4°C. The post-nuclear supernatant (PNS) was isolated by centrifugation followed by pre-clearing with Zysorbin for 1 hr. Cleared supernatant was incubated with S-protein agarose beads overnight and subsequently washed twice with MNT buffer. After the final MNT wash, glycosylation assays were performed by adding appropriate buffers and either no treatment, Endo H or PNGase F enzymes according to the manufacturer’s protocol. Finally, reducing sample buffer was added to all samples, and they were analyzed by SDS-PAGE.

Alkaline extraction

HEK293T cells were transfected as indicated for 16 hr followed by radiolabelling for 1 hr with $[^{35}S]$-Cys/Met. Cells were fractionated in an isotonic buffer and an untreated sample was retained (Whole cell lysate, WCL). Cell lysate is centrifuged at low speed to pellet nuclei (N). The remaining post nuclear supernatant (PNS) is ultracentrifuged to separate cytosol (C) from the total membrane fraction (TM). Alkaline extraction was performed on part of the TM fraction by incubation with Na$_2$CO$_3$ pH 11.5 for 30 min at 4°C. Finally, an ultracentrifugation step was
employed to separate luminal proteins in the supernatant (S) from integral membrane proteins in
the pellet (P). The pH was corrected in the alkaline extraction tube to ~ pH 7 by addition of HCl.
MNT was added in excess to all samples and affinity purification (AP) or immunoprecipitation
(IP) was performed as indicated.

**Trypsin protection assay**

HEK293T cells were transfected as indicated for 16 hr followed by radiolabeling with
$[^{35}S]$-Cys/Met. ER microsomes were purified by ultracentrifugation and resuspended in
homogenization buffer. Aliquots of the ER microsomes were treated with Triton X-100 and
Trypsin for 10 min at 27 °C as indicated. Trypsin was inhibited with soybean trypsin inhibitor
followed by an AP with S-protein agarose beads as described above and analysis on a SDS-
PAGE.

**Shotgun LC-MS/MS and silver stain**

HEK293T cells were transfected as indicated by isotonic fractionation and
ultracentrifugation to purify microsomes. These microsomes were then resuspended in a MS (50
mM Hepes pH 7.4, 300 mM NaCl, 25 mM EDTA) buffer supplemented with either 1% Triton X-
100 or 1% CHAPS detergent followed by AP with S-protein agarose beads. APs were washed
twice in MS buffer and twice in 50 mM ammonium carbonate before addition of reducing sample
buffer. A portion of the sample was loaded onto a SDS-PAGE and subject to silver staining to
verify enrichment of putative binding partners. Once appropriate enrichment had been observed, a
portion of the AP was loaded onto a SDS-PAGE gel, and the dye front was allowed to migrate ~
1 cm into the resolving gel. The sample containing area of the gel was excised and prepared for
in-gel trypsin digestion. Tryptic peptides generated by trypsinization were loaded on a LC-
MS/MS instrument followed by protein identification. Proteins were scored based on the number
of unique peptides that were identified from the corresponding protein.
Immunoblotting and affinity purification

HEK293T cells were transfected with TMTC3-S-tag or TMTC4-S-tag, and 16 hr post transfection cells were harvested. Total membranes were purified as described previously followed by either resuspension in gel loading buffer or affinity purification with S-protein agarose beads. Immunoblots were performed with antisera against indicated proteins.
<table>
<thead>
<tr>
<th>Name</th>
<th># of unique peptides</th>
</tr>
</thead>
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<tr>
<td>Fatty acid synthase</td>
<td>Mock: 2, TMTC3: 28, TMTC4: 34</td>
</tr>
<tr>
<td>DNA-dependent protein kinase catalytic subunit</td>
<td>Mock: 5, TMTC3: 21, TMTC4: 31</td>
</tr>
<tr>
<td>BiP</td>
<td>Mock: 0, TMTC3: 3, TMTC4: 26</td>
</tr>
<tr>
<td>HSPA8</td>
<td>Mock: 0, TMTC3: 2, TMTC4: 26</td>
</tr>
<tr>
<td>P97</td>
<td>Mock: 0, TMTC3: 1, TMTC4: 21</td>
</tr>
<tr>
<td>Calnexin</td>
<td>Mock: 0, TMTC3: 0, TMTC4: 20</td>
</tr>
<tr>
<td>Heat shock protein HSP 90-beta</td>
<td>Mock: 1, TMTC3: 6, TMTC4: 19</td>
</tr>
<tr>
<td>Heat shock 70 kDa protein 1A/1B</td>
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<tr>
<td>Leucine-rich PPR motif-containing protein, mitochondrial</td>
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</tr>
<tr>
<td>Tubulin beta chain</td>
<td>Mock: 3, TMTC3: 7, TMTC4: 16</td>
</tr>
<tr>
<td>Transcription intermediary factor 1-beta</td>
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</tr>
<tr>
<td>Tubulin alpha-1B chain</td>
<td>Mock: 5, TMTC3: 5, TMTC4: 14</td>
</tr>
<tr>
<td>Beta-actin-like protein 2</td>
<td>Mock: 9, TMTC3: 9, TMTC4: 13</td>
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<tr>
<td>Clathrin heavy chain 1</td>
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<tr>
<td>Pyruvate kinase isozymes M1/M2</td>
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<td>Importin</td>
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<tr>
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<td>Probable ATP-dependent RNA helicase DDX5</td>
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<tr>
<td>SERCA2</td>
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Table 3.1 Putative binding partners of TMTC3 and TMTC4.

Putative binding partners of TMTC1 and TMTC2 as determined by LC-MS/MS. Proteins are ranked by number of unique peptides identified that originates from the corresponding proteins compared to mock transfected cells.
Figure 3.1 Putative ER adapter proteins.

Our screen for novel secretory adapter proteins identified nine proteins. The number and position of tetratricopeptide repeat (TPR) (red rectangles), hydrophobic domains (green squares), N-linked glycans (black tree-like structures), proline rich domains (blue squares) and histone binding domains (brown rectangles) are color-coded.
Figure 3.1 TMTC3, TMTC4, TTC13 and TTC17 co-localize with an ER marker

Cellular localization of TMTC3, TMTC4, TTC9B, TTC13, TTC17 TTC35 and NASP was investigated by confocal microscopy. Cos7 cells were transfected as indicated with TMTC3, TMTC4, TTC9B, TTC13, TTC17 TTC35 and NASP harboring a C-terminal S-tag. Fixed cells were stained with S-tag, ERp57 (ER) or GM130 (Golgi) antisera. Nuclei were visualized by DAPI staining (blue). Scale bars represent 10 µm.
Figure 3.3 TMTC4, TTC9B, TTC13 and TTC17 are upregulated by oxidative stress.

HEK293T cells were treated with regular growth media or media containing 5 mM DTT, 1 μM thapsigargin, 5 μg/mL tunicamycin or lacking Cys and Met (-Cys/Met) for 8 hr prior to RNA purification. RNA was reverse transcribed to cDNA followed by qRT-PCR with appropriate primers, and changes in gene expression were calculated using beta actin as a reference. Statistical significance between treatment groups was determined using one-way ANOVA followed by Tukey multiple comparison tests. *, ** and *** indicates a P-value of less than 0.05, 0.01 and 0.001, respectively. Error bars represent standard deviation from at least three independent experiments.
Figure 3.4. TMTC3 and TMTC4 are ER membrane proteins whereas TTC13 and TTC17 are soluble.

(A) HEK293T cells were transfected with S-tagged TMTC3, TMTC4, TTC9B, TTC13, TTC17, TTC35, or NASP as indicated and radiolabeled for 1 hr with \[^{35}S\]-Cys/Met. Cells and media were collected. TMTC3, TMTC4, TTC9B, TTC13, TTC17, TTC35, or NASP collected from the media and lysed cells were affinity purified using S-protein agarose. Samples were then subjected to a glycosylation assay with either Endo H (lanes 2, 5, 8 and 11) or PNGase F (lanes 3, 6, 9 and 12) digestion as indicated. Reducing sample buffer was added, and the samples were analyzed by 7.5\% SDS-PAGE. (B) HEK293T cells expressing S-tagged TMTC3, TMTC4, TTC9B, TTC13, TTC17, TTC35, or NASP were radiolabeled for 1 hr followed by homogenization, fractionation and alkaline extraction. The fractions collected were whole cell lysate (WCL), nucleus (N), cytosol (C), total membrane (TM), as well as supernatant (S) and pellet (P) fractions upon alkaline extraction of the TM. Samples were resolved by reducing 7.5\% SDS-PAGE.
Figure 3.5. The TPR domains of TMTC3, TMTC4, TTC13 and TTC17 are facing the ER lumen.

TMTC3-S-tag, TMTC4-S-tag, TTC13-S-tag or TTC17-S-tag was expressed in HEK293T cells. After radiolabeling for 1 hr, cells were homogenized, and microsomes were purified by ultracentrifugation then resuspended in homogenization buffer. Aliquots of the ER microsomes were incubated for 10 min at 27 °C without (lanes 1, 5 and 9) or with 0.1% Triton X-100 and 10 µg trypsin (lanes 4, 8 and 12) as indicated. TMTC3, TMTC4, TTC13, and TTC17 were affinity purified with S-protein agarose beads. Samples were resolved on a reducing 9% SDS-PAGE.
Figure 3.6. TMTC4 interacts with the lectin chaperone calnexin.

(A) HEK293T cells were transfected as indicated and homogenized prior to purification of microsomes by ultracentrifugation. TMTC3 or TMTC4 was isolated with its associated factors from microsomes in MS buffer using S-protein agarose affinity precipitation. A portion of the samples was loaded onto a SDS-PAGE and silver stained to verify enrichment of putative binding partners. A separate portion of the affinity-purified sample was run on a short SDS-PAGE before excision of the sample and in gel trypsin digestion. Tryptic peptides were injected into a LC-MS/MS instrument followed by peptide identification. (B) HEK293T cells expressing TMTC3 or TMTC4 were harvested in homogenization buffer. A portion of the cell homogenate was subjected to ultracentrifugation and resuspended in reducing sample buffer. This was considered the total membrane fraction. An excess of MNT was added to an equal amount of cell homogenate and subjected to S-protein agarose affinity precipitation. Proteins were detected by immunoblotting with appropriate antisera directed against the S-tag epitope, calnexin (CNX), BiP and calreticulin (CRT).
CONCLUSIONS AND FUTURE DIRECTIONS

The endoplasmic reticulum (ER) consists of a continuous network of ER sheets and tubules that extend throughout the mammalian cell. Although continuous, a compartmentalization of the ER into sub-domains has been observed (Lynes and Simmen, 2011). It has been proposed that these ER sub-domains are maintained by adapter proteins that nucleate protein complexes. Tetratricopeptide repeats (TPR) are common adapter motifs, which are capable of nucleating multi-protein complexes (D'Andrea and Regan, 2003). To this end we conducted an *in silico* search for TPR containing proteins that are targeted to the secretory pathway. This search and subsequent experiments lead to the identification of TMTC1, TMTC2, TMTC3, TMTC4, TTC13 and TTC17 as novel ER TPR containing proteins (Figure 1.6).

**TMTC1 and TMTC2 are novel regulators of calcium homeostasis**

TMTC1 and TMTC2 were found to reside in protein complexes of 500-600 kDa in size by the sucrose sedimentation assay (Figure 2.5D). While the exact nature of the TMTC1 and TMTC2 complexes are not known, both the sucrose gradient and the co-affinity purification (AP) imply that SERCA2B is part of the TMTC1 and TMTC2 complexes and that calnexin is part of the TMTC2 complex. (Figure 2.5B and D). Additionally, some TPR proteins have been proposed to use regions of their TPR domains, which are not used for ligand binding, for dimerization (Jinek et al., 2004; Wu and Sha, 2006). Thus, it is possible that the complexes observed contain homoligomers of TMTC1 and TMTC2.

A short-coming of the current study is that we do not have an antibody against TMTC1 and TMTC2, which allows us to study the endogenous proteins. To circumvent this problem we could use the TALON system to create a cell line that stably expresses S-tagged TMTC1 and TMTC2 under their endogenous promoters (Gaj et al., 2013). First, this approach is more likely to
preserve the appropriate stoichiometry of the TMTC1 and TMTC2 complexes. Secondly, it would allow us to verify the interactions between TMTC1 and TMTC2 with SERCA2B, and TMTC2 with calnexin without relying on overexpression. Thirdly, it would allow us to investigate if TMTC1 or TMTC2 are targeted to an ER sub-domain, since the delicate organization of the ER might be distorted by over expressing TMTC1 and TMTC2. Since calnexin has been proposed to modulate SERCA2B activity at the mitochondria associated membranes (MAM) (Lynes et al., 2013) and TMTC2 interacts with calnexin whereas TMTC1 does not, an interesting hypothesis is that TMTC2 resides in the MAMs whereas TMTC1 could be interacting with the SERCA2B in areas beyond these contact points. Taken together, a clearer view of the functions of TMTC1 and TMTC2 would be available with either antibodies against the endogenous protein or with stable cell lines expressing tagged versions of TMTC1 or TMTC2.

While the TPR domains of TMTC1 and TMTC2 were important for binding SERCA2B (Figure 2.6), it is still unclear if this is the only intended ligand for the TPR motifs of TMTC1 and TMTC2. It is likely that some interactions were missed by our mass spectrometry analysis since the AP was performed in 1% Triton X-100. To circumvent this problem, the mass spec analysis could be repeated in a gentler detergent or with a cross linker. Alternatively, we could express the TMTC1^{TPR} and TMTC2^{TPR} constructs and perform a mass spectrometry analysis with these constructs to identify binding partners specifically of the TPR domains.

Live cell calcium measurements illustrated that cells transfected with either TMTC1^{GFP} or TMTC2^{GFP} released less calcium upon simulated release of intracellular calcium. Additionally, knock down of TMTC1 and TMTC2 caused an increased amount of calcium release upon stimulation. While the Fura2-AM experiments implicate TMTC1 and TMTC2 in calcium homeostasis, a clear understanding of the exact molecular events underlying this observation is missing (Figure 4.1). We favor the hypothesis that TMTC1 and TMTC2 affect calcium homeostasis by modulating SERCA2B, since both TMTC1 and TMTC2 readily interact with the
calcium pump (Figure 2.5B). However, it is important to note that Fura2-AM monitors changes in global cytoplasmic calcium. Since multiple calcium pumps and channels exist in a eukaryotic cell, it is possible that altering the levels of TMTC1 and TMTC2 also affect, directly or indirectly, calcium pumps or channels in the ER, Golgi, mitochondria or plasma membrane (Brini and Carafoli, 2009; Clapham, 2007). Transfecting cells with TMTC1\textsuperscript{GFP} or TMTC2\textsuperscript{GFP} and pretreating them with the SERCA2B inhibitor thapsigargin prior to the Fura2-AM based calcium measurements can address whether TMTC1 and TMTC2 act on other calcium pumps and channels. If SERCA2B is the only calcium pump affected, then no difference should be observed in the clearance of cytoplasmic calcium. Additionally, changes in ER calcium levels can be measured by using the chimeric cameleon protein, which consists of a cyan fluorescent protein (CFP), a calmodulin domain and yellow fluorescent protein (YFP) expressed as a single protein (Demaurex, 2005; Miyawaki et al., 1997). When calcium binds to the calmodulin domain, a structural re-arrangement allows for Förster resonance energy transfer (FRET) between the CFP and the YFP, thus the amount of FRET observed can serve as indicator of calcium levels. To test if ER calcium levels are modified by TMTC1 or TMTC2, we could express an ER targeted cameleon with either knockdown or over expression of TMTC1 and TMTC2 and assay for changes in ER calcium levels, before and after stimulation.

