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Determinants of intra-membrane client recognition by the molecular chaperone Calnexin

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Table of Contents

Summary

Integral membrane proteins (IMPs) are a ubiquitous class of proteins and account for around one-third of the human proteome. They are permanently embedded in the lipid membranes surrounding cells and the various intracellular organelles of a mammalian cell, where they serve numerous important cellular and physiological tasks. These include transporting hydrophilic molecules and signals through the lipid bilayers, functioning as metabolic enzymes, or maintaining cell-cell interactions. Accordingly, IMPs are indispensable for multicellular organisms in which individual cells function as part of the whole and thus have to communicate with one another and their environment.

Despite their importance, however, IMPs often cause disease for two reasons. First, IMPs are particularly vulnerable to incorrect folding and assembly. Their biogenesis requires several topologically distinct folding events that pose a particular challenge to the endoplasmic reticulum (ER) folding and quality control machinery. This includes folding of soluble domains and, defining a membrane protein, the correct integration and assembly of hydrophobic transmembrane domains (TMD) inside the lipid bilayer. In addition, mutations that introduce charges into the TMDs or abolish a polar binding partner, leaving the other unpaired and exposed, disfavor the integration of TM segments and can ultimately lead to misfolding and misassembly of a membrane protein. Therefore, intra-membrane chaperones must guide and control membrane protein structure formation inside the lipid bilayer. However, mechanistic insights into these processes remain very limited.

In this study, we use Calnexin (CNX), one of the most abundant ER chaperones, and putative intra-membrane chaperone as a model to define principles of intra-membrane chaperoning. We show that CNX binds non-glycosylated proteins and distinguishes the folded and misfolded state of its client. Furthermore, we demonstrate that CNX directly recognizes misfolded clients via its single TMD, regardless of its luminal lectin or C-terminal domain. By combining experimental and computational approaches, we finally reveal a protective function of CNX on membrane proteins by identifying an intra-membrane recognition motif within its TMD that protects clients from premature

degradation and appears to be conserved in other intra-membrane chaperones. At the same time, using an artificial minimal consensus membrane (CoMem) reporter system, which we have established in this study, we systematically dissect client signatures that CNX recognizes in the membrane.

In summary, using CNX as an example, we have revealed comprehensive insights into intra-membrane substrate recognition processes by molecular chaperones that provide a basis for understanding ER-mediated quality control of IMPs. As a result, our findings could contribute to a better understanding of numerous diseases caused by membrane protein-misfolding.

Zusammenfassung

Integrale Membranproteine (IMPs) sind weit verbreitet und machen etwa ein Drittel des menschlichen Proteoms aus. Sie sind fest in der Lipidmembran verankert, die sowohl Zellen als auch die verschiedenen intrazellulären Organellen einer Säugerzelle umgibt und erfüllen dort wichtige zelluläre und physiologische Aufgaben. Zu diesen gehören der Transport hydrophiler Moleküle und Signale durch die Lipiddoppelschicht, die Funktion als Stoffwechselenzym sowie die Aufrechterhaltung von Zell-Zell-Interaktionen. Daher sind IMPs unverzichtbar für vielzellige Organismen, bei welchen einzelne Zellen als Teil des Ganzen fungieren, was eine ständige Kommunikation der Zellen untereinander sowie mit der Umgebung erfordert.

Trotz ihrer großen Bedeutung verursachen IMPs jedoch häufig Krankheiten. Der Grund hierfür ist, dass ihre Biogenese mehrere topologisch unterschiedliche Faltungsereignisse erfordert, die für die Faltungs- und Qualitätskontrollmaschinerie des endoplasmatischen Retikulums (ER) eine besondere Herausforderung darstellen. Dies führt dazu, dass IMPs besonders anfällig gegenüber Fehlern bei der Faltung und Assemblierung sind, welche nicht nur die Faltung von Intramembrandomänen, sondern – der Definition von Membranproteinen folgend – auch die korrekte Integration und Assemblierung von hydrophoben Transmembrandomänen (TMD) innerhalb der Lipiddoppelschicht umfasst. Darüber hinaus können Mutationen die Integration von Transmembransegmenten beeinträchtigen, etwa durch das Einführen von Ladungen oder die Deletion eines polaren Bindungspartner, was schlussendlich Auswirkungen auf die korrekte Faltung hat. Um Fehlfaltungen entgegenzuwirken ist es deshalb essentiell, dass Chaperone des Intramembranraums die Ausbildung der nativen Struktur von Membranproteinen innerhalb der Lipiddoppelschicht unterstützen und überwachen. Mechanistische Einblicke in diese Prozesse verschiedener Intramembran-Chaperone sind allerdings sehr begrenzt.

In dieser Studie verwenden wir Calnexin (CNX), eines der am häufigsten vorkommenden ER-Chaperone und mutmaßliches Intramembran-Chaperon als Modellprotein, um die Grundlagen bei Chaperon-Vorgängen im Intramembranraum zu definieren. Wir zeigen, dass CNX nicht-glykosylierte

Membranproteine bindet und bei diesen den gefalteten und fehlgefalteten Zustand unterscheidet. Darüber hinaus zeigen wir, dass CNX fehlgefaltete Membranproteine ganz unabhängig von seiner luminalen Lektin oder C-terminalen Domäne durch die TMD erkennt. Durch die Kombination experimenteller und computergestützter Ansätze konnten wir schließlich ein Erkennungsmotiv innerhalb der TMD von CNX identifizieren, das Membranproteine durch Bindung vor dem vorzeitigen Abbau schützt und in anderen Chaperonen des Intramembranraums konserviert zu sein scheint. Gleichzeitig konnten wir mithilfe eines von uns in dieser Studie etablierten, künstlichen *minimal consensus membrane* (CoMem) Reportersystems Merkmale in Transmembrandomänen von Membranproteinen dechiffrieren, die von CNX innerhalb der Membran erkannt werden.

Zusammenfassend liefert diese Studie am Beispiel von CNX umfassende Einblicke in Substraterkennungsprozesse molekularer Chaperone innerhalb von Membranen, die als Grundlage bei der Aufklärung der ER-vermittelten Qualitätskontrolle von IMP dienen können. Die Erkenntnisse dieser Arbeit können dadurch zu einem besseren Verständnis zahlreicher Erkrankungen beitragen, die durch die Fehlfaltung von Membranproteinen verursacht werden.

1 Introduction

In multicellular organisms, each cell has to function as a part of the whole. Thus, cells have to communicate and interact with their environment while maintaining cellular and organismal homeostasis. These and other tasks such as transporting hydrophilic molecules and signals through the lipid bilayers, specific recognition events in an apolar environment, functioning as metabolic enzymes, or maintaining cell-cell interactions are mainly dependent on integral membrane proteins (IMPs), which reside within the lipid membranes that surround cells and the various intracellular organelles of a mammalian cell. Consequently, IMPs are indispensable for multicellular structures, which is furthermore highlighted by the fact that this particular class of proteins accounts for around one-third of the human proteome (von Heijne and Gavel 1988).

1.1 Membrane proteins – a brief overview

In eukaryotic cells, the biosynthesis of both IMPs and soluble secretory pathway proteins occurs at the endoplasmic reticulum (ER) (Shao and Hegde 2011). However, IMPs pose more complex challenges to the ER folding and quality control system than secreted soluble proteins. The formation of their native state requires multiple topologically distinct folding events. These include correct folding of soluble domains on both the ER luminal and cytoplasmic side and, defining a membrane protein, the correct integration as well as the intra and intermolecular assembly of hydrophobic transmembrane domains (TMD) in the lipid bilayer destination (Shao and Hegde 2011, Cymer, von Heijne et al. 2015). During structure formation, interactions between different TM segments throughout a TM protein significantly contribute to correct integration and topogenesis. Further complicating these processes, the TMDs of multi-pass membrane proteins are highly diverse. They can be short or long, only partially inserted, often contain polar residues, breaks, or kinks, and may exhibit flexibility in the membrane, which gives rise to complex membrane integration and folding pathways (Ota, Sakaguchi et al. 1998, Lu, Turnbull et al. 2000, Hessa, White et al. 2005, Sadlish, Pitonzo et al. 2005, Kauko, Hedin et al. 2010). However, especially these deviations from ideal hydrophobic membrane anchors allow

membrane proteins to fulfill their vast functional repertoire (Illergard, Kauko et al. 2011). At the same time, they render IMPs vulnerable to incorrect folding and assembly in the lipid bilayer. Moreover, many severe human pathologies are caused by mutations that disfavor the integration of TM segments by introducing charges and ultimately result in membrane protein misfolding (Partridge, Therien et al. 2004, Guerriero and Brodsky 2012, Marinko, Huang et al. 2019). Thus, intramembrane chaperones and quality control factors must exist that can recognize and counteract such threats. Remarkably, many factors located in the ER lumen, membrane, or cytosol facilitate the highly sophisticated biogenesis of IMPs from the very beginning and assist IMPs in reaching their native state or degrade upon failure, as will be described in the following.

1.2 Biosynthesis of membrane proteins

The biosynthesis of membrane proteins is a complex and precisely coordinated process that occurs either co- or post-translationally and can be divided into specific but coupled stages:

i) the accurate targeting of the growing polypeptide chain to the ER membrane
ii) the translocation and integration of TM structures into the lipid bilayer
iii) the formation of the final topology and structure.

This first step in the biogenesis of membrane proteins is generally initiated by recognizing hydrophobic segments as they emerge from the ribosome and the targeting of such signal sequences to the ER membrane (Figure 1) (Egea, Stroud et al. 2005). Despite their diversity in amino acid sequence composition and length, all ER signal sequences share the same common characteristic: they are hydrophobic. Importantly, hydrophobic segments once they emerge from the ribosome do not necessarily represent signal sequences, but can also function as ER targeting signals, which constitute the first TM helix of a membrane protein. In such a case, these segments exhibit a greater hydrophobicity than typical signal sequences, and lack a signal peptide peptidase cleavage. Either way, early identification and shielding of these hydrophobic segments from the aqueous cytosol by specific factors is important. One such factor is the nascent polypeptide-associated complex (NAC) which interacts with short nascent

polypeptide chains emerging from the ribosome-nascent chain complex (RNC). NAC can sense substrates directly upon synthesis by inserting a sensor domain deep into the ribosomal exit tunnel. As a consequence of polypeptide elongation, NAC is pushed out of the ribosomal tunnel allowing the recruitment and handover of the substrate to other targeting factors such as the cytosolic signal recognition particle (SRP) (del Alamo, Hogan et al. 2011, Gamerdinger, Kobayashi et al. 2019).

SRP is a ribonucleoprotein and one of the most studied protein biogenesis factors involved in co-translational handling the vast majority of ER-destined proteins. The most critical functions include recognizing and binding hydrophobic sequences once they emerge from the exit tunnel of a translating ribosome and its GTP-dependent interaction with the ER membrane-bound SRP receptor (SR). Structurally, the SRP consists of one RNA scaffold and six protein subunits, of which SRP54 is of particular relevance as this subunit mediates signal sequence recognition and binding. Mainly responsible for recognizing and binding hydrophobic sequences is a methionine-rich, hydrophobic groove located in the M-domain of SRP54. When bound to the ribosome, the M-domain is positioned precisely at the exit tunnel. Cryo-electron microscopy of SRP-RNC assembly complexes has revealed that in the absence of a substrate this groove is occupied and autoinhibited by an amphipathic helix originating from the carboxy terminus of SRP54. It seems conceivable that the helix functions as a hydrophobicity threshold, only allowing sufficiently hydrophobic signal sequences to be accommodated in the groove when they emerge from the ribosomal exit tunnel. Consequently, this leads to a displacement of the helix and repositioning above the hydrophobic groove whereby the signal sequence is efficiently shielded from the cytosol (Walter and Blobel 1980, Walter and Blobel 1982, Voorhees and Hegde 2015, Guna and Hegde 2018).

Beyond that, recognition and binding of SRP causes translational arrest and guides the RNC to the ER membrane. ER-targeting occurs *via* an interaction of the SRP with the membrane-bound heterodimeric SRP receptor (SR), which consists of a cytoplasmic α subunit that shows remarkable homology with SRP54 and an integral membrane β-subunit. The binding of the SRP to its receptor is followed by a direct interaction between the RNC complex and the central

translocation machinery in the ER membrane, the Sec61 translocon (Egea, Stroud et al. 2005, Zimmermann, Eyrisch et al. 2011, Dudek, Pfeffer et al. 2015). Notably, both SRP and SR contain GTPase domains, required for association and to target the ribosome-nascent chain complex to the Sec61 translocon. Only correct binding of SRP to the heterodimeric SR leads to extensive interactions of both GTPases and the formation of a complete active site that is crucial for reciprocal activation of GTP hydrolysis (Egea, Stroud et al. 2005). This provides a quality control mechanism that monitors the proper alignment of the ribosomal exit tunnel with the Sec61 translocation channel and thus shields the emerging polypeptide chain from the cytosol. Hydrolysis of GTP leads to the dissociation of the SRP from the ribosome-nascent chain complex, relieving translational arrest and enabling insertion of the nascent peptide into the translocation channel of Sec61 and integration of helices into a membrane.

Of note, during or after insertion into the membrane, signal peptides are cleaved by the membrane-bound signal peptidase (SP). Subsequently, the following TM segments are then integrated into the membrane. In contrast, hydrophobic segments functioning as signal anchors are not cleaved but move laterally out of the Sec61 translocon into the membrane (Egea, Stroud et al. 2005, Shao and Hegde 2011, Cymer, von Heijne et al. 2015, Dudek, Pfeffer et al. 2015).

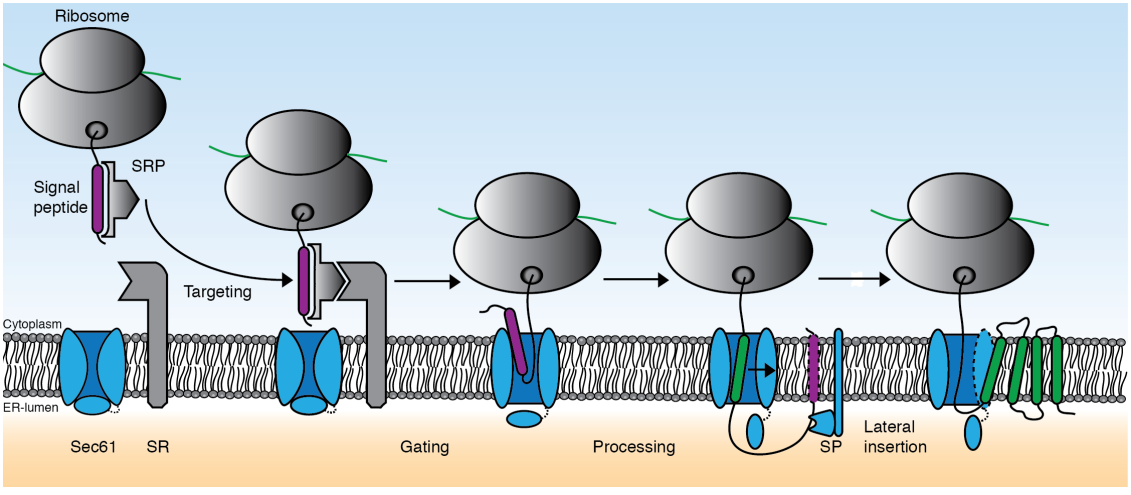


Figure 1: Biosynthesis of integral membrane proteins: Once they emerge from the ribosome exit tunnel, hydrophobic signal sequences are recognized by the signal recognition particle (SRP) and targeted to the ER membrane-bound SRP receptor (SR), which allows insertion of the nascent polypeptide chain into the Sec61 translocon. Following engagement by a signal sequence, the Sec61

translocon undergoes a rotational movement that induces the opening of the central pore through the membrane and into the lipid bilayer. During or after insertion into the membrane, signal peptides are cleaved by the signal peptidase (SP), and subsequent TM helices are integrated laterally into the membrane (Figure based on Guerriero and Brodsky 2012).

The second and most decisive step in the biogenesis of a large majority of integral membrane and soluble proteins is their integration into the membrane, mainly depending on the ER translocon Sec61. Although it is known that peptides can also spontaneously integrate into the membrane, this process only applies to short and highly hydrophobic peptides (Tissier, Woolhead et al. 2002, Brambillasca, Yabal et al. 2006). The Sec61 itself is a highly conserved heterotrimeric ER membrane protein complex composed of the essential α and γ subunits and the non-essential β subunit. It is characterized by an aqueous protein-conducting channel that spans the entire membrane and is formed by TM helices of Sec61α (Simon and Blobel 1991, Crowley, Liao et al. 1994, Mothes, Prehn et al. 1994). This channel enables the cell to fulfill two essential cellular functions: the translocation of soluble proteins across the ER membrane as well as the integration of TM segments into the surrounding lipid bilayer by lateral movement through a gate present in the central pore (Shao and Hegde 2011, Cymer, von Heijne et al. 2015).

In the absence of translation, the Sec61 translocon switches from an open to a closed conformational state. In this inactive state, the translocon is not only closed axially by a short Sec61α helix reaching into the central pore, functioning as a plug, but also laterally. Thus, the permeability barrier of the ER membrane for small molecules is maintained (Erdmann, Jung et al. 2010, Zimmermann, Eyrisch et al. 2011). Impermeability of the ER membrane is additionally ensured by the action of immunoglobulin heavy-chain binding protein (BiP), which binds and seals the ER luminal side of the translocon pore (Hamman, Hendershot et al. 1998). Conversely, the molecular understanding of how substrates activate the Sec61 translocon by causing channel opening for translocation or insertion is incomplete. It has been revealed that in complex with the RNC, Sec61 undergoes a rotational movement following the engagement by a signal sequence. As a consequence of this substrate-induced conformational shift, the Sec61α helix

responsible for closing the channel is displaced, which induces the opening of the central pore across the membrane and into the lipid bilayer (Figure 1). It seems plausible that for these processes, the hydrophobicity of signal sequences is a determining criterion since insertion into the lateral gate requires the disruption of interactions between Sec61α helices that contribute to the formation of the channel. In fact, this process would be energetically unfavorable for hydrophilic segments of a polypeptide (Voorhees and Hegde 2015, Voorhees and Hegde 2016).