The HEK293T cell line does not utilize calcium signaling for cellular maturation processes, nor do the cells reflect changes in calcium stimulation by oscillating internal calcium levels, which presents pitfalls in our current study. Thus, it would be more desirable to knockdown TMTC1 and TMTC2 and monitor defects in T-cell activation, as this processes is dependent on calcium signaling (Feske, 2007). Furthermore, the effect of TMTC1 and TMTC2 on oscillation of cytoplasmic calcium could be studied in the rat pancreatic AR42J cell line (Zhao et al., 1990). Recent work in AR42J cells implies that a depletion of calcium renders the ER environment more reducing, and this effect can be exploited to determine whether altering the
levels of TMTC1 or TMTC2 affect disulfide bond formation (Avezov et al., 2013). The interplay between calcium homeostasis and the oxidizing environment of the ER highlights that perturbations of TMTC1 or TMTC2 can have far reaching consequences for a cell.

Since elevated ER calcium levels may aid protein folding in the ER (Ong et al., 2010), knockdown of TMTC1 and TMTC2 could make the folding process more efficient. Moreover, it has been suggested that when misfolded proteins accumulate in the ER, calnexin dissociates from SERCA2B at the MAM in order to support protein folding rather than calcium homeostasis (Lynes et al., 2013). Therefore, TMTC2 knockdown could further enhance protein folding by liberating calnexin from SERCA2B. Taken together, TMTC1 and TMTC2 are novel regulators of calcium homeostasis, although the exact molecular mechanisms involved are unclear.

**TMTC3 and TMTC4 are two novel ER membrane TPR containing proteins**

TMTC3 and TMTC4 were identified as two novel ER adapter proteins with their TPR domains facing the ER lumen (Figure 4.2). While no clear binding partner has been verified for TMTC3, an interaction between TMTC4 and calnexin was observed (Figure 3.6). Furthermore, an interaction between TMTC4 and fatty acid synthase and p97, a cytoplasmic ATPase associated with diverse cellular activities, was suggested through mass spectrometry. The interaction with TMTC4 and fatty acid synthase or p97 would imply a role for TMTC4 in lipid synthesis or degradation of misfolded proteins, respectively (Jensen-Urstad and Semenkovich, 2012; Wolf and Stolz, 2012). Alternatively, TMTC4 could regulate the controlled degradation of an enzyme(s) important for certain lipids. This would be similar to the cholesterol regulation in the ER, wherein the rate limiting enzyme is degraded when cholesterol is abundant (Goldstein and Brown, 1990). To address if TMTC4 is indeed important for degradation of misfolded proteins, we will knockdown or overexpress TMTC4 and monitor the disposal of misfolded secretory proteins. Alternatively, TMTC4 could be important for the proper maturation of secretory proteins, by
virtue of the interaction with calnexin. To this end, secretion efficiency of a variety of secretory proteins will be addressed with either over expression of knockdown of TMTC4.

The mass spectrometry data did not show an interaction between TMTC4 and any known ER ligase. This could be attributed to using inadequate buffering conditions, since interactions between ER luminal factors and ER membrane E3 ligases are abolished in 1% Triton X-100 buffer (Christianson et al., 2012). Thus, the mass spectrometry approach could be retried by using gentler detergents or cross linkers, and verification of these interactions will then be tested in a gentler detergent buffer. Furthermore, our current list of putative TMTC4 binding partners (Table 3.1), likely contains other bona fide binding partners of TMTC4. To this end we intend to acquire the appropriate antisera against putative binding partners and address binding by co-AP and western blots.

To address if the TPR motifs of TMTC4 is important for recognizing calnexin, we could express a truncated form of the TMTC4 that contains a N-terminal signal sequence, the TPR motifs and a C-terminal tag and assess binding by AP and western blots. If an interaction is observed, the experiment will be performed in conditions that abolish the binding between calnexin and N-linked glycans (Hebert et al., 1995). Once additional binding partners have been verified from the mass spectrometry, binding to the TPR motifs of TMTC4 will be addressed as well. In conclusion, we have identified TMTC3 and TMTC4 as ER membrane proteins with luminally oriented TPR domains, and yet unknown functions.

**TTC13 and TTC17 are two novel ER luminal TPR containing proteins**

TTC13 and TTC17 where the only two luminal TPR containing ER proteins identified in our screen (Figure 4.2), which puts certain limitations on the function of these adapters. Since TTC13 and TTC17 are luminal, they are not expected to interact with any cytoplasmic components, unless part of a ternary complex. Additionally, most ER sub-domains tend to be
anchored in the ER membrane (Lynes and Simmen, 2011). This could mean that TTC13 and TTC17 could serve a more general ER function encountered throughout the domains, rather than a function in a specialized ER sub-domain. Anchoring a protein complex in a membrane can enhance complex formation by limiting diffusion to two dimensions (Good et al., 2011). It is also possible that the interactions between TTC13 and TTC17 and their respective ligands are less stable than what was observed for TMTC1, TMTC2 and TMTC4, which would hamper the detection of binding partners. This notion is supported by the fact that no enrichment of putative binding partners was observed on a silver stained SDS-PAGE.

Attempts to identify binding partners of TTC13 and TTC17 by AP followed by silver staining of a SDS-PAGE have failed to yield a significant enrichment of putative binding partners, thus the experimental conditions must be re-optimized. Specifically, we will perform the AP in more gentle detergents and we could employ cross linkers to covalently trap interactions in the ER. Although cross linking may increase the number of false positives, it could help us identify genuine interactions. Additionally, the TTC13 and TTC17 TPR domains could act as dominant negatives for their respective binding partners; which, if true, can be exploited to potentially trap the binding partners on the respective individual TPR domains. Finally, an amber suppressor codon could be utilized to label TTC13 and TTC17 on specific sites with a photoactivatable cross linker and identify binding partners via LC-MS/MS (Hino et al., 2005). Taken together, a number of experimental methods such as cross linkers and different detergents can be employed to identify the unknown binding partners for TTC13 and TTC17. These future experiments will be followed by experiments to determine the purpose of these interactions.

**TTC9B could be important for ER stress signaling**

Since TTC9B appears to be a cytoplasmic protein, it was unexpected that TTC9B was transcriptionally up-regulated by oxidative stress (Figure 3.3). This could imply that TTC9B is involved in ER stress signaling from a cytoplasmic aspect, and may be critical for maintaining
and restoring ER homeostasis upon insult. To investigate the contribution of TTC9B in ER stress signaling, we could knockdown TTC9B with or without various stress inducing agents and assess whether there is a change in activation of either of the three branches of the unfolded protein response (Hetz, 2012). This data highlights the importance of the crosstalk between the ER and other organelles and the role of cytoplasmic factors in shaping and relaying messages across the ER membrane.

**Summary**

In this thesis we have identified six novel ER proteins with multiple TPR motifs, which could aid in the compartmentalization of the ER. Subsequent experiments implied that TMTC1 and TMTC2 are novel regulators of calcium homeostasis, which opens up new avenues of studying calcium signaling and homeostasis. Conversely, no clear function can yet be assigned to TMTC3, TMTC4, TTC13 or TTC17, although preliminary studies are underway in our laboratory. Overall, this strategy was successful in identifying novel ER proteins with TPR motifs. The method can be applied to detect other common adapter motifs such as PDZ or ankyrin repeats in the ER or other organelles that have recognizable targeting sequences such as the mitochondria or chloroplast (Bhattacharyya et al., 2006; Good et al., 2011)
Figure 4.1 TMTC1 and TMTC2 are important for ER calcium homeostasis.

(A) Both TMTC1 and TMTC2 were found to interact with SERCA2B, and TMTC2 was also found to interact with calnexin. Overexpression of TMTC1 or TMTC2 caused a reduction in stimulated calcium release, implying a defect in the ability of SERCA2B to sequester calcium. Hydrophobic domains are denoted by green rectangles and TPR motifs are denoted by red hexagons. (B) Knockdown of either TMTC1 or TMTC2 caused an increase in stimulated calcium release, which is indicative of enhanced sequestering by SERCA2B resulting in elevated ER calcium levels. Simultaneous overexpression or knockdown of TMTC1 and TMTC2 did not provide any additive effect, implying that they serve redundant functions.
Figure 4.2 TMTC3, TMTC4, TTC13 and TTC17 are novel ER TPR proteins.

TMTC3 and TMTC4 are ER membrane proteins whereas TTC13 and TTC17 are ER luminal proteins. An interaction between TMTC4 and calnexin was verified by co-AP. Hydrophobic domains are denoted by green rectangles, TPR motifs are denoted by red hexagons and the asparagine (N)-linked glycan is denoted by the black branched structure on TMTC4.
APPENDIX A

EDEM1 RECOGNITION AND DELIVERY OF MISFOLDED PROTEINS TO THE SEL1L-CONTAINING ERAD COMPLEX.

(Cormier et al, 2009)
EDEM1 Recognition and Delivery of Misfolded Proteins to the SEL1L-Containing ERAD Complex

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SUMMARY

N-terminal misfolded or unassembled secretory proteins are retained in the endoplasmic reticulum (ER) and subsequently cleared by the ER-associated degradation (ERAD) pathway. The degradation of ERAD substrates involves mannose trimming of N-linked glycans; however, the mechanisms of substrates recognition and sorting to the ERAD pathway are poorly defined. EDEM1 (ER degradation-enhancing \alpha-mannosidase-like 1) protein has been proposed to play a role in ERAD substrate signaling or recognition. We show that EDEM1 specifically binds nonnative proteins in a glycan-independent manner. Inhibition of mannosidase activity with kitunensine or disruption of the EDEM1 mannosidase-like domain by mutation had no effect on EDEM1 substrate binding but diminished its association with the ER membrane and SEL1L. These results support a model whereby EDEM1 binds nonnative proteins and uses its mannosidase-like domain to target aberrant proteins to the ER membrane dislocation and ubiquitination complex containing SEL1L.

INTRODUCTION

Newly synthesized proteins that traverse the secretory pathway enter the endoplasmic reticulum (ER) and interact with a diverse set of chaperones and processing enzymes. These ER proteins assist in the maturation of secretory cargo and administer a quality control test that helps to sort native proteins for exit to the Golgi (Elggaard and Helenius, 2003; Hebert and Molinari, 2003). N-terminally misfolded proteins are sequestered away from the productive folding pathway and are eventually delivered to an ER membrane dislocation and ubiquitination complex for retrotranslocation and subsequent degradation by the cytosolic proteosome in a process termed ER-associated degradation (ERAD) (Venkitaraman, 1999). The partitioning and cleavage of aberrant proteins are essential for cellular homeostasis and survival. N-linked glycans can act as sorting tags during the maturation and quality control processes in the ER (Hebert and Molinari, 2007). As proteins emerge into the ER lumen, glycans comprised of 3 glucose, 9 mannoses, and 2 N-acetylglucosamines (Glc3Man9GlcNAc2) are attached to proteins. Generation of monoglucosylated glycans by successive cleavage of the first two glucoses by glucosidases I and II allows for the interaction between the immature glycoprotein and the lectin chaperone calnexin and its soluble paralog, calreticulin (Hebert and Molinari, 2007). Lectin chaperone binding to monoglucosylated glycans facilitates the efficient folding and ER retention of immature glycoproteins. In contrast, the identification of aberrant proteins for destruction appears to involve mannose trimming, as pharmacological or genetic inhibition of mannosidase activity stabilized glycosylated ERAD substrates (Jakob et al., 1998; Liu et al., 1999; Svedine et al., 2004). The ER contains a number of mannosidase-like or mannose-binding proteins that are involved in ERAD. Degradation appears to involve the extensive mannose trimming of substrates to Man3 (Hosokawa et al., 2009; Guan et al., 2008). The glycosyl-hydrodrolase family 47 contains two subgroups that reside in the ER, including \alpha,\beta-mannosidase I (ER ManI) and the EDEM (ER degradation-enhancing \alpha-mannosidase-like proteins) family. ER ManI removes a single \alpha,\beta-mannose on the B branch and can continue to remove additional mannose residues under higher nonphysiological concentrations of the enzyme (Harscromics et al., 2002). The EDEM family contains three ER proteins, EDEM1-3, and their corresponding genes are targets of transcriptional regulation in response to stress. Overexpression of EDEM1 increased the rate of misfold glycoprotein trimming and degradation, while the trafficking of correctly folded glycoproteins was unaffected (Molinari et al., 2003; Oda et al., 2003; Olivari et al., 2006). However, the mechanism by which EDEM1 assists glycoprotein quality control remains uncertain.

In the present study, we characterized the binding properties of EDEM1 to investigate how misfold glycoproteins are selected and targeted for degradation. EDEM1 binding to the model ERAD substrate \alpha1-antitrypsin (A1AT) null Hong Kong (NHK) did not require mannose trimming or glycosylation. However, EDEM1 was shown to associate with the downstream ERAD component SEL1L in a carbohydrate-dependent manner. Hence, we propose that EDEM1 serves as a quality control receptor that acts as a molecular link between misfolded proteins and the SEL1L-containing ER membrane dislocation and ubiquitination complex.

RESULTS

EDEM1 Selectively Binds Misfolded Proteins

To characterize the binding specificity of EDEM1, a cell-based binding assay was established. Wild-type (WT) and mutant forms
of A1AT, a model soluble glycoprotein, were specifically coexpressed in 293T cells with EDEM1 tagged with a C-terminal FLAG epitope. Cells were radiolabeled with [35S]Met/Cys for 15 min, chased for various times, and solubilized using a non-de-naturing detergent prior to immunoprecipitation with A1AT or FLAG antisera. Immune complexes were washed under harsh conditions to minimize nonspecific interactions. These conditions disrupt substrate interactions with calnexin and calreticulin (Hebert et al., 1995).

The ER form of WT A1AT was resolved as a sharp band of ~48 kD (Figure 1A, lanes 1–6). A slower migrating smear appeared after 1 hr of chase that corresponded to protein containing complex sugars received after passage through the Golgi. The complex carbohydrate-containing form of A1AT accumulated in the culture media in a time-dependent manner (Figure 1A, lanes 15–18). The stability of WT A1AT was unaffected by the coexpression of EDEM1 (Figure 1A, plot).

The NHK mutant variant of A1AT has a frameshift mutation resulting in a C-terminal truncation of 61 amino acids (Sifers et al., 1988). NHK has served as a model ERAD substrate and has been shown to colocalize with EDEM1 (Hosokawa et al., 2007; Liu et al., 1999; Zubler et al., 2003). As expected, the ER glycoprotein of NHK accumulated and was not found in the culture media (Figure 1B) (Sifers et al., 1988). In contrast to WT A1AT, these fractions (Figure 2A, α-FLAG). EDEM1-NHK complexes were found most predominately in fraction 4 (i.e.~75, Figure 2A, α-FLAG). The complex size and the presence of only the EDEM1-doublet and NHK in the A1AT immunoprecipitated fraction 4 supports the formation of a complex comprising largely of EDEM1 and NHK (Figure 2A). EDEM1 also efficiently bound and accelerated the turnover of two additional ERAD substrates, the Z-variant of A1AT and a mutant form of the type I membrane glycoprotein tyrosinase (see Figure S1 available online) (Termine et al., 2005) (data not shown). Together, these results indicated that EDEM1 differentiates between native and nonnative glycoproteins and transiently binds aberrant proteins.

**EDEM1 Binds Misfolded Glycoproteins in a Carbohydrate-Trimming-Independent Manner**

Mannose trimming of substrate carbohydrate side chains during maturation has been proposed to play a role in marking aberrant proteins for destruction (Cabral et al., 2001; Hebert and Mollnes, 2002). To investigate the requirement for carbohydrate trimming as a determinant for EDEM1 binding to misfolded proteins, EDEM1 was coexpressed with NHK in the absence or presence of various glycosidase inhibitors, and the binding of EDEM1 to NHK was analyzed by immunoprecipitation (Figure 2B and 2C). These different mannosidase inhibitors were employed to...
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EDEM1’s Binding Properties

explore the role of mannos trimming for EDEM1 binding. Kf/turnase (KIF) and 1-deoxynojirimycin (DNJ) inhibit class I mannosidases such as ER ManI (Vallee et al., 2000). In contrast, swainsonine (SWN) inhibits class II mannosidases such as Golgi α-mannosidases (Moremen, 2003).