Although the Sec61 translocon predominantly mediates the insertion of IMPs, many additional factors contribute to its function and assist on translocation and hence the biogenesis of IMPs (Conti, Devaraneni et al. 2015). Amongst these factors are the translocon-associated protein (TRAP) and the translocating-chain associating membrane (TRAM). The TRAP complex is formed by four different proteins that, through constitutive association with Sec61, affect both the structure of the ribosome-translocon complex and the function of the Sec61 translocon itself (Fons, Bogert et al. 2003, Shao and Hegde 2011). In addition, TRAP appears to control signal sequence recognition and transmembrane helix insertion in a substrate-dependent manner. Only recently has it been revealed that TRAP preferentially binds signal peptides whose amino acid composition shows a high content of glycine and proline and a slightly below average hydrophobicity (Nguyen, Stutz et al. 2018). Since hydrophobicity of a signal sequence, as described above, is a decisive factor for the opening process of the translocon channel, it is possible that TRAP assists in the channel opening for particularly those substrates which based on their low hydrophobicity, would only be inefficiently or improperly translocated (Fons, Bogert et al. 2003, Nguyen, Stutz et al. 2018). Similar to the function of TRAP, the multipass TM protein TRAM enhances the integration efficiency of different substrates, however, preferentially those with relatively hydrophilic TM segments in the early stages of synthesis (Heinrich, Mothes et al. 2000). Accordingly, it has been suggested that TRAM plays a vital role during the challenging integration process of multi-spanning TM proteins. Presumably, TRAM holds several TMDs of nascent translocating proteins in a temporary transition state before their coordinated integration into the lipid phase (Do, Falcone et al. 1996, Voigt, Jungnickel et al.

1996, Heinrich, Mothes et al. 2000, Shao and Hegde 2011). This process seems to be especially important for proteins with charged or polar residues in their TM regions as they need to interact with other TM segments to form hydrophobic complexes where polar residues are buried within the protein´s interior (Illergard, Kauko et al. 2011). In general, hydrophobicity and length but also helicity of TMDs of ER targeted IMPs are very diverse and influence the precise mechanism for insertion into the ER. Therefore, membrane proteins unable to use this default and highly conserved SRP-dependent Sec61 ER-insertion pathway, like small secretory protein, tail-anchored (TA) proteins, and proteins that insert into the ER membrane post-translationally, alternative routes exist, including the GET or the recently discovered SND pathway in yeast (Hegde and Keenan 2011, Johnson, Powis et al. 2013, Strzyz 2016).

1.2.1 Alternative routes - SRP-independent ER-targeting

TA proteins are characterized by a single TMD domain located near the C-terminus. As a consequence of this spatial proximity between the TMD and terminating stop codon, the translation terminates, and the TA-protein is released from the ribosome before the signal sequence can emerge into the cytosol. As a consequence, co-translational engagement by the SRP is no longer possible, which makes recognition and delivery of an emerging nascent chain by an alternative SRP-independent and post-translationally acting pathway indispensable. In such situations, the conserved ATPase GET (guided entry of TA proteins) enables transfers of tail-anchored proteins to the ER (Stefanovic and Hegde 2007). However, GET alone is not able to capture substrates effectively but requires assistance. Sgt2 is a chaperone-like protein that recognizes hydrophobic TA sequences soon after they are released from the ribosome and subsequently mediates transfer to the homodimeric targeting factor Get3. Interestingly, like SRP54, Get3 possesses a methionine-rich groove required to accommodate TA protein targeting signals (Mateja, Szlachcic et al. 2009, Wang, Brown et al. 2010, Chio, Cho et al. 2017). Finally, following interaction with its receptor Get1/2, a transmembrane insertase located in the ER membrane, Get3 releases its substrate and allows insertion of the TA protein into the membrane (Wang, Chan et al. 2014). This pathway, however, is not accessible for TA

substrate where the TM segment has only moderate hydrophobicity, which prevents interaction with Get3. In this case, insertion of the TA substrate is mediated by a different insertase, the transmembrane domain insertase EMC, as will be described later (Schuldiner, Metz et al. 2008, Borgese 2016, Guna and Hegde 2018, Shurtleff, Itzhak et al. 2018).

Another pathway for proteins that exhibit downstream instead of amino-terminal located TMDs and thus do not represent preferred SRP substrates is mediated by the SND (SRP-independent targeting) pathway. Proteins of this pathway, namely Snd1, Snd2, and Snd3, were initially discovered in a screen to identify novel factors that mediate ER targeting independent of SRP or GET. Unlike the previously described pathways, the SND pathway specifically recognizes centrally located TM segments within the polypeptide chain of its substrates. Most likely, once present in the cytosol, signal sequences are co-translationally captured by the ribosome-associated Snd1 and subsequently transferred to the ER membrane-embedded and translocon-associated Snd2/3 complex, which not only functions as a receptor for Snd1 but also enables translocation of the substrate (Aviram, Ast et al. 2016).

In summary, all SRP-independent pathways that have been uncovered share the same working principle: preinsertional shielding of proteins in the cytosol followed by rapid targeting to the ER membrane and the subsequent assistance during integration into the ER membrane. Moreover, the individual pathways can cooperatively work together or compensate for each other in loss of function events or as a consequence of cellular stress (Aviram, Ast et al. 2016, Hassdenteufel, Johnson et al. 2018). Together, this enables the most efficient ER targeting for the largest possible number of proteins. Interestingly, this characteristic of mutual compensation does not only seem to affect the various ways of ER targeting but has also been described for the translocation process via the Sec61 translocon. In addition to Sec61, yeast can rely on a further, alternative translocon, namely Ssh1 (SEC61A2 in humans), which functions as a backup mechanism when the canonical Sec61 is overloaded. Of note, the range of functions of Ssh1 seems to be far-reaching as specific substrates prefer translocation via Ssh1, which causes functional overlapping with Sec61 (Finke, Plath et al. 1996, Spiller and Stirling 2011, Ast and Schuldiner 2013).

However, regardless of whether ER targeting is based on an SRP-dependent or independent process, one thing becomes apparent: the nature of the primary protein sequence itself, the amino acid composition, and the degree of hydrophobicity dictate which mechanism comes into effect for membrane insertion.

1.2.2 Insertion and topogenesis of membrane proteins at the ER membrane

The final and most complex step in the biogenesis of membrane proteins following membrane insertion involves forming their topology. In general, the topology of an integral membrane protein relates to the number of TM helices present in a transmembrane protein (which allows classification into single- or multi-spanning TM proteins), and how these helices are oriented relative to each other and within the membrane with respect to the position of N- and C-terminus (Figure 2) (von Heijne 2006). In addition, luminal or cytoplasmic domains that protrude from the hydrophilic backbone of membrane phospholipids and connect the assembled transmembrane helices make a significant contribution to the topology of membrane proteins. Considering that fully assembled membrane complexes are highly dynamic structures, topology formation becomes even more complicated. Switching between conformations through positional changes or the partial folding and unfolding of helices within the structure of IMPs is not a rare phenomenon (von Heijne 2006).

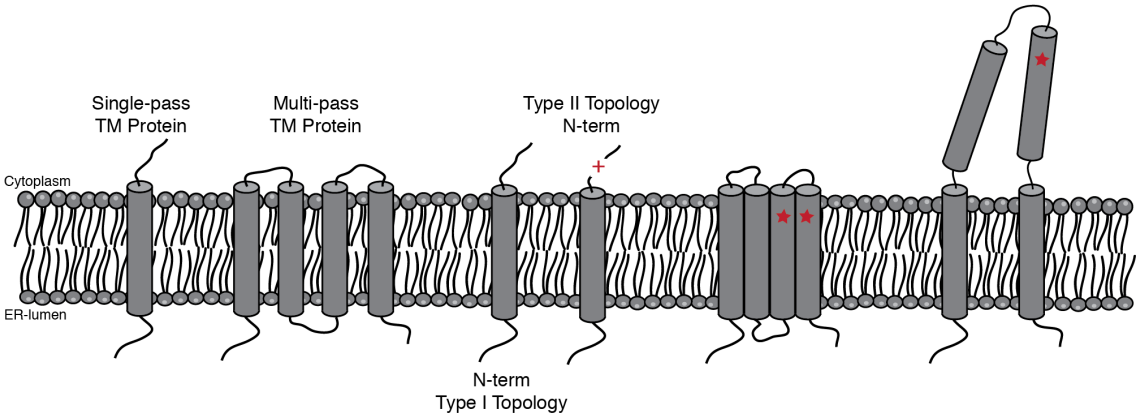


Figure 2: Schematic representation of TM proteins in different conformational states: Depending on the number of TM segments within the ER membrane, IMPs can be classified into single or multi-pass TM proteins. The distribution of charged residues mainly determines the orientation of TM

segments within the ER membrane. Particularly positively charged amino acids within the polypeptide sequence such as Arginine (indicated with a red "+") are preferentially localized in the cytoplasm. They can thus induce a type II topology in which the N-terminus of the IMP is located within the cytosol. This is in contrast to the type I topology where the N-terminus is located inside the ER lumen. Interactions of polar residues (indicated with a red star) allow for specific helix-helix interactions within the membrane and thus contribute to the formation of functional three-dimensional structures in membrane proteins. However, mutations that cause the introduction of polar residues into TM segments or abolish a polar binding partner, leaving the other unpaired can disfavor the integration of TM segments and alter their position within the membrane, thereby affecting the total membrane protein structure and function.

Multiple factors determine the orientation of a TM segment in the membrane. However, of particular relevance is the distribution of charged amino acids within the sequence. During membrane integration of the first TM segment, the N-terminus of the nascent chain can either remain in the cytosol or translocate across the membrane into the ER lumen, consequently affecting the orientation of the first TM segment and thus the orientation of downstream TM segments (Heinrich and Rapoport 2003). Of note, the insertion of TM segments is not a rigid process, and TM segments can be inverted after their insertion into the membrane. According to the "positive-inside rule", the orientation of the first TM segment mainly relies on the protein sequence itself. In general, the part of the polypeptide chain with a more positive charge becomes predominantly localized to the inside of a cell, the cytoplasmic side (Heijne 1986, Hedin, Ojemalm et al. 2010, Shao and Hegde 2011). Importantly, charged residues not only determine whether the first TM segment exhibits type I topology (N-terminus in the ER) or type II topology (N-terminus in the cytoplasm). Moreover, charged residues can also affect the localization of the nascent peptide stretch between two consecutive TM segments. As a result, the polypeptide stretch can be present either in the ER lumen or cytosol. However, the emergence of both conformations is not solely dependent on the orientation of the TM segment itself but additionally favored by altered conformational states between the ribosome and the Sec61 translocon: in contrast to a tight association state, which promotes translocation of the polypeptide chain into the ER lumen, a relatively loose degree of interaction allows the polypeptide chain to escape into the cytosol (Rapoport, Goder et al.

2004). Besides that, the orientation of a TM segment is furthermore affected by the distribution of specific upstream or downstream sequence, folding of flanking domains, and overall length of the hydrophobic sequence and hydrophobicity in general. For instance, rapidly folding structures in the N-terminal domain of a nascent polypeptide chain can influence the orientation of a downstream TM segment in such a way that a type-I orientation is prevented. The reason for this: In order to adopt this conformation, transport of the already folded domain through the narrow translocation channel would be required (Denzer, Nabholz et al. 1995).

In contrast, rather long and hydrophobic TM segments are often found in type-I orientation because the inversion of TM segments after insertion into the translocon head-first is slowed down and energetically unfavored with increasing hydrophobicity of the TM sequence. Ultimately, however, the rate of inversion is mainly determined by the number of charged amino acids close to the hydrophobic signal (Goder and Spiess 2003). Although the tremendous structural variability of TM helices is mainly caused by the distribution of specific amino acids along the sequence of an IMP, their occurrence and distribution within distinct TMDs are subject to a certain regularity. Whereas hydrophobic residues like Ala, Ile, Val, and Leu are more often found directly in the middle of the membrane, aromatic residues like Tyr and Trp are more likely to be found in the vicinity or within the lipid-water interface. In contrast, charged or polar residues are usually found less frequently in membranes, although they have important functions, as explained below (Ulmschneider, Sansom et al. 2005).

In general, it is assumed that membrane integration is based on a partitioning process between the hydrophilic environment inside the Sec61 translocon channel and the hydrophobic environment within the lipid bilayer. Thus, proteins whose TM segments are strongly hydrophobic should readily partition into the membrane one by one or in pairs (Rapoport, Goder et al. 2004). This mainly applies to single-spanning TM proteins (Hessa, Kim et al. 2005). However, as briefly discussed above, membrane integration is far more complex for multi-spanning TM proteins. The cause for that is that IMPs need to establish their correct topology during or after synthesis, which, among others, essentially depends on interactions between different TM segments.

1.2.3 TM helix-helix interactions and the role of sequence motifs in function and disease

Interactions between neighboring TM segments based on designated sequences and motifs are indispensable to establish correct IMP topologies and functions. Furthermore, these interactions can be an essential prerequisite that enables membrane integration in the first place. However, failures during assembly can lead to the exposure of these residues and motifs to the membrane´s hydrophobic environment, which is often the case with unpaired polar residues. In such a case, it seems conceivable that similar sequences and motifs, once exposed, could contribute to the process of substrate recognition by intra-membrane chaperones, which is also based on a direct interaction between TM segments. Therefore, helix-helix interactions based on designated sequences and motifs will be discussed in more detail in the following.

Two major driving forces underlie the folding and assembly of polypeptide segments within the lipid bilayer: saturation of hydrogen bond donors and acceptors within the peptide backbone and the interaction of polar residues, whose presence in the hydrophobic interior of a membrane would otherwise be unfavorable. Interactions of polar residues allow for specific helix-helix interactions within the membrane and thus contribute to forming functional three-dimensional structures in membrane proteins (Figure 2). Remarkably, the capability of polar residues mediating the association of membrane-embedded helices is differently pronounced. Whereas amino acids that contain two polar-side chains such as Asn and Gln strongly promote the interaction between different TM segments, amino acids like Thr or Ser, which exhibit only one polar side-chain atom, show a much weaker tendency (Finke, Plath et al. 1996, Choma, Gratkowski et al. 2000, Gratkowski, Lear et al. 2001, Zhou, Merianos et al. 2001). Responsible for this effect is the ability to simultaneously function as a hydrogen donor and acceptor, a characteristic that applies to amino acids containing two polar atoms such as Asn or Gln in their side chains. However, stabilization of TM helix-helix interactions through interhelical hydrogen bonds between polar residues is not always guaranteed. Quite in contrast, these interactions show a strong positional-dependency of polar residues along with the TMD (Dawson, Melnyk et al. 2003). Moreover, relying on polar residues for

the establishment of IMP topologies comes at a cost: TM segments with only marginal hydrophobicity show poor insertion efficiencies, which could consequently impair correct integration and folding of IMPs (Hedin, Ojemalm et al. 2010). However, this effect of polar residues on membrane insertion does not seem to be generally valid, but rather to be context-dependent. Using suitable in vitro translation systems, it could be shown that position-specific Asn- or Asp mediated interactions with an adjacent TM helix can also promote the membrane insertion efficiency of a marginally hydrophobic transmembrane segment (Meindl-Beinker, Lundin et al. 2006).

Strikingly, polar residues are very widespread and highly conserved in TM segments, although they can cause aberrant membrane integration (Illergard, Kauko et al. 2011). This further underlines the structural and functional relevance of polar residues in IMP TM segments. Indeed, previous work analyzing the integration efficiency of individual TM segments revealed that about 25 % of all human TM helices present in multi-spanning IMPs are predicted to exhibit unstable membrane integration ($\Delta G_{app} > 0$ kcal/mol) in isolation (Hessa, Meindl-Beinker et al. 2007). This argues for potentially very common folding and integration problems for IMPs posed by unpaired polar residues. Unfortunately, hardly anything is known about cellular control and response mechanisms dealing with such problems during IMP biogenesis. Polar residues in TM segments are not only a problem encountered during the biogenesis of a large number of naturally occurring IMPs – they are also a cause for disease (Partridge, Therien et al. 2004). In fact, mutations that cause the introduction of polar residues into TM segments or abolish a polar binding partner, leaving the other unpaired and exposed, are related to various human pathologies. In this regard, it has been shown that polar residues not only disfavor the integration of TM segments but can also alter their position within the membrane, thereby affecting the structure and function of the total membrane protein (Figure 2) (Partridge, Therien et al. 2004, Hutt, Powers et al. 2009, Guerriero and Brodsky 2012). This was shown for the Erb-B2 oncogene product, a receptor tyrosine kinase that belongs to the family of epidermal growth factor receptors. As a result of the occurrence of a single point mutation in the TMD of the protein in which Val is exchanged for Glu, Erb-B2 undergoes dimerization and activation, which causes

a large number of breast and ovarian cancers (Bargmann, Hung et al. 1986, Weiner, Liu et al. 1989).

As mentioned earlier, the distribution of charged amino acids within the sequence of a nascent IMP significantly contributes to the topology. In addition, positively charged amino acids such as Arg and Lys located in the areas between and within TM segments fulfill essential structural and functional roles in membrane proteins. Among others, they are required for substrate recognition and are involved in assembly processes (Oosawa and Simon 1986, Finke, Plath et al. 1996, Xu, Kakhniashvili et al. 2000, Ding and Wilson 2001). Necessary for assembly are dimerization processes mainly determined by the protonation state of the respective Arg or Lys residue residing in a TM segment. While the formation of hydrogen bonds stabilizes dimerization, an increasing repulsion contributes to a monomeric state and mainly occurs if positive amino acids are located directly in the interacting interface of the two helices (Finke, Plath et al. 1996, Therien and Deber 2002). Unfortunately, similar to polar residues, positively charged residues when introduced into TM regions by mutation are linked to a large number of genetic diseases such as cystic fibrosis, Charcot-Marie-Tooth disease, or Wilson´s disease to name just a few (Partridge, Therien et al. 2002, Partridge, Therien et al. 2004, Fink, Sal-Man et al. 2012, Ruskamo, Nieminen et al. 2017). In most cases, introducing a positive charge leads to an impaired interaction or assembly of TMD segments, which consequently affects the entire structure. Interestingly, most of the mutations that ultimately lead to diseases result from the introduction of Arg but not Lys residues into the TMDs (Fink, Sal-Man et al. 2012). However, the influence of positively charged residues on the structure of an IMPs does not necessarily have to be associated with adverse effects. Quite in contrast, it seems that ionic interactions of oppositely charged residues inside the membrane strongly promote the interaction between adjacent TMDs and thus are essential for the assembly of various IMPs (Herrmann, Fuchs et al. 2010).