EDEM1 binding to NHK was unaffected regardless of the mannosidase inhibitor employed (Figures 2B and 2C). Furthermore, the inclusion of the glucosidase inhibitor 1-deoxynojirimycin (DNJ) had no effect on EDEM1 binding. As observed previously, KIF moderately stabilized NHK after a 1 hr chase (Hosokawa et al., 2001). These results demonstrated that EDEM1 bound NHK irrespective of glycan trimming and suggest that EDEM1 recognizes misfolded regions of aberrant proteins.

**EDEM1 Binds Misfolded Proteins Independently of Glycans**

Human ER ManI and EDEM1 share 24% amino acid sequence identity, and the three active site residues of ER ManI (Glu330, Asp463, and Glu599) are conserved in EDEM1 (Glu225, Asp370, and Glu493) (Karaveg et al., 2003). Mutation of the acidic residues of ER ManI to neutral amino acids abolished mannosidase activity while either enhancing (Glu330) or diminishing (Asp370 and Glu599) its affinity for Man9 glycan (Karaveg et al., 2003). The similar acidic residues in the mannosidase-like domain of EDEM1 were mutated to neutral amino acids to investigate their role in substrate binding. The EDEM1 mutants possessed half-lives similar to WT-tagged and endogenous EDEM1 (Figure S3B). NHK was coexpressed with either WT EDEM1, EDEM1 single (E225Q, D370N, or E493Q), or triple (E225Q/D370N/E493Q, termed QNQ) site mutations, and the binding to NHK was monitored.

The binding level to NHK for all three EDEM1 single site mutations was indistinguishable from WT EDEM1 (Figures 3A and 3B). The peak binding level of ~35% was found after a 1 hr chase (Figure 3B). Similar results were also observed with the triple mutant EDEM1-QNQ. The three acidic residues were also exchanged for bulky and positively charged Lys residues (E225D/D370E/E493K to K16, termed KKK) to determine the effect on substrate binding using a more severe mutation that is expected to abolish any carbohydrate-binding activity. Interestingly, EDEM1-KKK binding to NHK increased to 43% after 1 hr of chase, indicating that an intact carbohydrate-binding domain was not required for substrate binding.

All the EDEM1 single and triple mutants accelerated the turnover of NHK, as the level of NHK observed after 1 hr of chase was decreased (Figures 3A and 3C). Furthermore, mutation of single or all three putative active site acidic residues combined caused a slight retardation in the mobility of NHK when compared to WT EDEM1 overexpression as observed by SDS-PAGE (Figure 3A, lanes 25–42, and Figure S3A). The difference in mobility was due to glycan trimming, as NHK migrated with identical mobilities after PNGase F treatment (Figure S3A). This observation is in agreement with previous findings using NHK and BACE as ERAO substrates that suggested that EDEM1 possesses mannosidase activity or works as an accessory protein for a mannosidase (Oliveri et al., 2006). These results indicate that the putative catalytic residues of the mannosidase-like domain of EDEM1 are not required for binding to misfolded glycoproteins.

Figure 2. Characterization of the EDEM1-NHK Complex and Its Glycan Trimming Independence

(A) NHK cells were co-transfected with EDEM1-FLAG and NHK. After pulse-labeling for 15 min, cells were chased for 1 hr and lysed with NTF buffer. The lysate was layered on a linear sucrose gradient in NTF buffer and centrifuged. Each fraction was subjected to immunoprecipitation using α-AAT or α-FLAG antibody, and samples were separated by reducing SDS-PAGE. Western blot analysis using antibodies to Man9 and Man6-P, respectively, illustrates that NHK migrates with identical mobilities as the unglycosylated forms of WT EDEM1 or EDEM1-QNQ. (B) EDEM1-FLAG was co-expressed with NHK in 293T cells. Cells were pulse labeled for 15 min and chased for the indicated times. NTF buffer was used to extract protein. The lysate was layered on a linear sucrose gradient in NTF buffer and centrifuged. Each fraction was subjected to immunoprecipitation using α-AAT or α-FLAG antibody, and samples were separated by reducing SDS-PAGE. Western blot analysis using antibodies to Man9 and Man6-P, respectively, illustrates that NHK migrates with identical mobilities as the unglycosylated forms of WT EDEM1 or EDEM1-QNQ. (C) Quantifications of the percentage of cellular NHK or percentage of EDEM1-bound NHK are displayed. Error bars represent the standard deviation for at least three independent experiments.

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Figure 3. EDEM1 Binding to Misfolded Glycoproteins Does Not Require Carbohydrates

(A) NHK was coexpressed with blank vector (-), WT, EDEM1-FLAG single (E225Q, D237N, or S493Q), or triple (D237N or K50Q mutants into 293T cells. Cells were metabolized for 15 min and chased for the indicated times. NHK and EDEM1 were isolated from the cell lysates with A1AT (a-A1AT) and FlAG (a-FLAG) antibodies. Quantification of percentage EDEM1-bound NHK B and percentage cellular NHK C are displayed. Blank vector (white), WT EDEM1 (black), and the EDEM1 active site mutants (E225Q [red], D237N [blue], S493Q [orange], QN [green], and K50Q [purple]) are represented in the bar graph. (B) NHK was coexpressed with either blank vector (-) or WT EDEM1 in 293T cells. NHK KO cells and EDEM1 were analyzed similar to (A). Secreted NHK KO was isolated from the cell culture media using A1AT antisera. Quantification of percentage EDEM1-bound NHK (C) and percentage cellular NHK (D) are displayed. All error bars represent the standard deviation for at least three independent experiments.

To further examine the carbohydrate requirement for EDEM1 binding to nonnative proteins, the ability of EDEM1 to associate with NHK lacking all three glycosylation sites (NHKΔG553) was analyzed. The three Thr in the N-linked glycosylation sites were mutated to Ala. EDEM1 bound 50% of NHKΔG553 after 1 hr of chase. The binding level of EDEM1 to NHKΔG553 was greater than that found for NHK containing all of its glycans (Figure 3D, lanes 10–12, and Figure S6 compared to Figure 1G), and it appeared to be found in a similar site complex to glycosylated

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cellular control proteins revealed that EDEM1 bound ∼15% of the total SEL1L (Figures 4A and 4B). EDEM1 binding to GRP94, BIP, or GAPDH was not observed under these conditions. The interaction between EDEM1 and SEL1L was investigated using glycosidase inhibitors to characterize the requirement for carbohydrate trimming. EDEM1 binding to SEL1L was only affected by the addition of KIF, which caused a 5-fold decrease in binding. The nature of the EDEM1-SEL1L interaction was further explored by determining if the putative active site residues of the mannosidase-like domain of EDEM1 were utilized for interacting with SEL1L. Mutation of the mannosidase-like domain acidic residues decreased the binding for SEL1L by 2- to 3-fold (Figures 4C and 4D). Allogether, these results suggest that the putative catalytic residues of the mannosidase-like domain of EDEM1 are involved in binding to SEL1L, and not the binding or recognition of ERAD substrates.

DISCUSSION

We characterized the binding properties of EDEM1 and found that EDEM1 is able to discriminate between mannose and native proteins during protein maturation and quality control. EDEM1 binding to ERAD substrates did not require the trimming of substrate glycans or for the substrate to be glycosylated. Instead, EDEM1 appeared to use its mannosidase-like domain to bind to the downstream ERAD machinery target SEL1L. These results support a role for EDEM1 in the recognition of aberrant proteins and their delivery to an ER membrane ubiquitin ligase complex, by binding to the adaptor protein SEL1L.

EDEM1 bound mutant variants of the soluble ERAD substrate A1AT (NHK and Z; Figure 1C and Figure S1A) and the membrane-integrated ERAD substrate tyrosinase (TYR-C71R, Figure S1B). Soluble substrates are solely reliant on luminal selection for ERAD. We chose to characterize the binding of EDEM1 to NHK in detail since it is arguably the most thoroughly studied soluble mammalian ERAD substrate. Previous studies have demonstrated that pharmacological mannosidase inhibition stabilized NHK, and its turnover was accelerated by the overexpression of mannosidases or EDEM family members (Honda et al., 2001; Liu et al., 1999). Short hairpin RNA knockdown of HRD1 or SEL1L also decreased the rate of NHK degradation, indicative of their involvement in NHK clearance (Christiansen et al., 2008). These results demonstrate that, after recognition in the ER lumen as an ERAD substrate, NHK is degraded through the ERAD pathway involving the HRD1/SEL1L complex.

The ERAD process in yeast and mammalian cells appears to utilize a number of protein complexes for recognition, dislocation, ubiquitination, and degradation (Carvalho et al., 2006; Christiansen et al., 2008; Denic et al., 2006; Mueller et al., 2008). EDEM1 was found recently to reside in a complex with ERdj5 and BIP (Ushio et al., 2008). ERdj5 possesses reductase activity and a J domain, which recruits the ER Hsp70 chaperone BIP. This EDEM1 ERdj5 BIP complex is predicted to select and maintain ERAD substrates in a translocation-competent form for dislocation to the cytoplasm. Interestingly, we found that the depletion of ERdj5 by siRNA did not affect NHK binding to EDEM1 (Figure S6).

The involvement of protein complexes complicates the interpretation of experiments where the effect on ERAD substrate turnover is monitored after the overexpression of a single subunit from a complex. The creation of potentially orphan subunits might disrupt the function of the complex, producing a dominant negative effect. For instance, OS 9 and XTP9 B, two putative ERAD receptors observed in complexes with GRP94 and/or BIP, stabilize NHK when they are individually overexpressed (Christiansen et al., 2008; Hosokawa et al., 2008). However, overexpression studies can be used effectively to facilitate the probing of the binding properties for the individual components of the ERAD network if the overexpressed factor directly binds the substrate. The ability of EDEM1 to bind NHK in the absence of ERdj5, combined with the size of the EDEM1-NHK complex as...
determined by ultracentrifugation, are consistent with EDE1 binding directly to NHK in a 1:1 complex.

The current glycoprotein quality control hypothesis predicts that the mannose trimming of ERAD substrate glycans by a mannosidase provides a deaminoxylated signal on the aberrant secretory cargo for ERAD (Cabral et al., 2001; Hebert and Mollari, 2007). Glycans trimmed to a glycoform ranging from Man9 to Man8 glycans are signal for the recognition by carbohydrate-binding quality control receptors (Jakob et al., 1998). Recent studies suggest that terminal α1,6-linked mannose oligosaccharides provide the signal for defective glycoproteins (Clerc et al., 2009; Hoshikawa et al., 2009; Quan et al., 2008). In this current ER quality control model, the substrate-receptor complex is then targeted to the ER membrane complex for dislocation, ubiquitination, and subsequent degradation by the cytosolic proteasome. EDE1 has been proposed to act as either the mannosidase that creates the deaminoxylated signal or the quality control receptor that recognizes and sorts mannose-trimmed proteins for ERAD by extracting them from the calnexin binding cycle (Moliniari et al., 2000; Oda et al., 2003; Olziv et al., 2006).

Our findings from the analysis of the binding properties of EDE1 are in conflict with the current model described above. We found that EDE1 appeared to directly recognize nonnative structures, as it associated with NHK irrespective of its glycosylation status. However, EDE1 did not bind or accelerate the turnover of two naturally nonglycosylated ERAD substrates, β-lactamase and mutant transthyretin (Figures S4 and S5) (Okuda-Shimizu and Hendershot, 2007; Sekijima et al., 2005). The ability of EDE1 to recognize aberrant substrates appears to be highly substrate dependent. If EDE1 can also act as a mannosidase, it must behave in a nontraditional manner as it selectively, efficiently, and persistently binds substrate, properties not shared by other glycosidases.

An intact mannosidase-like domain of EDE1, expected to be required for substrate recognition or signaling, was instead required for binding to the downstream ERAD machinery. EDE1 binding to SEL1L is disrupted by mutation of the acidic amino acids that are putative mannoside interacting residues in EDE1 or mannosidase inhibition with KIF, KIF and DME treatment both inhibit mannose trimming resulting in the accumulation of Man9 glycans (Avezov et al., 2008). However, only KIF treatment inhibited EDE1 binding to SEL1L. As KIF is a mannosone analog, binding inhibition may involve its direct association with the EDE1 mannosidase-like domain rather than the accumulation of untrimmed mannoside side chains.

Kopito and colleagues recently demonstrated that OS-9 and XTP3B interacted with SEL1L (Christianson et al., 2008). Mutations to their mannos-6-phosphate receptor homology (MPR) domains perturbed associations with SEL1L, indicating that the MPR domains of OS-9 and XTP3B are used for binding to SEL1L, likely through the glycans of SEL1L. Human SEL1L possesses five N-linked glycosylation sites, and the bulk of its luminal domain is comprised of 11 tetrapeptide repeats (TPR). TPR domains mediate protein-protein interactions supporting the recruitment of a variety of proteins, including chaperones from the Hsp70 and Hsp60 families (D’Andrea and Regan, 2003). Therefore, EDE1 binding to SEL1L may involve bipartite interactions including its mannosidase-like domain binding the large and flexible glycans of SEL1L, and protein-protein association with the TPR domains. Interactions with the TPR domains of SEL1L may be mediated directly through EDE1 or a member of the EDE1 complex such as BIP or ERp72. Together, these results suggest a modified model whereby the mannosidase-like domain or the MPR domains of these quality control receptors mediate interactions with the downstream ERAD apparatus, providing an alternative glycan-dependent mechanism for targeting and delivery of aberrant secretory cargo through the ERAD pathway.

A detailed understanding of the recognition and delivery process for the ERAD pathway will require the reconstitution of these processes using purified components. In vitro activity studies are needed to understand whether the additional trimming observed after EDE1 overexpression was due to EDE1 directly possessing mannosidase activity or the potential association of EDE1 with a mannosidase. In support of the latter possibility, EDE1 was recently found to bind and stabilize ER Mani (Termea et al., 2009). As most of the proteins involved in the ER quality control selection and delivery process are themselves glycosylated, the interpretation of results using chemical glycosidase inhibitors or the modulation of glycosidase levels through overexpression or RNAi approaches is complicated. It has been widely assumed that perturbations to the glycan status are mediated through substrate glycans. However, results presented here suggest that the glycan status of the ERAD machinery itself also appears to play an important role in shaping ERAD substrates and factors through the ERAD pathway.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**

Human embryonic kidney (HEK) 293T cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin and incubated at 37°C ± 5% CO₂. Single and cotransfections of plasmid into cells were accomplished using Lipofectamine 2000, following the manufacturer's instructions. For all cotransfections, EDE1 and its mutants were transfected at a plasmid molar ratio of 2:1 to the plasmid encoding the substrate protein. Transfections were incubated 16 hr before pulse labeling or harvesting.

**Western Blotting**

Pulse-chase labeling, immunoprecipitation, and immunoblotting were performed as previously described, with a modified resolution of the starvation period from the pulse-chase experiments (Sivivone et al., 2004). Isolation of polysomal supernatant (PNS) and all subsequent immunoprecipitation (IP) steps were conducted at 4°C. Cells were lysed in NTE buffer (0.5% Triton X-100, 20 mM MOPS, 100 mM NaCl, 20 mM Tris-HCl pH 7.5) containing protease inhibitors. The PNS was cleared with 1% Zymolase for 1 hr. The clarified supernatant was incubated with the indicated antigen complexed to protein A-Sepharose beads and treated for 1 hr. Immunocomplexes were washed twice with 0.05% Triton X-100, 0.1% SDS, 300 mM NaCl, 10 mM Tris-HCl (pH 8.0), resuspended in reducing sample buffer, and resolved on SDS-PAGE.

**Statistical Analysis**

Quantification of percentage of inhibition at an indicated chronic treatment was performed with the Mann–Whitney U test. Percentage EDE1 bound = [AAT] – [AAT at the indicated chase time] × 100. Percentage EDE1 bound = [AAT] × 100. Percentage SEL1L bound = [SEL1L] × 100.
Molecular Cell
EDEM1's Binding Properties

protein after γ-FAG (Ψ) (SELUL cell lysates × 10) Error bars represent the standard deviation for at least three independent experiments.