In addition to polar and charged amino acids, aromatic amino acids also play a role in the interaction of helices. Albeit their role is comparatively low, aromatic residues show a highly conserved occurrence in TM segments. Due to their unique structure, they are crucial for many molecular recognition and assembly

processes in IMPs, such as in amyloid polypeptides or bacterial toxins (Belbeoc'h, Falasca et al. 2004, Ramachandran, Tweten et al. 2004, Fink, Sal-Man et al. 2012). Responsible for the interaction between helices containing aromatic residues is the formation of different high-order clusters, which are also referred to as non-covalent π – π interactions between planar aromatic rings (Bernstein and Sun 1996, McGaughey, Gagne et al. 1998, Gazit 2002). Interestingly, TM-self-assembly is not exclusively promoted by π – π interactions alone but also by cation – π interactions, which seem to promote tertiary structure formation and function greatly. Although these interactions are only well documented for soluble proteins thus far, they also appear to be crucial for the folding and stability of IMPs. This is because dimerization of TM segments is greatly enhanced by the interaction of charged residues and hydrophobic residues such as Lys with Trp, Tyr or Phe (Johnson, Hecht et al. 2007).

Ultimately, besides individual amino acids of different hydrophobicity, charge, or aromaticity, which cause non-covalent interactions of TM helices alone or in combination with others, helix-helix interactions are mediated by various highly conserved interaction motifs present in the TM regions of IMPs. The most prominent example of many well-known interaction motifs is the GxxG motif, first discovered in glycophorin A, a membrane protein of the erythrocytes (Lemmon, Flanagan et al. 1992, Lemmon, Treutlein et al. 1994, Russ and Engelman 2000).

As outlined above, helix-helix interactions are highly diverse and form the basis for the correct integration and stable interaction of TM segments within the membrane. Remarkably, however, it is assumed that interaction of TM segments and the formation of functional structures may occur even before membrane insertion. Either within the ribosomal exit tunnel, which is capable of promoting structure formation by providing a confined compartment, or inside the Sec61 translocon channel, which can accommodate more than two TM segments at the same time and thus in principle enables the promotion of interactions (Rapoport, Goder et al. 2004, Tu, Khanna et al. 2014, Cymer, von Heijne et al. 2015). In line with this, it is assumed that TM segments of nascent polypeptide chains can be retained within the translocation channel of Sec61, even until the translation is terminated, and then be collectively delivered into the surrounding lipid bilayer. Although this model contradicts the aforementioned “linear insertion model”, it

highlights the need for alternative integration mechanisms that can account for polar or charged residues within helical TM segments that do not follow the canonical view of a thermodynamically favorable integration of individual TM segments.

1.2.4 The secretory pathway

In order to fulfill their function, most newly made proteins in the membrane or the lumen of the ER, once correctly folded and assembled, exit the ER and travel via the secretory pathway to their target destination (Figure 3A). Of particular relevance for the journey of proteins along this pathway: The Golgi apparatus. The Golgi is composed of flattened, membrane-enclosed cisternae and represents an important site for synthesizing oligosaccharides. Furthermore, it mainly contributes to the sorting and trafficking of ER-derived proteins. In general, the secretory pathway refers to the selective and efficient trafficking of coated vesicles loaded with the protein to be transported between the ER and the Golgi as well as the cell membrane and the lysosomes or any other intracellular compartment. Although little is known about signals that initiate exit and guide proteins out of the ER, the exit process itself is a better understood and highly regulated process restricted to specific, randomly dispersed, ribosome-free subdomains within the ER membrane, the ER exit sites (ERES) (Figure 3B). (Watson and Stephens 2005, Peotter, Kasberg et al. 2019). In mammalian cells, the ER-to-Golgi transport is mediated by Coat Protein complex II (COPII)-coated vesicles. Depending on whether transmembrane or soluble molecules are exported from the ER, selection and loading of the respective cargo mediated by COPII occurs either directly or indirectly with the help of specific cargo receptors. In both cases, however, COPII causes deformation of the membrane at the ERES and, consequently, the generation of budded transport vesicles. Following scission, loaded cargo vesicles are delivered to the ER-Golgi intermediate compartment (ERGIC) located between the ER and the cis-site of the Golgi apparatus and mainly responsible for facilitating the sorting of cargo between these two organelles. Importantly, delivery of the cargo content to the ERGIC primarily requires the shedding of the COPII coating and subsequently fusion of the transport vesicles. Thereby, vesicular tubular clusters (VTC) are formed that

constitute a new compartment and lack ER proteins. (Kuehn, Herrmann et al. 1998, Appenzeller-Herzog and Hauri 2006). In contrast to the first COPII dependent transport step, forward transport from the ERGIC to the cis-Golgi network, representing the Golgi complex´s receiving cisternae, does not depend on COPII. However, migration of the carriers occurs in a microtubule-dependent process (Watson and Stephens 2005, Peotter, Kasberg et al. 2019).

Once present in the Golgi, secretory cargo traverses the different compartments of the Golgi and can undergo an ordered series of covalent modifications, amongst others, by a distinct set of glycosyltransferases and glycosidases. These enzymes, which are localized in the different compartments of the Golgi, mainly contribute to the processing and formation of complex oligosaccharides of glycoproteins originally glycosylated in the ER (Ohtsubo and Marth 2006). Finally, cargo undergoes repackaging in order to be released and delivered to the target destination. Although intra-Golgi trafficking is highly debated, two prevailing models are discussed. On the one hand, the cisternal maturation model and alternatively the vesicular transport model. According to the highly dynamic cisternal maturation model, the cargo is stably located in a given compartment, a newly formed cis cisterna following vesicle fusion, which in turn functions as a transient carrier and gradually traverses the Golgi stack. During progression through the stack, various Golgi enzymes of the medial and then trans cisternae are acquired through COPI vesicles that move in a retrograde traffic fashion from later to earlier cisternae and cause maturation of the initial cargo-loaded cisternae. Ultimately, the oldest cisternae, namely the trans-Golgi network (TGN), disintegrates into secretory vesicles or other types of carriers (Glick and Luini 2011, Luini 2011). In the vesicular model, the different compartments of the Golgi from cis to trans are primarily static, and the characteristic set of Golgi processing enzymes remains unchanged while cargo proteins in transit move through the cisternae. Unlike the previously described model, the transport of cargo mediated by COPI vesicles mainly occurs in an anterograde traffic fashion where cargo is moved forward from one cisterna to the next in the direction of the TGN (Glick and Luini 2011).

From here, proteins can take different trafficking routes and are transported either to the endolysosomal system, the plasma membrane, or beyond to the

extracellular region (Figure 3C). However, not all proteins continue their journey along the secretion pathway. Some, which reside in the Golgi are retained and subsequently return to their appropriate cisterna, or, if resident in or escaped the ER, are transported in a COPI-dependent fashion from the cis-Golgi back to the ER (Figure 3D)(Schekman and Orci 1996). Due to the numerous possible destinations a protein can be targeted to after exiting the ER, it becomes evident that the sorting process at the TGN essentially requires many specialized vesicle carriers and trafficking pathways.

Required for the retrieval pathway is the presence of an ER retrieval sequence, which can be a C-terminal cytosolic dilysine (e.g., KKxx or KxKxx) or luminal KDEL motif, which allows interaction with the COPI coat either directly via distinct repeat domains or is mediated by a specific KDEL receptor (KDELR) that can be found in the membrane of the cis-Golgi compartment (Lewis and Pelham 1992, Orci, Stamnes et al. 1997, Aoe, Lee et al. 1998, Jackson, Lewis et al. 2012). The binding and release of proteins carrying a KDEL sequence is mainly facilitated based on varying pH-values established in the different compartments of the secretory pathway. Whereas the comparatively more acidic pH-value inside the lumen of the cis-Golgi increases the affinity of the KDELR for the KDEL sequence and consequently promotes the incorporation of cargo into COPI vesicles, the neutral pH-value in the ER contributes to the release from the receptor once the vesicle complex arrives at the ER (Brauer, Parker et al. 2019).

However, besides the KDEL retrieval pathway, other models enable the anchoring of proteins already in the ER or determining their location in the cell. For instance, it is assumed that in order to remain in their organelle, ER-resident proteins, independent of their KDEL signal, bind to each other and form larger complexes that vesicles can no longer transport. Since the concentration of ER-resident proteins inside the ER is very high, comparatively low-affinity interaction would be sufficient for such complexes to arise. Interestingly, Golgi processing enzymes that function together in the same compartment prevent their passaging into transport vesicles by the exact same aggregation mechanism (Nilsson, Slusarewicz et al. 1993, Bruce, Johnson et al. 2002). Retention of Golgi-localized proteins is furthermore facilitated based on distinct retention motifs as recently, a conserved KXD/E motif has been discovered that directly interacts with COPI

(Gao, Cai et al. 2014). Another sorting mechanism, relevant if the cargo involves membrane proteins, is mediated by the length of their TM region. The TMD of membrane domains dictates the partitioning process into the membrane of vesicles and different compartments, but additionally, it seems that the length of a TMD is optimized to match the thickness of the lipid bilayer in which it resides (Sharpe, Stevens et al. 2010). Thus, whereas shortening of the TMD can cause retention, lengthening of this region allows Golgi-localized membrane proteins to progress in the secretory pathway and relocate via vesicular transport to the plasma membrane (Bonifacino and Traub 2003, Borgese 2016).