SUPPLEMENTAL DATA
Supplemental Data include Supplemental Experimental Procedures and six figures and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1977-7959(09)00351-7.

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APPENDIX B

THE ROLE OF UDP-GLC:GLYCOPROTEIN GLUCOSYLTRANSFERASE 1 IN THE
MATURATION OF AN OBLIGATE SUBSTRATE PROSAPOSIN

(Pearse et al, 2010)
The role of UDP-Glc:glycoprotein glucosyltransferase 1 in the maturation of an obligate substrate prosaposin

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A n endoplasmic reticulum (ER) quality control system assists in efficient folding and disposal of misfolded proteins. N-linked glycans are critical in these events because their composition dictates interactions with molecular chaperones. UDP-glucose:glycoprotein glucosyltransferase 1 (UGT1) is a key quality control factor of the ER. It adds glycans to N-linked glycans of nonglycosylated substrates that fail a quality control test, supporting additional rounds of chaperone binding and ER retention. How UGT1 functions in its native environment is poorly understood. The role of UGT1 in the maturation of glycoproteins at basal expression levels was analyzed. Prosaposin was identified as a prominent endogenous UGT1 substrate. A dramatic decrease in the secretion of prosaposin was observed in ugt1-1 cells with prosaposin localized to large juxtaplauomic aggregates-like inclusions, which is indicative of its misfolding and the essential role that UGT1 plays in its proper maturation. A model is proposed that explains how UGT1 may aid in the folding of sequential domain-containing proteins such as prosaposin.

Introduction

Protein maturation in the mammalian secretory pathway largely occurs in the ER, where maturation intermediates and products are subjected to an evaluation or quality control process (Ellgaard and Helenius, 2003). The majority of the proteins that traverse the eukaryotic secretory pathway receive multiple N-linked glycans (Apweiler et al., 1999). These covalent modifications can act as maturation and quality control tags with their composition providing information about the fitness of the modified protein (Helmis and Abe, 2004; Hebert and Molinari, 2007). The UDP-glucose:glycoprotein glucosyltransferase 1 (UGT1) is a key ER quality control folding sensor that modifies the composition of glycans based on the structural integrity of the modified protein (for review see Caramelo and Faroldi, 2008). UGT1 reglucosylatior is proposed to support folding assistance and ER retention of maturing proteins by directing their persistent binding by ER-resident carbohydrate-binding molecular chaperones (Van Leeuwen and Kearse, 1997; Wada et al., 1997; Molinari et al., 2005; Pearse et al., 2008).

The ER lumen chaperone system is comprised of the type I membrane protein calreticulin and its soluble parologue calnexin. These two chaperones bind proteins possessing monoglycosylated glycans (Glc; Man:GlcNAc;), Hammad et al., 1994). The initial round of binding to the chaperones is triggered by the rapid, sequential, and frequently co-translational trimming of two glycans from the glycans by glucosidase I followed by glucosidase II to generate the monoglycosylated substrate (Chen et al., 1995; Hebert et al., 1997). Chaperone binding also supports the recruitment of the chaperone-associated oxidoreductase ERP57 that can assist in disulfide bond formation or rearrangement (Oliver et al., 1997; Zapun et al., 1998; Soldà et al., 2006). Glucosidase II cleavage of the terminal glucose abrogates lectin chaperone association by generating oligoglycosylated proteins (Hebert et al., 1995). If the protein folds and assembles properly, it
is free to be transported out of the ER to the Golgi. In contrast, proteins possessing nonglycosylated domains are recognized by UGT1, reglycosylated, and targeted for chaperone re-binding and ER retention.

UGT1 is a large soluble ERI protein (170 kDa) that consists of an N-terminal domain responsible for the recognition of the nonglycosylated substrates and a C-terminal catalytic domain, which transfers glucose residues to the nascent protein. The ERI domain of UGT1 recognizes native folded globular substrates via a combination of hydrophobic patches (Sauve and Parodi, 1995; Caramelo et al., 2003, 2004). Deletion of the ERI domain is lethal for mice; however, UGR1−/− mouse embryonic fibroblast (MEF) cells have been generated (Molnari et al., 2005). Overexpression of the temperature-sensitive misfolded VSVG (vesicular stomatitis virus G protein) in UGR1−/− cells results in an accumulation of intracellular and cell-surface aggregates. A study involving the heterologous expression of influenza haemagglutinin demonstrated that the magnitude of substrate misfolding dictates the degree of reglycosylation (Pearse et al., 2008). Understanding the cellular localization of UGT1 has been hindered by the inability to follow its activity in live cells and the dependence on overexpressed substrates, and in vitro studies have relied largely on the analysis of nonnatural or engineered substrates (Sauve and Parodi, 1995; Caramelo et al., 2003, 2004; Taylor et al., 2003, 2004; Keith et al., 2005). The biological significance of these studies underscores the importance of understanding the physiological role of UGT1 in the maturation and quality control of endogenous cellular proteins in the natural environment.

In this study, the role of UGT1 in endogenous protein maturation and quality control was investigated. Using a cellular assay to isolate endogenous substrates of the enzyme, prosaposin was identified as a primary endogenous substrate for UGT1 reglycosylation. In the absence of UGT1, there was a significant decrease in monoglycosylated prosaposin and prosaposin secretion. Prosaposin formed nonnative conformations and accumulated in cytoplasmic perinuclear aggregates-like inclusions in UGR1−/− cells. Together, these results provide evidence for an integral involvement of UGT1 in glycoprotein maturation, as well as a novel role in the folding, trafficking, and secretion of glycoproteins.

Results

Prosaposin is an endogenous UGT1 substrate

To understand the role of UGT1 in the maturation and quality control of glycoproteins within the cell, the identification of endogenous cellular substrates of UGT1 was explored. The oligosaccharide transferase activity of MIR-8 CHO cells transfected with truncated unglycosylated N-linked glycosidase (Mano,GlcNAc) as the result of a deficiency in the dolichol-P-glucose-dependent glycosyltransferase (Algs; Quelle, 2001) was used. This permits the isolation of oligosaccharides reglycosylated by UGT1 because in these cells, reglycosylation is the exclusive mechanism by which proteins can reach a monoglycosylated state (Fig. 1 A; Pearse et al., 2008). As CHO cells lack an endomannosidase activity (Karaikanavan et al., 1998), glucosidase inhibition with N-butyl deoxynojirimycin (DNP) traps reglycosylated glycans in their monoglucosylated state in MIR-8 CHO cells. Monoglycosylated proteins can then be isolated based on their affinity for GST-catalytic domain (Pease et al., 2008), a soluble carbohydrate-binding chaperone which binds oligosaccharides with high mannose glycans (Pease et al., 1995; Kapoor et al., 2003).

To isolate endogenous substrates of UGT1, MIR-8 cells were pulse labeled in the presence of [14C]Met/Cys for 2 h, and total monoglycosylated glycoproteins were isolated from the cell lysates and media using GST-Catalytic domain. Several distinct bands were visible in the cell lysate (Fig. 1 B, lane 2), whereas the cell media was devoid of monoglycosylated proteins (Fig. 1 B, odd numbered lanes). The addition of DNP prevented the removal of the UGT1-transfected glucose by glucosidase II, resulting in the accumulation of monoglycosylated substrates (Fig. 1 B, lane 4). After a 2-h chase period, several potential substrates were cleared from the cell lysates, whereas others continued to be recognized by GST-catalytic domain in the presence of DNP. An ~60 kDa protein was readily distinguishable by its abundance, and it remained bound to GST-catalytic domain after a 2-h chase, which is suggestive of its being a prominent UGT1 substrate (Fig. 1 B, lane 8; and Fig. S1 A). Treatment with jack bean α-examniosidase confirmed the presence of monoglycosylated glycans on the 60 kDa protein because only a slight shift was observed upon jack bean α-examniosidase digestion, which is indicative of the protection of A-branch mannose residues by glucosidase (Fig. S1 B; Hammond et al., 1994; Hebert, 1995).

Numerous biochemical characterizations were performed to gain insight into the identity of the 60 kDa protein. First, alkaline fractionation revealed that it was a soluble protein (Fig. S3 A), likely bearing three to four N-linked glycans as predicted by the size of the mobility shift observed after endoglycosidase digestion (Fig. S1 B). In addition, two-dimensional isoelectric focusing and gel electrophoresis yielded α of ~5 for the native UGT1 substrate (Fig. S2, A and B). The 60 kDa protein associated with both calnexin and catalytic domain, as determined by immunoprecipitation experiments using antibodies directed against the two lectin chaperones (Fig. S3, A and B). After a bioinformatics search using these various experimentally determined parameters, potential matches were screened with specific antisera during pulse-chase analysis. The 60 kDa protein immunoprecipitated with antisera recognizing prosaposin. Prosaposin migrated at the same position as the 60 kDa protein (Fig. 1 C, compare lane 6 with lane 7). Sequential pull-down of monoglycosylated substrates with GST-catalytic domain followed by neso D antiserum revealed prosaposin as the 60 kDa UGT1 substrate. This was confirmed by a reciprocal sequential pull-down of prosaposin followed by GST-catalytic domain, demonstrating that prosaposin contained monoglycosylated glycans in MIR-8 cells (Fig. 1 C, lanes 8 and 9).

Prosaposin is a soluble secretory protein that contains an N-terminal signal sequence. It is comprised of four sequential homologous domains, each containing three disulfide bonds and a single N-linked glycan (Fig. 1 D; Kishimoto et al., 1992). After passage through the ER, prosaposin has dual fates: it can
traffic to lysosomes where it is processed by cathepsin D into four separate proteins, or it can be secreted as a full-length protein lacking its signal sequence (LeFrancová et al., 2003; O’Brien et al., 1994).

**Prosapasin reglucosylation supports persistent lectin chaperone binding**

Reglucosylation by UGT1 drives the lectin chaperone binding cycle or chaperone re-binding (Hebert et al., 1995; for reviews see Helenius, 1994; Curamela and Parodi, 2008). This is believed to increase the fidelity of the glycoprotein folding process and support the ER retention of immature or aberrant proteins (Rajagopal et al., 1994; Hebert et al., 1996; Vasiliakos et al., 1998).

The topology of a substrate and the location of its glycans typically dictate whether it interacts with the membrane-bound calnexin, the soluble calreticulin, or both chaperones (Wada et al., 1995; Hebert et al., 1997; Danilczyk et al., 2000). To determine whether reglucosylated prosaposin was redirected for calnexin and/or calreticulin binding, radiolabeled substrates of the lectin chaperones were isolated with calnexin and calreticulin antibodies followed sequentially by a pull-down with prosaposin antibodies. Reglucosylated prosaposin was bound by both calnexin and calreticulin in M8-5 cells (Fig. 2 A, lanes 5 and 7).

**Prosapasin is an endogenous UGT1 substrate** (Pearse et al., 2001).
Prosaposin is reglucosylated by UGT1 and targeted to the lectin chaperones. To determine the extent to which prosaposin is reglucosylated by UGT1, ugt1^-/- MEF cells were used (Molinari et al., 2005; Soldà et al., 2007). Wild-type and ugt1^-/- MEF cells were pulse labeled and chased for the designated times (Fig. 2 B). Total monoglucosylated glycoproteins were isolated at each time point with GST-calreticulin. In wild-type cells, 50% of monoglucosylated prosaposin remained after ~22 min. However, in ugt1^-/- cells, there was a significant reduction in monoglucosylated prosaposin, exhibiting a monoglucosylated half-time of 8 min. A portion of reglucosylated prosaposin could be observed in wild-type cells when DNJ was added at the 30-min chase time point (39%). The DNJ-trapped prosaposin fraction was largely absent from ugt1^-/- (77%; Fig. 2, B and C). These results indicate that UGT1 plays a significant role in regenerating monoglucosylated endogenous prosaposin. In addition to prosaposin, several other potential endogenous substrates displayed significant alterations in their monoglucosylated state in ugt1^-/- cells. As an unknown protein of ~100 kD exhibited a dramatic decrease in GST-calreticulin binding from ugt1^-/- cells, a similar phenotype was observed for prosaposin, which is indicative of reglucosylation. Interestingly, an additional protein of ~80 kD displayed an increase in GST-calreticulin binding in the absence of UGT1 (Fig. 2 B). This phenotype has been observed previously in an analysis of endogenous calnexin substrates (Soldà et al., 2007). However, the mechanism by which glucose trimming is slowed is not understood. In summary, prosaposin is efficiently reglucosylated by UGT1 during its maturation.

UGT1 is critical for the proper maturation of prosaposin.
Prosaposin is reglucosylated by UGT1, and this reglucosylation occurs readily during the maturation of prosaposin. A portion of prosaposin is secreted from cells as full-length protein (Grinsfeld et al., 1986; Collard et al., 1988). To investigate the importance of UGT1 in prosaposin secretion, wild-type and ugt1^-/- cells were pulse labeled with [35S]Met/Cys, and prosaposin was isolated from cell lysates and media. Secreted prosaposin was detected after 0.5 h of chase and reached ~72% secretion after 2 h of chase (Fig. 3 A and B). In ugt1^-/- cells, secretion was greatly reduced, reaching a maximum of only 24% after 1 h of chase (Fig. 3 A, compare lane 6 with lane 12). Only a minimal level of prosaposin was processed to saposin in either cell type after a 1-h chase (Fig. 3 A and Fig. S5 A, low molecular bands in cell lysates labeled SAPs).
To ensure that the decreased secretion of prosaposin observed in ugt1Δ/Δ cells was caused by the absence of UGT1, human UGT1 tagged at its C terminus with a protein C epitope (pUGT1-C) was transfected into ugt1Δ/Δ cells (Fig. 3 E). Cells transfected with pUGT1-C or empty vector were subjected to pulse-chase analysis. As observed previously (Fig. 3 A and B), only ~20% of total prosaposin was secreted from ugt1Δ/Δ cells (Fig. 3 C and D). However, expression of pUGT1-C restored prosaposin secretion to a level similar to that observed in wild-type cells (Fig. 3 C and D).

To confirm that the deficient secretion of endogenous prosaposin in ugt1Δ/Δ cells was the result of the lack of UGT1 exoglycosylation activity, a catalytic mutant of UGT1 (D1369A) was expressed, and the trafficking of prosaposin was analyzed. The mutation of the DxD motif in the C-terminal domain of UGT1 has been shown to prevent the catalytic transfer of glucose in vitro (Tressier et al., 2000). Protein C epitope-tagged UGT1D1369A was transfected into ugt1Δ/Δ cells. Expression levels were similar to those observed for wild-type UGT1 (Fig. 3 E). Whereas overexpression of pUGT1-C was able to rescue the
secretion defect of endogenous prosaposin, the expression of the corresponding catalytic mutant was unable to increase the levels of prosaposin found in the media (pUGT12090C; Fig. 3, C and D). Together, these results implicate UGT1 in playing a critical role in prosaposin maturation, as the absence of UGT1 resulted in a significant decrease in the secretion of prosapo-
sin, and its secretion can be efficiently rescued by overexpression of the folding sensor. This defect arises as the result of the absence of the catalytic reglucosylation activity of UGT1 because expression of a catalytic mutant of UGT1 was incapable of improving secretion levels of endogenous prosaposin in 17.7/cells.

ProSaposin is located in juxtanuclear aggresome-like inclusions in 17.7/cells. Because there was a striking decrease in the level of secreted prosaposin in 17.7/cells, the subcellular localization of prosa-
sopin was investigated by immunofluorescence microscopy using a saposin antibody. In wild-type cells, prosaposin was found in punctate structures dispersed throughout the cell, with a portion of prosaposin colocalizing with the ER marker protein dixulose isomerase (PDI; Fig. 4A). Only minimal levels of saposin were localized to lysosomes in wild-type or 17.7/cells (Fig. S4A). Surprisingly, in approximately one-fifth of the 17.7/cells, prosaposin was located in an additional region that corresponded to large juxtanuclear structures. These structures did not colocalize with the ER markers PDI or cal-
secin (Fig. 4A and Fig. S4B).