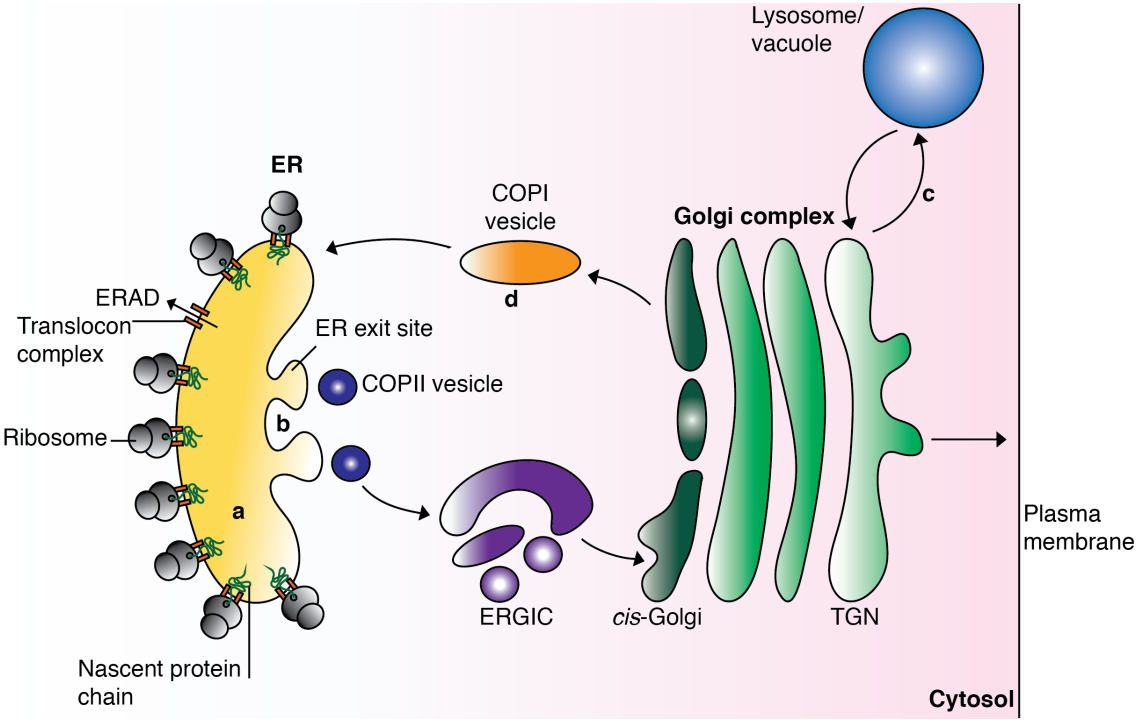


Figure 3: Intracellular protein traffic – the secretory pathway. (A) Once correctly folded and assembled, native membrane and soluble proteins enter ribosome-free subdomains within the ER membrane, the ER exit sites **(B),** from where their journey along the secretory pathway begins. At the ER exit sites, coatomer protein (COP) II causes deformation of the membrane and, consequently, the budding of cargo-loaded transport vesicles. Following scission, COPII coated cargo vesicles traffic via the ER-Golgi intermediate compartment (ERGIC) to the cis-site of the Golgi apparatus. From here, secretory cargo traverses the different compartments of the Golgi and undergoes an ordered series of covalent modifications mediated by glycosyltransferases and glycosidases. **(C)** From the TGN, proteins are either transported to the plasma membrane and beyond or, delivered to the endolysosomal system for degradation. **(D)** In certain cases, however, proteins that are misfolded and

escaped the ER or ER-resident proteins do not proceed their journey along the secretion pathway, but are instead transported in a COPI-dependent manner from the cis-Golgi back to the ER (Figure based on Ellgaard and Helenius 2003).

Upon arrival of the transport vesicles at the plasma membrane, IMPs are embedded in the plasma membrane and fulfill their specific functions. In contrast, soluble proteins are secreted to the extracellular space in a process called exocytosis. The reverse process, internalization, is carried out amongst others by endocytosis and includes the uptake of extracellular material and ligands as well as plasma membrane proteins that have just arrived at the membrane. Although endocytosis can be divided into clathrin-dependent and clathrin-independent pathways, where proteins based on a cytoplasmic recruiting and internalization sequences are packaged either into clathrin-coated or uncoated carriers, both pathways result in the formation of the early endosome through the fusion of the endocytic cargo carriers. From the early endosome system, which functions as a major sorting site, cargo is either sorted for degradation or retrieved from this decision and guided into recycling processes. Notably, these fateful sorting decisions can already be made during endocytosis or mediated by pre-early endosome compartments (Lakadamyali, Rust et al. 2006, Jean-Alphonse, Bowersox et al. 2014). Thus, recycling selected cargo back to the cell surface can occur directly through uncoated vesicular carriers or indirectly. In this case, cargo traverses to an endocytic recycling compartment first. Moreover, recycling back to the cell surface can also be carried out by delivering the cargo back to the TGN. This process is essentially dependent on Rab protein family members and allows cargo to enter the secretory pathway again (Grant and Donaldson 2009, Jovic, Sharma et al. 2010). However, cargo that fails to be recycled back is retained in the early endosomes and sorted into specialized vesicles. Following the maturation of the early into the late endosomes, fusion with the lysosome contributes to the formation of an endolysosome where cargo is ultimately degraded (Maxfield and McGraw 2004, Cullen and Steinberg 2018).

Remarkably, lysosomal degradation and the role of the Golgi apparatus in the early secretory pathway are important checkpoints in a highly sophisticated and redundant network of quality control pathways. Although these quality control checkpoints do not represent the first line of defense, they contribute to protein

homeostasis (Arvan, Zhao et al. 2002, Sun and Brodsky 2019). Nevertheless, quality control already starts inside the ER and ensures that only correctly folded proteins can leave the ER. Consequently, this should prevent misfolded or misassembled proteins from interfering with the function of native proteins if they are transported onward. Of note, the complex machinery involved in IMP biogenesis immediately highlights that this process is not straightforward and error-prone. Therefore, quality control appears to be particularly relevant for this protein class. Remarkably, however, only very little is known about cellular quality control mechanisms that detect, signal and repair or degrade intermediates during the biogenesis of IMPs.

1.3 Cellular quality control mechanisms for membrane proteins

Proteins of the secretory pathway, including membrane proteins, are key for cellular communication. Since they are involved in fundamental cellular functions, including transmitting information regarding cell division, migration, differentiation, or even cell death, it is crucial that only correctly folded proteins exit the ER and reach the cell surface – where control measures are mostly absent. Once a nascent polypeptide chain that emerges from the ribosome exit tunnel is translocated from the cytosol across the membrane and inside the ER lumen, it is received by a unique environment that perfectly suits folding, assembly, and post-translational modifications (PTMs). Besides a variety of folding factors important for guiding and monitoring protein folding, the ER itself provides an exceptional oxidative environment that supports the formation of disulfide bonds, which contribute to stabilizing the structure of a protein. Folding factors present in the ER can be classified into folding enzymes that promote or stabilize protein folding and molecular chaperones, which actively prevent misfolding by binding and masking aggregation-prone regions. Regardless of their specialized functioning, however, all folding factors have in common that they cause newly synthesized proteins to fold more efficiently. In addition, the ER is equipped with enzymes that mediate N-linked glycosylation (Wei and Hendershot 1996, Kleizen and Braakman 2004). Notably, the decoration of a nascent polypeptide chain with sugar moieties is not just a “simple” PTM that is supposed to increase proteomic diversity but, in the ER, glycosylation is linked to

the folding status of a protein and functions as a quality control mechanism. This ensures that only proteins that have reached their native conformation can reach their target destination, and those that fail and persist in their non-native or incompletely assembled state either be retained or sent to degradation.

Compared to soluble proteins, the topology of IMPs provides an extra dimension since these proteins are exposed to at least two very distinct environments, making correct folding a complex and challenging task. While one

part of a membrane protein resides within a membrane´s lipid environment, its soluble domains on one or both sides of the TM region can face either luminal structures, the cytosol, or the extracellular space. In addition, the simultaneous assembly of TMD segments within the membrane involves multiple intermediate states and can comprise more than 20 TM helices (Houck and Cyr 2012). For this reason, it is of particular relevance that different types of machinery and quality control mechanisms exist that ensure functionality in different environments.

1.3.1 Glycan-dependent ER quality control

N-linked protein glycosylation in the ER is the most common modification of both membrane and soluble proteins in eukaryotic cells and is catalyzed by a single enzyme, the oligosaccharyltransferase (OST). This highly conserved process is characterized by assembling a lipid-linked uniform precursor oligosaccharide and the subsequent transfer of this glycan to the nascent polypeptide chain emerging into the ER lumen. Essentially required for this glycan transfer to occur is the presence of an Asn residue present in an N-X-S/T sequon, where X can be any amino acid except proline (Figure 4). Consequently, this allows the formation of an N-glycosidic linkage between the glycan and the side chain of the Asn residue (Mohorko, Glockshuber et al. 2011, Aebi 2013)

The human OST is a hetero-oligomeric membrane protein complex composed of seven different subunits. The active site for glycan transfer is formed by the two paralogous forms STT3A and STT3B, of which only one is incorporated into the complex (Kelleher, Karaoglu et al. 2003). Even though all STT3 proteins share structural similarities, both homologous subunits differ significantly in their catalytic activity and substrate selectivity. Whereas STT3A is associated with the

translocon, can scan for consensus sites and perform glycosylation co-translationally, STT3B is in a somewhat distant position and carries out glycosylation post-translationally once the nascent polypeptide is released into the ER. This is particularly relevant for very C-terminal glycosylation sites (Ruiz-Canada, Kelleher et al. 2009, Shrimal, Trueman et al. 2013). Thus, STT3B can modify skipped sequons or compensate for failed transfer reactions missed by co-translational scanning of the STT3A complex (Shrimal, Cherepanova et al. 2015). This effect is reinforced by the observation that STT3B is more active in terms of glycopeptide formation and, at the same time, exhibits a reduced selectivity towards the oligosaccharide donor substrate that is transferred from the dolichol-pyrophosphate-activated carrier to the substrate protein (Mohorko, Glockshuber et al. 2011). Although most of the other subunits are highly conserved, their function, except the N33 subunit, is unknown. Interestingly, N33 can slow down protein folding due to the formation of transient disulfide bonds with OST substrates. This process is mediated by a membrane-bound thioredoxin domain facing the lumen of the ER and presumably contributes to an increase in glycosylation efficiency (Mohorko, Owen et al. 2014).

Besides primarily assisting in protein folding of newly synthesized proteins, glycosylation serves a multitude of functions inside the ER (Paulson 1989, Helenius 1994). For example, N-linked glycosylation enables sorting of glycoproteins, improves solubility, and at the same time reduces aggregation or dictates degradation as a consequence of trimming. Of particular relevance, however, N-linked glycans, after processing of the primary oligosaccharide, represent distinct recognition motifs that can direct folding chaperones such as Calnexin (CNX) or Calreticulin (CRT) (Mohorko, Glockshuber et al. 2011, Guerriero and Brodsky 2012).

Glycan processing begins shortly after a precursor oligosaccharide, which is assembled first in the cytosol and then the ER lumen by several glycosyltransferases is transferred from its lipid pyrophosphate donor residing in the ER membrane, dolicholpyrophosphat, to a selected Asn residue present in the nascent polypeptide chain (Helenius and Aebi 2004, Caramelo and Parodi 2008). In the following, the sequential action of glucosidases I and II results in the trimming and removal of the two outermost glucose residues of $Glc_3Man_9GlcNAc_2$

whereby the $Glc_1Man_9GlcNAc_2$ epitope is uncovered. This monoglucosylated polymannose glycan structure is of particular relevance as it represents a unique and high-affinity recognition motif for the two lectin chaperons CNX and CRT, which will be discussed in more detail below (Ou, Cameron et al. 1993, Zapun, Petrescu et al. 1997, Deprez, Gautschi et al. 2005, Caramelo and Parodi 2008).

Although CNX and CRT share a high degree of sequence identity, they differ significantly in terms of activities and substrate specificities (Molinari, Eriksson et al. 2004). Whereas CNX is a translocon-associated membrane protein and can co-translationally bind substrates, the soluble protein CRT primarily interacts with secreted proteins after being released from the ribosome. Both chaperones, however, when bound to the glycosylated substrate, fulfill similar functions. They mediate ER retention, prevent aggregation and degradation, and beyond that, recruit different folding enzymes such as PDI or ERp57, which in turn assist and catalyze maturation and folding of the non-native glycoprotein (Guerriero and Brodsky 2012, Kozlov and Gehring 2020). This enhanced catalysis has been nicely demonstrated in the maturation process of monoglucosylated RNaseB, where the interaction of this glycoprotein with CNX and ERp57 significantly promotes disulfide bond formation (Zapun, Darby et al. 1998). Dissociation then occurs once further action of glucosidase II removes the remaining innermost glucose moiety. Consequently, this renders substrate glycoproteins decorated with $GlcNAc_2Man_9$ unable to bind to CNX or CRT and ends the first folding cycle. In the following, glucose-free glycoproteins, if folded correctly, are guided out of the ER and progress in the secretory pathway (Figure 4).

However, if folding is delayed at this point, and the protein still displays a non-native structure, reglucosylation of the glycoprotein by UPD-glucose:glycoprotein glucosyltransferase (UGGT) occurs and allows the glycoprotein to re-enter the CNX and CRT cycle. Thereby, several cycles of CNX/CRT-glycoprotein binding and release catalyzed by the opposite activities of glucosidase II and UGGT can be completed until the glycoprotein has attained its native conformation, which consequently prevents reglucosylation by UGGT (Sousa and Parodi 1995, Trombetta and Helenius 2000, Caramelo and Parodi 2008). Notably, within this cycle, the soluble enzyme UGGT is the only component that recognizes exposed hydrophobic surfaces of non-native substrate glycoproteins and partially

assembled subunit components and thus able to sense protein conformations (Sousa and Parodi 1995, Keith, Parodi et al. 2005). To date, however, it is not clear how the cell distinguishes whether a protein is just delayed in folding or permanently misfolded, which would require the protein to exit the cycle and allow proteasomal degradation to proceed.

Nevertheless, two proteins are known which mediate the exit of terminally misfolded proteins from the CNX/CRT cycle by trimming of mannose residues: The membrane-bound ER α1,2-mannosidase I and the soluble ER degradation-enhancing mannosidase-like proteins (EDEMs) (Frenkel, Gregory et al. 2003, Zuber, Cormier et al. 2007). Based on the finding that these enzymes are relatively slow in terms of mannose trimming, a mannose timer model was proposed that allows slowly folding proteins to reach their native conformation before they are sent to glycoprotein endoplasmic reticulum-associated degradation (ERAD) (Figure 4) (Caramelo and Parodi 2015). Responsible for sending substrates to ERAD are two different ER lectins, namely OS-9 and its functional homolog XTP3-B, which only bind to the glycoprotein following mannose trimming and recognize the resulting terminal mannose residue as a degradation signal (Hosokawa, Wada et al. 2008, Hosokawa, Kamiya et al. 2009, Yamaguchi, Hu et al. 2010). Subsequently, both proteins deliver the permanently misfolded glycoprotein for ERAD degradation to the HMG-CoA reductase degradation 1 (Hrd1) protein, which was first identified in a genetic screen for mutants that exhibit impaired HMG-CoA reductase degradation activity (Hampton, Gardner et al. 1996, Hosokawa, Kamiya et al. 2009, Hwang, Walczak et al. 2017). Importantly, besides the N-glycan-dependent one, alternative mechanisms exist that monitor quality control and increase folding efficiency. These mechanisms do not apply to glycoproteins only but to all proteins, regardless of their origin and individual characteristics.

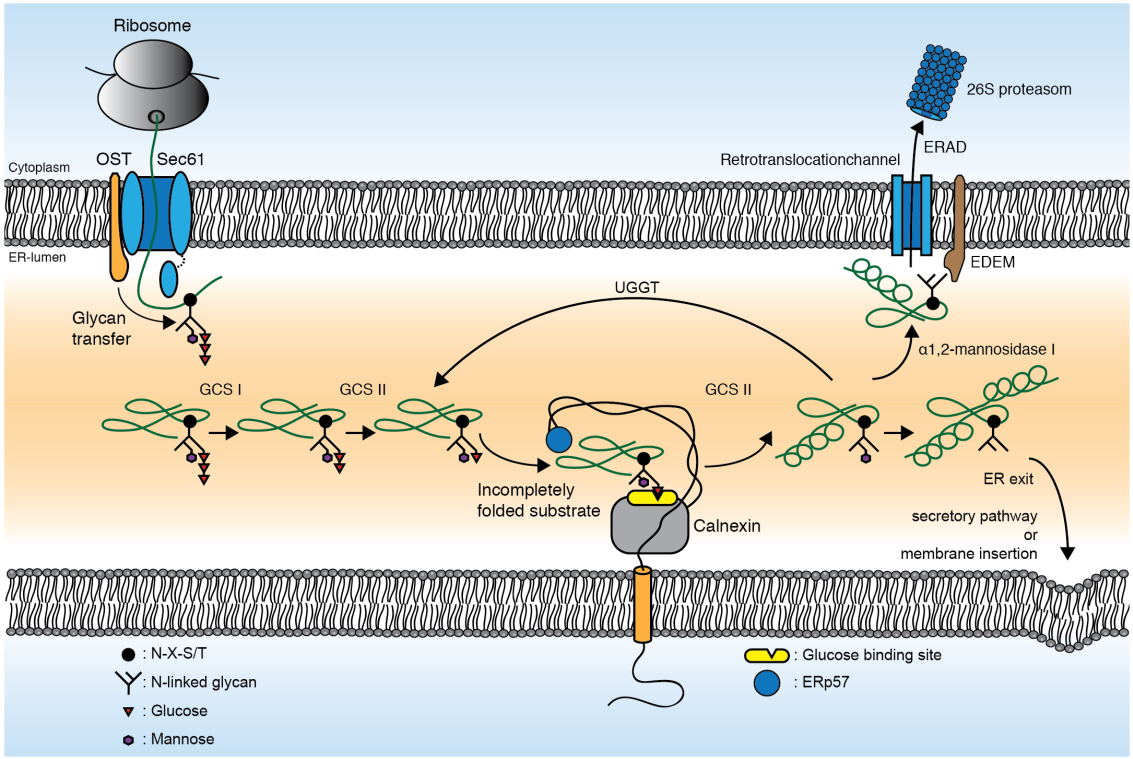


Figure 4: Calnexin mediated folding and quality control of glycosylated proteins inside the ER: Once a nascent polypeptide chain emerges into the ER lumen, the oligosaccharyltransferase (OST) enzyme complex mediates N-linked protein glycosylation. Following the sequential action of glucosidases I and II, the two outermost glucose residues are removed, and CNX recognizes monoglucosylated oligosaccharides. By recruiting different folding enzymes such as ERp57 to the arm domain, CNX assists and catalyzes the maturation and folding of the non-native glycoprotein. Further action of glucosidase II, which removes the remaining innermost glucose moiety, renders the substrate glycoproteins unable to bind to CNX and initiates ER exit of correctly folded glycoproteins. However, if folding is delayed at this point, and the protein still displays a non-native structure, reglucosylation of the glycoprotein by UPD-glucose:glycoprotein glucosyltransferase (UGGT) occurs and allows the glycoprotein to re-enter the CNX cycle. In contrast, permanently misfolded glycoproteins are processed by ER α1,2-mannosidase I, which leads to the removal of the mannose residue in the middle branch of the oligosaccharide. Subsequently, mannose trimmed glycoproteins are recognized by the ER degradation-enhancing mannosidase-like proteins (EDEMs), which targets glycoproteins for ER-associated degradation (ERAD) by the 26S proteasome.