Because the trafficking of properly folded prosaposin in-
volved passage through the Golgi, colocalization of prosapo-
sin with the Golgi marker GM130 was analyzed. In wild-type cells, a portion of prosaposin was found colocalized with GM130. The large juxtanuclear prosaposin structures observed in 17.7/cells did not colocalize with GM130 (Fig. 4A). In addition, neither the ER nor the Golgi appeared to be morphologically disturbed in the 17.7/cells.

There are several studies that have revealed intracellular inclu-
sions to sequester malformed proteins from the cellular milieu in mammalian cells. One such inclusion is the aggresome, a peri-
cellular structure generated by misfolded proteins delivered by microtubule motor proteins and localized to the microtubule-organizing center (Johnston et al., 1998, 2002). Unlike Russell bodies (Valletti et al., 1991) or the ER-derived quality control compartment (Kamhi-Neher et al., 2001), aggresomes are not surrounded by a membrane coat. Instead, aggregates are encap-
sulated by the reorganized intermediate filament protein vimentin (Johnston et al., 1998). Because the prosaposin inclusions ob-
served in 17.7/cells were juxtanuclear and did not colocalize with PDI, calnexin, or GM130 (Fig. 4A and Fig. S4B), their co-
localization with vimentin was investigated (Fig. 4A). In wild-
type cells, colocalization with prosaposin and vimentin was not observed as vimentin was mainly localized to the cell periphery. However, in 17.7/cells, vimentin localized just outside the nu-
cles in large punctate structures, which colocalized with the pro-
saposin inclusions.

To further examine the aggresome-like inclusions visualized by immunofluorescence microscopy, 17.7/cells were examined by differential interference contrast (DIC) microscopy and com-
pared with the localization of prosaposin using saposin anti-
sera. A large juxtanuclear punctate structure was found in 17.7/cells, which colocalized with endogenous prosaposin (Fig. 4B). Altogether, endogenous prosaposin at steady-state in 17.7/cells existed in dense punctate inclusions that were distinct from the ER and Golgi. These inclusions shared the characteristic vimentin colocalization with aggresomes, intracellular sequestera-
tions that function to compartmentalize malformed protein sub-
strates from the active folding/maturational events occurring in the cell.

ProSaposin exhibits antrusive conformational modifications in 17.7/cells. The localization of protein to intracellular inclusions is usually the result of a folding defect. Because prosaposin failed to be secreted in significant quantities and was found in aggresome-
like inclusions in 17.7/cells, the folding status of endogenous prosaposin was analyzed. To investigate the extent of the non-
native conformations found in prosaposin derived from 17.7/cells, the accessibility of free sulfhydryl groups was probed by poly-
ethylene glyco (PEG)-maleimide modification.

ProSaposin contains several rapidly forming disulfide bonds (Fig. 1D; Ruiz-Canada et al., 2009). Wild-type and 17.7/cells were radiolabeled, lysed, and subjected to PEG-
maleimide treatment. After quenching with DTT, radiolabeled prosaposin was immunoprecipitated. In wild-type cells, pro-
saposin was nearly completely resistant to PEG-maleimide modi-
ification with or without a chase period, indicating that there were no exposed cysteines on Cys residues (Fig. 5A, lanes 2 and 4). Interestingly, prosaposin was highly sensitive to PEG-maleimide addition in 17.7/cells (52% resistant), and this sensitivity increased with chase time, with only 37% resistant to modification after 0.5 h of chase (Fig. 5A, lanes 6 and 8).

To further evaluate the status of prosaposin in 17.7/cells, the detergent solubility of prosaposin was analyzed. In a previous study, proteins localized to aggresomes have been found to be detergent insoluble (Johnston et al., 1998). Wild-type and 17.7/cells were pulse-chased for 15 min, and endogenous prosaposin was isolated from Triton X-100-soluble and -insoluble fractions under nonreducing conditions after various times of chase. The detergent solubility levels of prosa-
posin were similar in wild-type and 17.7/cells under non-
reducing conditions (Fig. 5B, lanes 1-10). This is in agreement with the analysis of intracellular prosaposin levels under reducing conditions (Fig. 3, A and B). However, in 17.7/cells, there was a visible amount of prosaposin found in the detergent-insoluble fractions, as some of the prosaposin resided in high molecular weight disulfide-associated aggregates that did not enter the re-
-solving gel regardless of the duration of the chase period (Fig. 5B, lanes 16-20). Therefore, endogenous prosaposin in 17.7/cells possessed exposed reactive Cys residues, which resulted in a portion of prosaposin forming large disulfide-linked detergent-
insoluble aggregates.

To verify that the prosaposin maturation defect and the re-
sultant deficiency in secretion was not the result of a pleiotropic
Figure 4. Prosaposin is localized to aggresome-like inclusions in ugpt1/−/− cells.
Prosaposin localization in wildtype (WT) and ugpt1/−/− cells was determined by confocal immuno-fluorescence microscopy. Cells were fixed in 4% paraformaldehyde/5% and permeabilized in 0.1% Triton X-100. (A) Samples were double labeled with prosaposin (SapC) antibodies and PDI (ER), GM130 (Golgi), or vimentin (aggresome) antibodies. The white boxed regions denote the areas magnified in the panels to the right. (B) Samples labeled with full-length prosaposin (Psap) antibodies were compared with DIC images. Bars, 10 μm.

Effect of the ugpt1/−/− cells, affinity-tagged human prosaposin was transfected in wild-type and ugpt1/−/− cells. Cells were pulse labeled with [35S]Met/Cys, and the overexpressed human prosaposin was isolated from cell lysates and media using antisera directed against their C-terminal myc tag. Nearly 50% of the overexpressed prosaposin was secreted from wild-type cells after 1 h of chase (Fig. 5, C and D). In contrast, there was a significant decrease in the secretion efficiency of prosaposin in ugpt1/−/− cells (~17%; Fig. 5, C and D). In conclusion, the secretion defect that was observed with endogenous prosaposin in ugpt1/−/− cells (Fig. 3, A and B) was also observed for overexpressed human prosaposin, indicating that the folding defect that arises in the absence of UGT1 is robust and not caused by a defect in the prosaposin gene in ugpt1/−/− cells.
Discussion

A complete picture of the role of UGT1 in glycoprotein maturation within the mammalian cell has remained elusive since its discovery more than 20 yr ago in pioneering studies by Parodi et al. (1984), Trombetta et al. (1985), and Souza et al. (1992). This can be attributed in part to technical difficulties in following its activity within the mammalian ER. In this study, we have identified an endogenous substrate of UGT1 reglucosylation using a mutant mammalian cell line that allows the monitoring of the reglucosylation reaction and the isolation of UGT1 substrates. Prosaposin was efficiently reglucosylated by UGT1, and in its absence, prosaposin was unstable. Endogenous prosaposin secretion was drastically reduced without UGT1 as it existed in nonnative conformations sequestered in large intracellular perinuclear aggregates. Together, these results demonstrate the importance of UGT1 in glycoprotein maturation.
Identifying cellular substrates of UGT1 has proven difficult because monoglycosylated glycoproteins that have been generated via glucosidase II trimming versus those generated by reglucosylation are indistinguishable. To circumvent this issue, we have used the afg6-deficient mammalian cell line MIB-S, which allows for the isolation of products of UGT1 reglucosylation (Quelhotrist et al., 1999; Pearse et al., 2008). Substrates of reglucosylation were enriched by the inhibition of glucosidase II to prevent glucose removal and isolated with purified GST-catalytic. Furthermore, cellular maturation studies commonly rely on the use of overexpressed proteins (Woda et al., 1997; Johnston et al., 1998; Danilczuk et al., 2000; Kuhl-Nenker et al., 2001; Kawaguchi et al., 2003; Molinari et al., 2005; Soldà et al., 2007; Pearse et al., 2008). The use of overexpressed proteins can be problematic because the ER homeostasis is tightly controlled by the unfolded protein response (Schröder and Kaufman, 2005). The unfolded protein response pathway can be activated by protein overexpression, supporting a change in the balance of the ER-resident proteins, which are frequently the proteins under study (Schröder and Kaufman, 2005; Farhan et al., 2008). The ngtt^{	ext{Cys}} cells have previously been shown to possess an adaptive stress response (Rutkowski et al., 2006). Furthermore, aggregation is a concentration-dependent reaction, which can be nonphysiologically favored by protein overexpression, and quality control/sorting steps can be saturated, resulting in the aberrant trafficking of overexpressed proteins. Therefore, it is of importance to study the reglucosylation of endogenous proteins by this critical quality control factor in the intact ER.

A prominent endogenous cellular UGT1 substrate identified was prosaposin. Prosaposin is a soluble glycoprotein comprised of four domains that contain three overlapping disulfide bonds and a single N-linked glycan (Fig. 1 D; Kashimoto et al., 1992). After passage through the ER, prosaposin has multiple fates. It can be secreted as a full-length protein, whose function appears to be wide ranging, with proposed involvement in neurotrophic development, stress and antioxidant signaling, reproductive development, and cancer (O'Brien et al., 1994; Hiraia et al., 1997; Morales et al., 2000; Minasi et al., 2001). Alternatively, prosaposin can traffic to lysosomes via a mannose-6-phosphate receptor-independent mechanism involving the Golgi receptor sortilin (Lefrancois et al., 2003). Cathepsin D, a lysosomal protease, cleaves prosaposin into four individual proteins called saposin A–D (Fig. 1 D). In the lysosome, the individual saposin acts as cofactors for different lipid hydrolyases (Hiraia et al., 1997; Gopalakrishnan et al., 2004).

Each of the saposins exists as a stable protein, displaying resistance to elevated temperatures, low pH, and several proteases (Kondoh et al., 1991; Hiraia et al., 1993; Varela et al., 1995). In wild-type MEF cells, prosaposin was largely secreted with only a minor fraction being diverted to the lysosomes (Figs. 3 and 4 and Figs. S4 A and SS A). Prosaposin secretion was significantly reduced in ngtt^{	ext{Cys}} MEF cells (Fig. 3, A and B). This is likely caused by the persistent conformational instability of prosaposin found in the absence of UGT1, as disulfide bonding was found to be incomplete and absent, and prosaposin displayed an increase in detergent insolubility (Fig. 5). A recent study in mice, in which the 5th Cys of saposin C and D was mutated, revealed that the combined CD mutation resulted in increased ER retention of prosaposin and decreased processing to the individual saposin domains (Sun et al., 2007). Surprisingly, the loss the fifth or fourth Cys of the saposin D and B domains, respectively, supported trafficking to the lysosome and normal propocessing of prosaposin with the exception that the saposin containing the mutation was unstable and absent from the lysosomes (Sun et al., 2007, 2008). The remaining three saposins were present at normal levels. Therefore, it appeared that the folding defect in prosaposin that was evident in ngtt^{	ext{Cys}} cells was more severe than the loss of a single prosaposin Cys residue. Evidently, only one of the two disulfides that connect the two saposin helices are needed to pass the ER quality control test; however, both disulfides appear to be required to survive the harsh conditions encountered in the lysosome.

Like the decreased secretion of endogenous prosaposin in ngtt^{	ext{Cys}} cells, the overexpressed human protein also failed to traffic out of cells in significant quantities. This indicated that the folding defect that occurs in ngtt^{	ext{Cys}} cells was evident even at elevated levels of the substrate protein, illustrating the effectiveness of the ER quality control process and the necessity of the folding sensor of UGT1 for the proper folding of prosaposin.

In the secretory pathway, monomeric proteins are targeted for ER-associated degradation by quality control machinery, resulting in their subsequent degradation by the cytoplasmic proteasome (Hebert and Molinari, 2007). The aberrant prosaposin created in ngtt^{	ext{Cys}} cells was not efficiently turned over by the proteasome but rather was sequestered in large intracellular nuclear inclusions. These inclusions colocalized with a known aggresomal marker protein, vimentin. Aggresomes have been defined as a cellular response to protein misfolding (Johnston et al., 1998). A driving force for aggresome formation appears to be evasion of proteasomal degradation. Aggresomes are generally observed after cells overexpressing a mutant protein are treated with proteasome inhibitors (Johnston et al., 1998; Kawaguchi et al., 2003). In the present study, aggresome-like inclusions were found to accumulate in ngtt^{	ext{Cys}} cells in the absence of proteasome inhibitors (Hebert et al., 2008). The large number of hydrophobic residues within prosaposin, which are packed into folded domains and also involved in lipid binding in the lysosome, may contribute to the inability of the proteasome to degrade the aberrant protein (Rossman et al., 2008). Because the aggresome-like structures were sufficiently large to be viewed by DIC microscopy, they likely contain additional secretory cargo that is defective in the ngtt^{	ext{Cys}} cells.

The loss of secreted prosaposin in ngtt^{	ext{Cys}} cells implicate a role for the calnexin/calreticulin cycle in the normal maturation of prosaposin. Because calnexin and calreticulin binding can slow the folding of a protein in a domain-specific manner, it has been proposed that glycans can help to direct the molecular choreography of the maturation process (Hebert et al., 1997);
Daniels et al., 2003). Carbohydrate trimming and reglucosylation determine which glycans will be bound by the lectin chaperones and the timing and duration of the interaction. Glycans reglucosylated by UGT1 support persistent binding to a given domain, which in turn can delay the folding of the reglucosylated domain. We have previously analyzed the reglucosylation of a maturing model glycoprotein, influenza hemagglutinin, in the intact mammalian IER of M8-5 CHO cells (Pearse et al., 2008). UGT1 preferentially targets slow-folding domains of a glycoprotein to specifically direct chaperone binding to immature regions. In the case of hemagglutinin, this involved the reglucosylation of the N-terminal portion of a nonsequential domain comprised of two distal regions, thereby providing protection to the N-terminal domain until its C-terminal portion was translated.

UGT1 appears to recognize and reglucosylate near-native structures, as demonstrated using purified enzyme (Caramelo et al., 2003, 2004; Taylor et al., 2004; Keith et al., 2005). Studies in Schizosaccharomyces pombe and Trypanosoma cruzi demonstrated that UGT in these organisms recognizes substrates with near-native disulfide bonds and not fully reduced proteins (Fernández et al., 1996; Labriola et al., 1999). That UGT1 does not appear to recognize grossly misfolded substrates is in agreement with our results (Fig. 5B). The hydrophobicity of the regions C-terminal to the glycans of prosaposin found that the two C-terminal glycans on saposin C and D possess the signature hydrophobic patches associated with UGT1 recognition (Fig. 6).  Protein folding for secretory proteins is initiated co-translationally and co-translationally, supporting a vectorial folding reaction whereby N-terminal domains can fold first, helping to taper the number of available folding conformations or the width of the associated protein folding funnel (Clark, 2004). As prosaposin is comprised of four sequential homologous folding domains, a single round of calnexin/calreticulin binding to the N-terminal domains directed by glucose trimming of the oligosaccharides may minimize their time encumbered by chaperone binding and support their rapid folding. It is of interest to note that saposin A in humans contains an additional N-linked glycan that increases the overall hydrophobicity of this domain, which would presumably further favor the reglucosylation of its glycans by UGT1 and ensure transient binding to the lectin chaperones (Wang et al., 2008). Persistent chaperone binding to the C-terminal domains as dictated by UGT1 reglucosylation could aid in this vectorial reaction by extending the time permitted for the N-terminal domains to fold before being hindered by exposure to the immature C-terminal domains.

The identification of the prosaposin as a prominent substrate of UGT1 implicates UGT1 and the calnexin-binding cycle assisting in the proper folding of sequential domain-containing proteins. The discovery of additional UGT1 substrates and their sites of reglucosylation will provide us with a more comprehensive knowledge of the magnitude of the role of UGT1 in cell homestasis. UGT1 is not only a critical quality control factor that evaluates the maturation process for the ER retention and proper sorting of secretory cargo, but it also appears to be a critical protein folding factor that determines the timing and duration of chaperone binding to help optimize the folding of the following.
Materials and methods

Buffers and reagents

Wild-type and MIR-I CHO cells were a gift from S. King (Johns Hopkins University, Baltimore, MD; Quellhorst et al., 1999). Wild-type and p53−/− MEFs and the protein C-tagged UGT1 expression plasmid were described previously (Koshevoy et al., 2005; Soldo et al., 2007). The pRSV5X GST-catalytic domain plasmid was a gift from M. Michalk (University of Alberta, Edmonton, Alberta, Canada). The following reagents were obtained as indicated: aspirin D antiserum (K. Sandhoff, Université Laval, Bonn, Germany), aspirin C antiserum (Santa Cruz Biotechnology, Inc., cat. no. sc-49136), catalytic domain (Pharmacia Fish Scientific), GST (Pharmacia Fish Scientific), CM 120 (BS), protein C epitope (Roche) and vimentin and myc (Sigma-Aldrich). Additional reagents and reagent concentrations were as described previously (Sun et al., 2007). Cell culture materials and Lipofectamine 2000 were purchased from Invitrogen. [35S]Met/Cys and reduced glutathione Sepharose 4B beads were purchased from Fischer Scientific and GE Healthcare, respectively. All other reagents were purchased from Sigma-Aldrich.

Transfection, metabolic labeling, and immunoprecipitation

Cells were pulse labeled with [35S]Met/Cys and chased for the indicated times as described previously (Pearse et al., 2008). For the overexpression of GST-Catalytic protein, nearly confluent cells were transfected with Lipofectamine 2000 according to the manufacturer's instructions. GST-catalytic domain pull-down was performed as previously described (Pearse et al., 2008). In brief, briefly expressed GST-catalytic domain was purified according to a previous protocol used for the purification of reduced glutathione Sepharose beads (Bashk and Michalk, 1997). After cell lysis in MNT (20 mM 2-NaCl, 30 mM NaPO4, 1 mg/ml heparin, 1 mg/ml phosphatase inhibitor cocktail) containing 20 mM iodoacetate, 1 mM PMSF, PMEDC, and PMDEA, postnuclear supernatants were incubated with reduced glutathione Sepharose beads, which were preblocked by 8 µg GST-catalytic domain. Samples were rotated overnight at 4°C, centrifuged at 10,000 g for 5 min, and washed twice. Immunoprecipitations were performed as previously described (Pearse et al., 2008). In case of the sequential immunoprecipitation, pull-downs samples were eluted from the initial isolation by incubation in 1% SDS at 50°C for 10 min and pooled in excess buffer containing 2% Tween X-100. Radiolabeled samples were resolved via SDS-PAGE and visualized by autoradiography (Fuji-500 Fujifilm). Quantifications were determined using Multi-gage version 2.0 (Fujifilm).

Confocal immunofluorescence microscopy

Cells were fixed on coverslips and were fixed with 4% paraformaldehyde in PBS at room temperature and then permeabilized with 0.1% Triton X-100 in PBS at 4°C for 10 min. Cells were blocked in 1% BSA in PBS and then probed with goat anti-human IGFBP4 (Santa Cruz) and mouse anti-actin (Sigma). At a ratio of 1:200 dilution, covered cells were mounted on slides with Vectashield (Vector Laboratories) and sealed with nail polish. Images were taken with a confocal microscope (LSM510, Carl Zeiss, Inc.) and processed with Photoshop software (Adobe).

PEG-nondegrable and detergent insolubility

The sensitivity of porcine PEG-nondegrable was determined as previously described (Koshevoy et al., 2009). In brief, after metabolic labeling, cells were lysed in MNT containing the aforementioned radioactive substrates with or without 5 mM PEG-nondegrable 5K (Sigma-Aldrich) and re-cubated on ice for 30 min. Samples were then diluted in MNT containing 20% Triton X-100 (v/v) and immunoprecipitated as described in the section Translation, metabolic labeling, and immunoprecipitation. For detergent solubility assay, cells were lysed in MNT containing protease inhibitors after metabolic labeling. After complete lysis, samples were centrifuged at 10,000 g for 15 min at 4°C. Detergent-soluble supernatants were immunoprecipitated with protein G beads. Detergent-insoluble pellets were solubilized in 1% SDS in 100 mM Tris, pH 8.0, for 10 min at 100°C. The samples were then diluted in MNT and subjected to immunoprecipitation with protein G beads.

Online supplemental material

Figs. S1 and S2 above the paragraph of the prominent UGT1 substrate treated PUGST1 and its identification as proopio. Fig. S3 demonstrates that PUGST1 binds to both catalytic core and carboxyl terminus in MIR-I cells treated with DNI. Fig. S4 displays the intracellular localization of proopio in wild-type and p53−/− cells. Fig. S5 shows partial proopio processing to proopio and that AZC does not increase the reduction of glycosylation of proopio.

References


Ploasposin is an endogenous UGT1 substrate • Piester et al. 541

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APPENDIX C

SORTING THINGS OUT THROUGH ENDOPLASMIC RETICULUM QUALITY CONTROL.

(Tamura et al, 2010)
Sorting things out through endoplasmic reticulum quality control

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Abstract
The endoplasmic reticulum (ER) is a highly organized and specialized organelle organized for the production of proteins. It is comprised of a highly interconnected network of tubules that contain a large set of resident proteins dedicated to the maturation and processing of proteins that traverse the endoplasmic reticulum’s protein transport pathway. As protein maturation is an imperfect process, frequently resulting in misfolding and/or aggregation, proteins are subjected to a series of quality control processes within the ER. Proteins deemed native are sorted for retrograde trafficking, while immature or non-native proteins are targeted for degradation. Terminal mistranslated substrates are eventually targeted for turnover through the ER-associated degradation (ERAD) pathway to protect the cell from the release of a defective product. A clearer picture of the identity of the machinery involved in these quality control evaluation processes and their mechanisms of actions has emerged over the past decade.

Keywords: Protein folding, carbohydrates, molecular chaperones, ERAD

Introduction
Ribosome-attached secretory pathway cargo are cotranslationally targeted to the endoplasmic reticulum (ER) translocase Sec61 by the signal recognition particle or SRP. Nascent chains cotranslationally emerge into the ER lumen through this ER membrane translocation pore in a vectorial manner (Figure 1A). As protein maturation begins cotranslationally and translationally, the vectorial nature of these processes helps to target the ensemble of possible intermediates by starting maturation with shorter nascent chains. These early events, which are assisted by a group of factors localized in close proximity to the Sec61 entry site, include protein folding, processing, and modification. The majority of the secretory pathway proteins are modified by N-linked glycosylation (Figure 1A), and these modifications are used extensively as maturation and quality control markers that assist with the sorting process. This review will focus on the machinery and mechanisms for how these sorting decisions are mediated with the help of N-linked glycans and their related factors.

Getting started: Translocation and early maturation events
Proteins containing the consensus N-linked glycosylation site Asn-X-Ser/Thr are cotranslationally modified with a pre-assembled 14-member polypeptide comprised of three glucose, nine mannose, and two N-acetylglucosamine (GlcNAc) Figure 1A) through the action of the oligosaccharyl transferase (OST). The addition of these large bulky hydrophilic modifications greatly alters the fundamental properties of the protein. The enzymatic transfer of the glycan requires that the Ser or Thr, situated two residues C-terminal to the Asn attachment site, position their hydroxyl side chain to render the Asn amide group more nucleophilic to support the progression of the transfer reaction. Therefore, this reaction mechanism favors the transfer of glycans that are situated in flexible regions. These regions are frequently located on the aqueous exposed surface of the protein. This optimally positions glycans for their role in maturation and quality control factors, and the recruitment of protein folding and degradation factors.
The composition of the carbohydrate tag is controlled by a series of glycosidases and glycosyltransferases that line the secretory pathway (Table 1). In the ER, glucosidase I initially removes terminal glucose residues creating diglucosylated (Glc₂Man₉GlcNAc₂) side chains, which have recently been shown to support the recruitment of the ER-resident protein maltexin [Schallus et al. 2008]. Malexin was identified as a protein immunoisolated in aquaporin-2 containing vesicles, suggestive of it possibly playing a role in its maturation [Batle et al. 2005]. As glucosidase I cleavage occurs rapidly co-translationally [Chen et al. 1995], this implies that mallein acts early in the maturation process perhaps during translocation (Figure 2). However, further studies are required to understand the spectrum of mallein substrates, the timing and role in its assistance in cargo maturation.

The second glucose is trimmed by glucosidase II to generate monoglucosylated glycans. These carbohydrates serve as attachment sites for the lectin chaperones calnexin, a type I membrane protein, and its soluble parologue calreticulin [Ou et al. 1993; Peterson et al. 1995]. The action of glucosidase II also occurs co-translationally supporting early co-translational binding to the lectin chaperones [Chen et al. 1995, Hebert et al. 1997, Deprez et al. 2005] (Figure 2). Calnexin and calreticulin binding serves a number of roles. First, as both chaperones are ER retained through ER retention signals, binding to immature proteins keeps client proteins in the ER [Rajagopalan et al. 1994]. Second, both chaperones are associated with an oxidoeductase ERP57, so binding recruits an oxidoeductase to the maturing chain that can assist in disulfide formation, reduction or rearrangement [Oliver et al. 1997, Zapan et al. 1998, Solda et al. 2006]. Third, chaperone binding constrains or delays the folding of a protein in a region-specific manner; therefore, chaperone binding can direct the folding of a polypeptide by controlling the temporal accessibility of regions to fold [Daniels et al. 2003]. Finally, the lectin chaperones protect vulnerable nascent chains from aggregation and degradation [Hebert et al. 1996, Vassilakos et al. 1996]. Inhibition of glucose trimming using competitive inhibitors of glucosidases I and II such as castanospermine or deoxyribosylalanin abolished calnexin and calreticulin binding and accelerated the turnover for a number of glycoproteins through the endoplasmic reticulum-associated degradation (ERAD) pathway [Kearse et al. 1994, Kellner et al. 1998, Ayalon-Soffer et al. 1999, Liu et al. 1999, Chung et al. 2000, Molinari et al. 2002, Svedline et al. 2004]. While stable cell lines lacking calnexin, calreticulin, or ERP57 have been established, the lectin chaperones and their associated oxidoeductase are essential for the survival of multicellular metazoans underscoring their importance in organismal homeostasis [Mecasell et al. 1999, Dimcel et al. 2002, Coss et al. 2010].

The cleavage of the final glucose by glucosidase II disrupts binding to the lectin chaperones [Hebert et al. 1995] (Figure 2). Glucosidase II cleavage of the second and third glucose is not processive as the substrate appears to require repositioning between cleavage events [Mackeen et al. 2009]. Glucosidase II is comprised of α- and β-subunits. The α-subunit contains the catalytic activity, while the β-subunit possesses the ER retention signal as well as a Mannose-6-phosphate Receptor Homology (MRH) domain that influences substrate specificity [Deprez et al. 2005, Sigliano et al. 2009]. The MRH domain helps in substrate recruitment and perhaps in its activity as the β-subunit appears to be critical for the accelerated cleavage of the final glucose to generate unglucosylated glycans [Deprez et al. 2005, Watanabe et al. 2009]. As small proteins may be able to fold without the assistance of chaperones, it would...
Table 1. ER maturation and quality control factors. The mammalian components are listed with *Saccharomyces cerevisiae* homologues denoted in parentheses.

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<tr>
<th>Category</th>
<th>Component</th>
<th>Description</th>
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<tr>
<td>Hsp70/90</td>
<td>BiP/OPRE2/2 (Kar2)</td>
<td>Hsp70 molecular chaperone</td>
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<tr>
<td>Glycan processing and binding</td>
<td>ERP54</td>
<td>Hsp90 molecular chaperone</td>
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<tr>
<td>Glucosidase I (Glu1/CWH1)</td>
<td>Trimming of terminal glucose residue</td>
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<td>Glucosidase II (Glu2/Sol1)</td>
<td>Trimming of second and third glucose residues</td>
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<td>UGT1</td>
<td>Quality control sensor and glucose translocase</td>
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<td>ER mannosidase I (Mns1)</td>
<td>Trimming of mannose residues</td>
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<td>Malted</td>
<td>Association with Glc2 Man glycan</td>
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<td>C75 (Cen1)</td>
<td>Membrane integral chaperone that binds monoglycosylated glycans</td>
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<tr>
<td>Retromer (Htm1/Mtn1)</td>
<td>Soluble chaperone that binds monoglycosylated glycans</td>
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<td>OS-6 (Yoe6)</td>
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<td>XTP3-B (Yuu9)</td>
<td>ERAD receptor</td>
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<td>Oxido-reductase</td>
<td>PDI (Pdi1)</td>
<td>Disulfide bond formation, reduction and rearrangement</td>
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<td>Arranging cargo disulfide bonds with calnexin/calreticulin</td>
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<td>Reduction of ERAD substrate disulfide bonds, associated with EDDM1 and BIP</td>
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<td>ERFAD</td>
<td>Facilitator of ERAD substrate dislocation</td>
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<td>Sec61 (Sec5/Sec1)</td>
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<td>Deflin-3 (Der1)</td>
<td>Putative ERAD translocation channel</td>
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<td>Ubiquitination</td>
<td>G67 (Idd48)</td>
<td>AAA-ATPase involved in ERAD substrate extraction</td>
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<td>RNFS/ORMA1</td>
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<td>TEA3 (MARCh4V)</td>
<td>E3 ligase</td>
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Figure 2. Glucose-dependent early protein maturation. After the entry of polypeptides into the ER, glucosidase I trims the first glucose. Disglycosylated glycans mediate interactions with malted and glucosidase II trims the second glucose. This monoglycosylated glycan is the target of lectin chaperones, calnexin (CNX) and calretilcin (CFT). Glucosidase II removes the final glucose to generate an unglycosylated glycan. Mature polypeptides are sorted for anterograde trafficking, while still immature or non-native proteins are reglycosylated by UGT1 to support calnexin/calreticulin rebinding. Eventually, terminally misfolded proteins are targeted to ERAD pathway for destruction. This figure is reproduced in colour in the online version of Molecular Membrane Biology.

be of interest to determine if glucosidase II trimming can be controlled to by-pass lectin chaperone binding so that these chaperones can focus their attention on more complex proteins that require their assistance for proper maturation. The number and location of glycans appears to dictate to some extent the molecular choreography or the chaperone binding profile for the individual chaperones (Hebert et al. 1997, Molinari
and Helenius 2000, Wang et al. 2005, 2008). Molinari and Helenius (2000) proposed the cotranslational model wherein prior interaction with BIP before the lectin chaperones only occurs if there is no glycan located within 50 amino acids of the N-terminus of the protein. While non-glycosylated proteins or regions may be aided by the Hsp70 chaperone BIP and protein disulfide isomerase family members such as PDI, glycans appear to provide the dominant chaperone signal. Together, the region-specific recruitment of the carbohydrate binding chaperones and the bulky hydrophobic nature of the glycan, likely inhibit Hsp70 binding as this family of chaperones recognizes hydrophobic patches in extended conformations [Biond-Elguindi et al. 1993]. BIP can also serve as a back-up chaperone system in cases where lectin chaperone binding has been inhibited [Zhang et al. 1997].

The positioning of BIP at the translocon by the J-domain of Sec63 and its ability to directly bind polyepetides supports the early action of BIP in the protein maturation process. However, N-linked glycans route glycoproteins to the lectin chaperone assistance program. The lectin chaperone binding profile involves the initial binding to calnexin, the membrane integrated chaperone [Wang et al. 2005]. In many cases, this is followed by association with calreticulin, if the substrate is not soluble or if its glycans eventually extend deep into the lumen from the membrane. The level of calnexin associated with the translocon complex can be regulated by the phosphorylation of its C-terminal cytoplasmic tail, which supports ribosome binding and translocon association [Chevet et al. 1999]. The evolution of larger more complex proteins in multicellular organisms appears to have resulted in the development of an additional sophisticated chaperone system based on carbohydrate binding properties. A better understanding of chaperone recognition of the translocon-associated factors will aid in our knowledge of the maturation assembly line that appears to assist in the efficient maturation of secretory cargo.

**UGT1 as an early quality control gatekeeper**

Glucose trimming can support a single round of lectin chaperone binding, however re-binding to these chaperones or the so-called ‘calnexin cycle’ is controlled by the UDP-glucose:glycoprotein glucosyltransferase I (UGT1) (Figure 2). UGT1 is a ~ 170 kDa soluble ER resident protein comprised of two functional domains. The C-terminal domain provides the glucosyltransferase activity of the enzyme that uses the ER UDP-glucose pool as a source to transfer glucose onto the A-branch of glycans attached to immature or non-native substrates (Figure 1A and Figure 2). The majority of the enzyme is comprised of the N-terminal folding sensor domain for which the structure is unknown. Together, these activities are critical for its role as an important quality control gatekeeper that directs proteins for ER retention through re-binding to the lectin chaperones.

Biochemical studies using purified UGT1 have demonstrated that UGT1 recognizes surface-exposed hydrophobic regions on maturing proteins that are associated with non-native or unassembled proteins [Sousa and Parodi 1995, Trombeta and Helenius 2000, Keith et al. 2005]. The analysis of glycopeptides glucosylated by purified UGT1 discovered that regions immediately C-terminal to efficiently glucosylated sites possessed oscillating extended regions of hydrophobicity [Taylor et al. 2003]. Positioning of the hydrophobic region closer to synthetic glycans has also been shown to increase glucosylation levels [Totani et al. 2009]. These results suggest that UGT1 optimally acts on glycans proximal to hydrophobic structural perturbations.

In addition to retaining proteins in the ER, chaperone binding has also been proposed to perturb or delay the folding of proteins in a region-specific manner [Daniels et al. 2003, Wang et al. 2005, Pearse et al. 2006, Pearse and Hebert 2009]. For multidomain proteins, it can be the optimal result of domain folding. The non-sequential domain protein influenza hemagglutinin (HA) has an N-terminal segment that interacts with a C-terminal region, linked through a large loop disulfide bond. Efficient calnexin binding to the three glycans located on the N-terminal segment protects this domain from immature oxidation to form non-native disulfide linkages, and tethers it to the membrane until a medial domain folds and the C-terminal interacting region is translated [Daniels et al. 2003]. The efficient glucosylation of the N-terminal region of HA supports persistent chaperone binding, which helps to direct its efficient folding [Pearse et al. 2008]. These results are suggestive of a larger role for UGT1 beyond targeting proteins for ER retention, which includes actively playing a role in directing the folding of large multidomain proteins by controlling region-specific chaperone binding.

UGT1 glucosylation appears to be positioned within the ER to act at a late stage during ER maturation and quality control. Quantitative immunogold electron microscopy found UGT1 to be predominantly located in transitional ER exit sites or pre-Golgi intermediates [Zuber et al. 2001]. These results were consistent with more recent proteomics results that observed UGT1 levels to be the highest in smooth ER membrane preparations [Gilchrist et al. 2006]. Finally, glucosylation of HA was observed to solely
Mannosidase activity [Jakob et al. 1998, Liu et al. 1999, Svedine et al. 2004, Bhamidipati et al. 2005, Avesar et al. 2008, Terng et al. 2009]. An important caveat to note is that these approaches supported the global retention of mannose residues on all glycoproteins and was not limited to ERAD substrates. These findings led to the hypothesis that mannose trimming generalized an ERAD signal that was recognized by downstream carbohydrate-binding proteins that aided in sorting aberrant proteins for degradation. Mannosidase activity was proposed to act slowly; therefore, only glycoproteins that possessed non-native structures and had been retained in the ER would be trimmed of these particular mannose residues [Helenius 1994, Cabral et al. 2001]. Initially, trimming was thought to only involve the removal of a single mannose from the B-arm to generate the so-called Man9GlcNAc2 glycoforms (Man9GlcNAc2; Figure 1B).

More recent results suggest that the putative ERAD carbohydrate signal involves more extensive trimming of mannose residues to glycoforms from Man7 to Man5 in which B and C branch mannoses are trimmed (Figure 1B). The involvement of truncated mannose residues in ERAD is supported by the analysis of the carbohydrate binding specificities for downstream quality control receptors, which will be discussed more thoroughly below. Importantly, when proteasome degradation is inhibited, ERAD substrates were retained in the ER with mannose residues trimmed to Man5-7 glycoforms [Ermonval et al. 2001, Frenkel et al. 2003, Hosokawa et al. 2003, Kitzmüller et al. 2003, Avesar et al. 2008]. As inhibiting degradation resulted in the prolonged accumulation of the ERAD substrates in the ER, it cannot be ruled out that the block supported more extensive mannosidase access and trimming. Further complicating the identification of the ERAD signal, cytosolic free oligosaccharides apparently created by cytoplasmic endoglycosidase cleavage of retrotranslocated ERAD substrates accumulated as Man3Glc glycans in yeast [Hirayama et al. 2010]. And in cells that transfer Man5GlcNAc2 glycans, mannosidase inhibitors still support the stabilization of ERAD substrates even though the glycans should not require further trimming for ERAD recognition [Ermonval et al. 2001].

As mannose deprived side chains appear to mask substrates for ERAD, the next question becomes what is the identity of the mannosidase(s) responsible for the remodeling of mannose arms of ERAD substrates? And how does this mannosidase distinguish non-native from native proteins? The ER possesses a small group of mannosidase-like proteins, which includes ER mannosidase I and the EDEM family of proteins. In budding yeast, these families are comprised of two proteins termed Msn1p (ER α-Mannosidase I...
orthologue) and Hm1p (the EDEM1 orthologue, also called Mtn1p) [Quan et al. 2008, Clerc et al. 2009]. These two proteins appear to cooperate to support the extensive de-mannosylation of ERAD substrates. The initial trimming to Man8B by Mtn1p created a substrate, which then could be further trimmed by Hm1p to generate the ERAD signal that involved the exposure of α1,6-branched mannose residues of the Man7 side chains (Figure 1B) [Clerc et al. 2009]. Deletion of Mtn1 supported ERAD substrate stabilization as the protein was no longer a substrate for the downstream mannosidase activity of Hm1p.

The trimming of mammalian ERAD substrates appears to be more complex as the ER glycosyl hydrolase family is expanded, and the characterization of the mannosidase activity using purified proteins has been enigmatic. The overexpression of ER α-Mannosidase I in cells facilitated the turnover of misfolded proteins, and resulted in the accumulation of Man8B, as well as more extensively trimmed glycans (Man7 or Man6) [Hosokawa et al. 2003]. In vitro studies with ER α-Mannosidase I have found that at biological levels a single mannose is trimmed to create Man8B; however, at higher levels of the enzyme and 24 h of incubation additional α1,2-mannoses can be trimmed to produce Man3-C [Skowyra et al. 2002]. As exogenously expressed ER α-Mannosidase I have been observed to accumulate in a perinuclear region upon proteasome inhibition, this has led to an alternative hypothesis that aberrant proteins are sorted to an ER quality control compartment where ER α-Mannosidase I is concentrated sufficiently to support more extensive trimming of the mannose residues on ERAD substrates [Azezov et al. 2006, Leckerremer 2009]. However, obtaining elevated levels of ER α-Mannosidase I would appear to be problematic as it is not induced by the unfolded protein response (UPR) in yeast or mammals, and its turnover has been reported to be rapid [Travers et al. 2000, Hosokawa et al. 2001, Wu et al. 2007]. Intriguingly, ER α-Mannosidase I has been found to be stabilized by EDEM1 suggesting that ER α-Mannosidase I levels may be tightly regulated [Termine et al. 2009].

Recent evidence suggests that the mammalian ERAD system might work in a similar manner to that proposed for the yeast system as EDEM family members may act as mannosidases situated downstream of the activity of ER α-Mannosidase I. EDEM1 was first discovered as a mammalian orthologue to the yeast Hm1p protein that contained a mannosidase-like domain but was believed to lack mannosidase activity [Hosokawa et al. 2001]. The overexpression of EDEM1 accelerated the turnover of glycosylated ERAD substrates, whereas its knock down stabilized them [Molinaro et al. 2003, Gnaau et al. 2004, Gong et al. 2005]. Elucidating the role of EDEM1 in the ERAD process has been problematic. While the overexpression of EDEM1 in cells has been found to be associated with the accelerated trimming of mannose residues, attempts to identify any mannosidase activity using purified proteins have failed [Oliveri et al. 2006, Cormier et al. 2009, Mikami et al. 2010, Hosokawa et al. 2010b]. This has led to a couple of contrasting models for the function of EDEM1 in ERAD.

In the first model, EDEM1 possesses specialized mannosidase activity that acts on aberrant proteins to aid in the generation of an ERAD signal. The overexpression of EDEM1 in cells was associated with increased mannose trimming as determined by an increase in mobility of glycosylated ERAD substrates by SDS-PAGE or the composition of glycans released from ERAD substrates as analyzed by HPLC [Oliveri et al. 2006, Cormier et al. 2009, Hosokawa et al. 2010b]. EDEM1 overexpression also enhanced A-branch mannose trimming in B3F7 cells, as this cell lines transfers Man5 glycans lacking B- and C-branch mannose residues [Oliveri et al. 2006]. In contrast, the overexpression of wild type but not mannosidase domain mutant forms of EDEM1 supported the trimming of C-glycans (Hercegovac et al. 2002). We found that expression of alpha-1,3-mannosidase with an RGD motif (R-G-E) bound to a mannose glycosylated form of the mannose-binding lectin, LAMP-1, and that this interaction was inhibited by mannose oligosaccharides, indicating that EDEM1 may act as a mannose-binding lectin. However, the mechanism by which EDEM1 is involved in ERAD remains unclear.
Kosmaoglou et al. 2009). However, while EDEM1 efficiently binds to ERAD substrates lacking glycans, it did not accelerate the turnover of these non-glycosylated substrates [Cormier et al. 2009, Kosmaoglou et al. 2009]. Furthermore, mutation of the mannosidase domain of EDEM1 still supports the accelerated degradation of ERAD substrates [Cormier et al. 2009, Terziman et al. 2009, Hosokawa et al. 2010a]. These results are suggestive of a bipartite binding function for EDEM1 that involves both protein–protein interactions involving protein defects (perhaps exposed hydrophobic domains) and mannoside trimmed glycans. While one form of binding might support interactions as probed by co-immunoprecipitation of lysed cells, apparently both binding forms must be engaged for EDEM1 to accelerate the turnover of an ERAD substrate.

There are a number of additional mysteries in regard to EDEM1 characterization and function. First, EDEM1 was original identified as a type II membrane protein that contained an N-terminal hydrophobic signal anchor sequence [Hosokawa et al. 2001]. More recently a soluble form of EDEM1 has been observed generated by the cleavage of its N-terminal sequence [Olvari et al. 2005, Zuber et al. 2007]. Second, EDEM1 has been shown to associate with calnexin, directly positioning it to extract ERAD cargo from the calnexin binding cycle [Oda et al. 2003]. In contrast, morphological studies that explored the localization of endogenous EDEM1 found that it did not co-localize with calnexin and it could be found in the vesicle-like structures that exit the ER [Zuber et al. 2007]. Third, the half-life of EDEM1 is short and autophagy or an autophagy-like pathway has been implicated in its rapid turnover [Cutl et al. 2008, Le Fourn et al. 2009]. The significance of this turnover route and accelerated rate is unknown but it may help explain why EDEM1 can be found in vesicles exiting the ER. Finally, the mannosidase-like domain of EDEM1 that has been proposed to mediate substrate recognition or trimming, has also been implicated in supporting EDEM1 binding to a downstream ER membrane adapter protein, SELIL [Cormier et al. 2009]. EDEM1 binding to endogenous SELIL was diminished by mutating conserved residues in the mannosidase-like domain of EDEM1, or treatment with the mannosidase inhibitor kifensine. These results suggest that the mannosidase-like domain may be deployed to target EDEM1-substrate complexes to ER membrane complexes involved in dislocation and ubiquitination. Future studies using purified proteins to recapitulate the substrate selection and targeting events would help to further understand these processes.

Mammalian EDEM1 has two paralogues termed EDEM2 and EDEM3 that are also upregulated by UPR and possess ER α-Mannosidase I homology domains [Mast et al. 2005, Olvari et al. 2005, Hiroz et al. 2006]. EDEM2 and EDEM3 have N-terminal cleavable signal sequences that generate soluble ER resident proteins. Only EDEM1 and EDEM3 have been found to be associated with increased mannoside trimming when overexpressed in cells [Mast et al. 2005, Olvari et al. 2006, Hiroz et al. 2006, Cormier et al. 2009, Hosokawa et al. 2010b]. The overexpression of EDEM3 enhanced the mannoside trimming of N-glycan of ERAD substrates to Man6 and Man7 [Hiroz et al. 2006, Hosokawa et al. 2009]. However, as the demonstration of mannosidase activity for isolated EDEM family members has not been demonstrated, it cannot be ruled out that EDEM interacting or stabilized proteins are responsible for the observed increase in cellular mannoside trimming. In support of this possibility, ER α-Mannosidase I has been found to be stabilized by EDEM1 [Terziman et al. 2009].

Quality control sorting receptors

Mannose trimming appears to generate an ERAD signal that must then be recognized by quality control receptors to target the defective cargo for ERAD. The family of possible mannose-dependent quality control receptors includes EDEM1 (as discussed above) and a group of ER resident MRH domain containing proteins that include Yos9p/Os-9 and XTP3-B.

These MRH domain proteins bind to mannosides and interact with downstream ER membrane complexes involved in ERAD, implicating them in playing critical roles in the selection and delivery of defective secretory cargo to the ERAD pathway.

Yos9 was initially identified as an ERAD-related gene by genome screening of Saccharomyces cerevisiae as a Yos9p deletion strain stabilized CPY* [Buschhorn et al. 2004]. It appeared to act specifically on glycosylated proteins since it did not stabilize nonglycosylated CPY* [Buschhorn et al. 2004, Ranchara et al. 2010]. Its MRH domain is important for ERAD as mutating this domain suppressed CPY* turnover [Bharadwaj et al. 2005]. Yos9p recognized ERAD substrates bearing Man8B and Man3 N-glycan [Saxthmey et al. 2005]. In contrast, frontal affinity chromatography analysis, a quantitative method developed to analyze carbohydrate-protein interactions, demonstrated that the MRH domain of Yos9p has high affinity for Man3 glycoforms but not Man8B [Quan et al. 2008]. This later result suggested that Yos9p binds proteins that have been extensively trimmed by Man1p and Hr1p to capose
of the linker that connects the two MRH domains. The C-terminal MRH domain appears to be involved in substrate recognition as it is required for glycan-dependent binding to null Hong Kong alpha-1-antitrypsin [Yamaguchi et al. 2010]. However, knockdown of XTP3-B does not affect the degradation of null Hong Kong alpha-1-antitrypsin, suggestive of redundant cellular factors being involved in its turnover [Christianson et al. 2008, Hosokawa et al. 2008]. The C-terminal MRH domain of XTP3-B preferentially binds to Lec1 cells, which express proteases bearing Man1-4GlcNAc2 glycans on their surface [Yamaguchi et al. 2010]. Inhibitory studies using various disaccharides revealed that Man1-4Man efficiently inhibited substrate binding consistent with the second MRH domain of XTP3-B binding to terminally exposed 0-1,6-linked mannose residues. These results suggest that OS-9 and XTP3-B possess similar glycan binding requirements but as the two proteins are not associated with one another their roles appear to be distinct [Christianson et al. 2008]. Interestingly, the simultaneous knockdown of both OS-9 and XTP3-B by RNAi specifically stabilized soluble proteins possessing luminal lesions [Bernasconi et al. 2010]. Future studies will be required to determine if the two proteins are involved in redundant pathways or have different binding properties.

Similar to Yos9p, OS-9 and XTP3-B were found to associate with the mammalian Hrd3p homologue SEL1L, an ER membrane ERAD adapter protein [Christianson et al. 2008, Hosokawa et al. 2008, Mueller et al. 2008]. Therefore in addition to selecting glycosylated ERAD substrates for degradation, these luminal quality control receptors also appear to support delivery of the defective substrates to the dislocation site in the ER membrane. The nature of the quality control receptor-adaptor protein interaction is uncertain. It was reported that OS-9 and XTP3-B are recruited to the SEL1L complex by their MRH domains as mutation of the MRH domain of OS-9 and XTP3-B decreased their interaction with SEL1L but did not affect its association with ERAD substrates [Christianson et al. 2008] (Figure 3B). However, contradicting results are also available that suggest that OS-9 and SEL1L associate through protein-protein interactions [Hosokawa et al. 2009, Yamaguchi et al. 2010]. Similar uncertainty has also been observed with EDEM1 binding to SEL1L. The mannosidase-like domain of EDEM1 supports binding to the glycosylated SEL1L and not null Hong Kong alpha-1-antitrypsin as mutations of the mannosidase-like domain or kifunensine diminished EDEM1 binding to SEL1L and not the ERAD substrates [Cormier et al. 2009].
Quality control in the endoplasmic reticulum

Preparation of ERAD substrates for retrotranslocation

Once marked for ERAD and selected by quality control receptors for degradation, the non-native and possibly aggregated substrates need to be presented to an ERAD complex in a translocation competent form for their dislocation to the cytoplasm. As ERAD substrates can contain disulfide bonds, these events can require the protein-assisted reduction of disulfide bonds and unfolding.

The ER contains a family of oxidoreductases of which a number of these enzymes appear to play important roles in the reduction of intra- and inter-molecular disulfides to create ERAD substrates that are competent for dislocation. The extensively oxidized ERAD substrate BACE457 was found in a complex with PDI and BIP prior to dislocation to the cytoplasm [Molnari et al. 2002]. Furthermore, PDI disassembled cholera toxin dimers to facilitate their retrotranslocation [Tsai et al. 2001]. PDI has also been observed in a complex with Hsp110 in yeast further implicating it in the ERAD process [Clerc et al. 2009, Sakoh-Nakatogawa et al. 2009]. Interestingly, PDI stimulated the retrotranslocation of glycan and cysteine-free prepro (α) factor using mammalian and yeast microsome systems [Gillece et al. 1999, Wahlman et al. 2007]. PDI appears to aid in substrate recognition and preparation for dislocation by both reduction, as well as by utilizing its peptide binding properties to unfold misfolded and aggregated proteins.

The ER also contains a newly identified thioredoxin-domain containing flavoprotein called ERFAD [Kiemer et al. 2009]. The knockdown of ERFAD expression by RNAi stabilized ERAD substrate turnover and decreased the cellular level of poly-ubiquitinated proteins. ERFAD also interacted with a number of ERAD factors including OS-9, SEL1L and ERD5 as shown by co-immunoprecipitation. ERD5 possesses four thioredoxin domains, and a J-domain that supports the recruitment of BIP. ERD5 was also identified as an EDEM1 interacting protein in a modified yeast two-hybrid and co-immunoprecipitation procedures [Ushioda et al. 2008]. Its overexpression accelerated turnover of ERAD substrates by suppressing aggregation of misfolded proteins possessing non-native disulfide bonds. The activities of ERFAD, ERD5, and their interacting partners, support them playing roles in the reduction and possibly unfolding of ERAD substrates so that they can be efficiently threaded through an ER translocon.

Adenine nucleotide regulated ER chaperones also appear to be involved in generating translocation competent ERAD substrates. BIP has been localized to ERAD complexes containing EDEM1 and ERD5, as well as with XTP3-B [Christianson et al. 2008, Ushioda et al. 2008]. In addition, the ER Hsp90 family member GRP94 was observed in a complex with OS-9 using mass spectrometry to analyze interacting partners of overexpressed tagged OS-9 [Christianson et al. 2008]. The knockdown of GRP94 stabilized null Hong Kong alpha-1-antitrypsin. As both BIP and GRP94 are molecular chaperones, they may play a role in ERAD substrate recognition and/or their ATP-dependent binding cycle may aid in the unfolding of the misfolded substrates.

ER membrane ERAD adapter complexes

Once a protein has been deemed misfolded by an ERAD receptor, the misfolded protein needs to be targeted to a dislocation complex in a translocation competent form for the extraction of the misfolded protein to the cytoplasm. This ERAD complex appears to consist of a number of proteins that aid...
in the delivery, translocation and ubiquitination processes [Carvalho et al. 2006, Denic et al. 2006, Gauss et al. 2006, Christiaan et al. 2008]. Central to the formation of this ER membrane complex are adapter proteins that are integrated into the ER membrane and serve as the link between substrate recognition/delivery machinery and factors involved in dislocation and ubiquitination.

The yeast and mammalian ER contain ER membrane adapter proteins that possess a number of luminal-exposed tetrastricopeptide repeat (TPR) domains [D’Andrea and Regan 2003]. The yeast protein termed Hrd3p has nine TPR domains while the mammalian protein, SEL1L, contains a total of eleven. Structures of known TPR domains display helical folds comprised of two helices connected by a short linker. Adjacent TPR domains come together to form concave and convex surfaces, with protein-protein interactions generally occurring on the concave surface [Das et al. 1998]. The well studied cytoplasmic TPR-containing protein Hop provides a link between Hsp70 and Hsp90 chaperones by using two separate clusters of TPR domains to recruit the individual chaperones to form a large protein folding complex [Schuetler et al. 2000]. This cytoplasmic complex facilitates the passage of a maturing substrate from the Hsp70 to the Hsp90 chaperone system. Similarly, these ER membrane TPR domain proteins appear to create a connection between quality control receptors and dislocation/ubiquitination machinery to support the selective translocation of ERAD substrates to the cytoplasm for eventually destruction.

Affinity purification of different ERAD components followed by mass spectrometry was used to discover that Hrd3p resides in a large molecular weight complex interacting with luminal (Yos9p), ER membrane (Usa1p, Hrd1p, Ubx2p) as well as cytosolic ERAD components (Cdc48p). This implicates Hrd3p serving as a scaffold or adapter protein that nucleates an ER translocation complex [Carvalho et al. 2006, Denic et al. 2006]. Truncation studies with Hrd3p suggest that it binds Hrd1p and Yos9p through two distinct domains, implying that the TPR domains of Hrd3p direct distinct interactions with ERAD machinery [Gauss et al. 2006]. Furthermore, Hrd3p also has the ability to directly bind misfolded proteins independent of Yos9p [Denic et al. 2006]. The interaction between Hrd3p and Hrd1p may have multiple functions. First, Hrd3p appears to aid the delivery of misfolded proteins to the ER membrane E3 ligase, Hrd1p. Second, deletion of Hrd3p destabilizes Hrd1p [Plenemer et al. 1999, Gardner et al. 2000, Gauss et al. 2006], implying that Hrd3p might also be needed to stabilize the translocation complex, insuring that uncomplexed ligases do not accumulate.

The 11 TPR domains of SEL1L are arranged into three clusters comprised of four, five and two TPR domains moving from the N- to the C-terminus. SEL1L is N-linked glycosylated and has an N-terminal fibronectin type II domain [Bruno et al. 2006]. SEL1L is known to interact with a large number of proteins including Hrd1, Herp, Derlin-1/2, EDEM1, OS-9 and XTP3-B (see below) [Li et al. 2006, Christiaan et al. 2008, Hosokawa et al. 2008, Mueller et al. 2008, Cormier et al. 2009]. Kopito and co-workers showed that SEL1L can bind to both OS-9 and XTP3-B without its transmembrane domain, but deletion of the two C terminal membrane proximal TPR domains abolished binding [Christiaan et al. 2008]. The role of SEL1L in ERAD was initially discovered by studying the cytomegalovirus-induced degradation of major histocompatibility complex class I heavy chains [Mueller et al. 2006]. This study also demonstrated that knockdown of SEL1L stabilized a broader range of misfolded proteins.

As previously mentioned, both EDEM1 and XTP3-B have the ability to bind nonglycosylated misfolded proteins [Christiaan et al. 2008, Hosokawa et al. 2008, Cormier et al. 2009]. Mutations in their respective MRH or mannosidase-like domains abolished binding between OS-9/XTP3-B/EDEM1 and SEL1L [Christiaan et al. 2008, Cormier et al. 2009]. This suggests that the MRH or mannosidase-like domains of the ERAD machinery help support SEL1L binding, rather than substrate recognition (Figure 3B). The glycans of SEL1L are all positioned either within or in close proximity to the TPR domain clusters, consistent with the possibility that the glycans and TPR domains work in concert to support protein interactions. Unlike for Hrd3p, SEL1L, does not appear to directly bind ERAD clients [Christiaan et al. 2008]. Moreover the repertoire of SEL1L interacting proteins was recently expanded with the addition of UBCE (an ER membrane anchor for an E2 ligase), UBXD88 (receptor of the cytoplasmic AAA-ATPase p97) and AUP1 (ubiquitin-binding protein) [Mueller et al. 2008].

The Hrd3p complex in yeast also appears to contain an additional adapter protein termed Usa1p [Carvalho et al. 2006]. Usa1p is needed for the degradation of luminal ERAD clients and serves as a linker for membrane proteins including Hrd1p and Der1p [Carvalho et al. 2006, Hori et al. 2009]. The mammalian homolog of Usa1, Herp, is required for degradation of kappa light chain, a non-glycosylated subunit that is recognized by B2B rather than ER quality control lectin receptors [Okuda-Shimizu
and Hendershot 2007). Like Usa1p, Herp is an ER membrane protein with both termini residing in the cytoplasm [Kokame et al. 2000]. Interestingly, Herp was also shown to bind the H3 and S1 subunits of the proteasome, implying that Herp connects the translocation and ubiquitination processes to the proteasome [Okauda-Shimizu and Hendershot 2007]. Together, these results indicated that adapter proteins play critical roles in the recruitment of ERAD machinery within the lumen, cytoplasm, as well as the ER membrane.

Translocation and ubiquitination

The final ER events for ERAD include the translocation of the ERAD substrate across the ER membrane and its poly-ubiquitination at the cytoplasmic face of the ER membrane by membrane localized E3 ligases. Machinery involved in these important processes appears to be components of the large membrane protein complex nucleated by the ER membrane adapter proteins (Figure 4). The organization of these complexes insures the efficient transfer of ERAD substrates from quality control receptors, to translocons and their ubiquitination upon arrival in the cytoplasm.

The polytopic ER membrane protein Derlin-1 and its yeast homologue Der1p associate with the SEL1L/HRD1 and Hrd3p/Hrd1p adapter protein complexes, respectively [Lilley and Ploegh 2005, Schulze et al. 2005, Ye et al. 2005, Carvalho et al. 2006]. The human cytomegalovirus initiated turnover of major histocompatibility class I heavy chain involves the use of Derlin proteins, as Derlin-1 was discovered as an US11-interacting protein that accelerated the degradation of heavy chain [Lilley and Ploegh 2004]. Two additional homologues of Derlin have been discovered termed Derlin-2 and Derlin-3. The overexpression of the Derlin family members accelerated the degradation of ERAD substrates, whereas its knockdown stabilized them [Oda et al. 2006, Sun et al. 2006]. Additional studies will be required to determine if Derlins serve as translocation pores, as this role is largely speculative and based on their topology that includes four putative transmembrane domains.

The Sec61 translocon that is responsible for the insertion of proteins into the ER has also been proposed to play a role in the translocation of ERAD substrates in the reverse direction [Wiertz et al. 1996]. This demonstration is complicated by the need to delineate the protein import and export processes. However, yeast strains possessing mutations in Sec61p have been described that support efficient translocation into the ER but are deficient in the retrotranslocation of proteins out of the ER [Pilon et al. 1997, Plumper et al. 1997, 1998, Scott and Schekman 2000]. Furthermore, yeast and mammalian ERAD substrates have been observed to associate with Sec61 during late stages of quality control [Wiertz et al. 1996, Pilon et al. 1997, Plumper et al. 1997]. Future studies will be required to determine the range of proteins that can serve as retrotranslocons, and if each of the translocons supports the transit of different types of substrates. Additionally, while the AAA-ATPase p97 (Cdc48p in yeast) has been implicated in driving the translocation of the substrate once the termini of the substrate reaches the cytoplasm, it is unclear what the mechanism is for the initial threading of the ERAD substrate through the pore. Cotranslational translocation is aided by chain elongation driven by the ribosome, which is nestled on the translocon. However, a

Figure 4. ER membrane ERAD complexes. Proteins that reside in the yeast and mammalian ERAD translocation complex are designated. This figure is reproduced in colour in the online version of Molecular Membrane Biology.
post-translocation event would appear to require an initial push from the lumen for the chain to reach the cytoplasmic AAA-ATPase involved in pulling the chain into the cytoplasm.

During the arrival in the cytoplasm, ERAD substrates are rapidly ubiquitinated by a family of ER localized E3 enzymes. The topology of the ERAD substrate and the location of the defect appear to determine the identity of its modifier. In yeast, membrane proteins possessing cytoplasmic defects or soluble cytoplasmic proteins were modified by Dso1p [Vasanthi and Ng 2004]. In contrast, soluble or membrane proteins possessing luminal lesions used Hrd1p [Carvalho et al. 2006, Denie et al. 2006]. The transmembrane region of Hrd1p also appears to support the direct recognition of proteins with defects that lie within the membrane [Sato et al. 2009]. Altogether, Hrd1p appears to manage the formation of a number of ERAD complexes that support the processing of different substrate classes [Kanehara et al. 2010].

In mammalian cells, the family of E3 ligases involved in ERAD has expanded beyond the small set of yeast modiﬁers. In addition to the yeast Hrd1p homologue HRD1, mammalian E3 ligases currently include GP78, RNF5/RMA1, TEB4/MARCHIV, TRG8 and RFP1 [Tang et al. 2001, Hassink et al. 2005, Lerner et al. 2007, Motton et al. 2008, Stagg et al. 2009]. The selectivity of many of the ER E3 ligases is poorly deﬁned. The selection process appears to differ from the yeast system where Hrd1p ubiquitinates substrates with defects within the membrane or luminal compartments [Vasanthi and Ng 2004]. Recent results suggest that soluble ERAD substrates that are dependent on HRD1, can be rendered HRD1-independent by simply tethering them to the membrane with a single hydrophobic domain [Bernasconi et al. 2010]. The change in topology apparently alters the E3 ligase involved in directing turnover. The larger spectrum of ERAD substrates found in multicellular systems has resulted in the expansion of E3 ligases and diversification of the selection process. In some cases, the association of ligases with speciﬁc adapter complexes appears to dictate the substrate selection process, whereas for other ligases substrates may be selected directly by the ligase.

Conclusion

The quality control process evaluates mutant proteins that are defective and targets them for degradation. In addition, as one-third of the genome products traverse the secretory pathway and maturation is an error prone process, the quality control machinery must also test all the secretory client proteins. While many quality control components have been identiﬁed along with the complexes in which they reside, how this relatively small group of proteins efﬁciently screens the thousands of different cargo proteins is still poorly understood. In the cytoplasm, hundreds of E3 ligases are responsible for the recognition, modiﬁcation and turnover of cytosolic factors [Deshai and Joazeiro 2009]. Since recognition and modiﬁcation of secretory pathway proteins occurs in two separate locations, this would appear to provide an additional layer of complexity; however, the machinery involved appears to be simpliﬁed. Future studies will be required to determine how a glycan-based quality control process can efﬁciently manage such a large group of diverse components that includes wild type and mutant proteins, misfolded or unassembled cargo, and factors that are degraded through regulated proteolysis.

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