1.3.2 Chaperoning in the ER

Inside the ER lumen, molecular chaperones and folding modulators are highly abundant and play a central role in the conformational quality control of the

proteome and folding of immature proteins. Whereas natively folded protein conformers are not detected by molecular chaperones and free to leave the ER, only slight deviations from the native conformation can lead to the protein being recognized as defective or immature, causing transient binding by one or more of these factors. ER-associated chaperones, however, significantly differ in their substrate specificity and how they assist in folding and degradation or prevent aggregation. In the past, numerous ER chaperones have been identified, with the soluble immunoglobulin heavy chain binding protein (BiP) being a particularly central player. BiP is mainly involved in ER protein folding and assembly by shielding hydrophobic residues from the aqueous environment (Buck, Wright et al. 2007, Behnke, Mann et al. 2016). Thus, masking of hydrophobic patches that are normally buried inside the hydrophobic core of a native protein by BiP is required since these unstructured polypeptides are prone to aggregation when they emerge in the aqueous environment of the ER. Interestingly, however, if the emerging substrate is a glycoprotein, the association of BiP is often preceded by an association of the substrate with CNX or CRT, as these chaperones recognize already short nascent chains of approximately 12 residues length once they emerge in the ER lumen (Andersson, Nilsson et al. 1996, Lilley and Ploegh 2004, Hebert and Molinari 2007). Except for glycoproteins that bear a very N-terminally located N-glycan, this prevents BiP´s assistance to the nascent chain until the substrate is released by CNX or CRT (Molinari and Helenius 2000, Hebert and Molinari 2007). However, it is undisputed that BiP is a master regulator because of its unique role in various processes and functions in the ER (Hendershot 2004).

BiP belongs to the Hsp70 chaperone family and was first discovered based on its specific interaction with antibody heavy chains (Haas and Wabl 1983). In addition, it was shown shortly thereafter that BiP preferentially binds relatively short peptides or aliphatic residues and accommodates them in an energetic environment that resembles that of a folded protein (Flynn, Pohl et al. 1991). BiP is formed by two functional domains: a highly conserved N-terminal nucleotide-binding domain (NBD), with ATPase activity, and a substrate-binding domain (SBD) that at its C-terminal end contains an α-helical domain that functions as a lid. Both domains are connected by a flexible hydrophobic linker allowing for allosteric interaction. Like all Hsp70 family proteins, the binding of BiP to unfolded

substrates and its release is tightly regulated by its nucleotide bound state. In the ATP-bound form, BiP favors a conformation in which its NBD and SBD are close to each other with its lid open. This conformation results in a high on/off rate for substrate binding, which means that binding of substrates occurs with only low affinity and allows them to be released and fold. In contrast, following ATP hydrolysis, BiP reaches a state with low on/off rates for substrate binding, characterized by a conformational change, whereby NBD and SBD are separated from one another. In addition, the lid undergoes a relative movement over the SBD and causes high-affinity binding of the substrate to this domain (Otero, Lizak et al. 2010, Behnke, Feige et al. 2015).

Notably, the activity of all chaperones is accompanied by numerous co-chaperones and folding enzymes. This also applies to BiP, whose chaperone cycle is not only controlled by ATP but also by ER-localized DnaJ (ERdj) proteins and the nucleotide exchange factors (NEFs) that perform the transition from the ADP to the ATP bound state of BiP (Misselwitz, Staeck et al. 1998, Behnke, Feige et al. 2015, Kityk, Kopp et al. 2018). In the ER, this ATPase cycle is regulated by the NEFs SIL1 and glucose-regulated protein of 170 kDa (Grp170). By stimulating the release of ADP and allowing new ATP molecules to bind to the BiP complex, these proteins facilitate the release of substrates due to an opening of the lid on the SBD (Chung, Shen et al. 2002, Behnke, Feige et al. 2015). Interestingly, the activity and function of Grp170 seem to be more far-reaching. Due to its intrinsic chaperone property, Grp170 exhibits important cytoprotective activity by binding to many unfolded proteins, similar to BiP. In contrast to BiP, however, Grp170 does not mediate the release of its unfolded substrate in the presence of ATP (Behnke and Hendershot 2014). Furthermore, it has been shown that this chaperone is involved in ERAD, where it can function as a holdase that does not depend on its ATPase activity or interaction with BIP. Besides that, it has been demonstrated that Grp170 binds to suppressor/enhancer of Lin-12-like (SEL1L) which is an indispensable component of the HRD1 ubiquitin ligase complex and consequently links Grp170 activity to the degradation of misfolded substrates (Buck, Kolb et al. 2010, Buck, Plavchak et al. 2013, Inoue and Tsai 2016).

Within the BiP cycle, ERdj proteins perform the exact opposite function. Following binding to ATP-bound BiP, ERdj proteins stimulate ATP hydrolysis and thus cause greater interaction between BiP and its substrate. In addition, ERdj proteins can also bind unfolded substrates themselves and assist in the initial delivery of these to BiP (Misselwitz, Staeck et al. 1998, Braakman and Hebert 2013). Important for these functions and binding of BiP, is a conserved domain of approximately 70 amino acid residue length, the J domain. Thus, this protein family is also referred to as J-domain proteins. Importantly, within the J-domain, a conserved HDP motif is responsible for stimulating the ATPase activity of BiP, (Liberek, Marszalek et al. 1991, Tsai and Douglas 1996). In the mammalian ER, at least seven J-domain proteins (ERdj1-7) are known.

Despite all of them supporting the ER´s various functions, their range of substrates and effect on protein folding and degradation or preference for distinct binding sites is diverse (Otero, Lizak et al. 2010, Behnke, Mann et al. 2016). The integral membrane J-proteins ERdj1 and ERdj2 are often associated with the Sec61 translocon and assist maturation of nascent chains as they connect ER-luminal BiP functions with the cytosolic translation machinery. While ERdj1 assists in recruiting ATP-bound BiP and support positioning at the translocon pore to ensure that BiP is available for emerging nascent polypeptide chains following translocation, ERdj2 together with BiP and Sec62 controls opening of the Sec61 translocon pore. ERdj3 and ERdj6 function as pro-folding co-chaperones because they recognize unfolded substrates and maintain them in a soluble state. In contrast, ERdj4 and ERdj5 have the opposite effect as they bind aggregation-prone proteins and enhance their degradation. Interestingly, ERdj5 is not only a BiP cofactor but at the same time, also belongs to the family of protein disulfide-isomerases (PDI), which will be discussed in the next paragraph. Due to its high reductive potential, ERdj5 has a unique function in reducing disulfide bonds in ERAD substrates. ERdj7 is the most recently discovered and less studied ER J-domain protein. However, recent findings indicate that it might be involved in translocation processes and the degradation of misfolded or unfolded proteins (Braakman and Hebert 2013, Pobre, Poet et al. 2019, Ohta and Takaiwa 2020).

However, the activity of BiP is not only regulated by cofactors or rapid expression following protein-folding stress but also by various PTMs, including phosphorylation, acetylation, ubiquitination, AMPylation, and ADP-ribosylation. Although the understanding of how most of these PTMs events impact the functions of BiP remains elusive, it has been demonstrated that they are predominantly localized to the NBD and SBD (Freiden, Gaut et al. 1992, Nitika, Porter et al. 2020).

Besides the Hsp70 family member BiP, the ER also contains a chaperone of the Hsp90 family, glucose-regulated protein of 94 kDa, Grp94. Similar to BiP, nucleotide binding to the N-terminal domain (NTD) of Grp94 and hydrolysis leads to conformational changes, which influences the opening and closing and, therefore, the chaperones activity (Argon and Simen 1999, Braakman and Hebert 2013). Interestingly, Grp94 seems especially important for those substrates that represent complicated folding intermediates since Grp94 only recognizes some proteins after their release from BiP (Melnick, Dul et al. 1994)

Another important group of ER-resident folding factors is represented by folding catalysts, of which the oxidoreductase PDI is the most abundant and best characterized (Wallis and Freedman 2013). This family of enzymes is defined by the presence of a conserved CXXC active-site motif that serves to form, isomerize or reduce disulfide bonds. To this end, the cysteines in the CXXC motif itself can be found in either a reduced or oxidized state (Ellgaard and Ruddock 2005). Disulfide bond linkages within and between constituent chains of a polypeptide are generally formed in the ER and therefore primarily found in extracellular, periplasmic, and secreted proteins where they confer structural stability. Moreover, selective redox regulation of disulfides can be an important mechanism to control the activity of a protein. Of note, the ER lumen is more oxidizing than the cytosol and thus provides ideal conditions for formation of disulfide bonds within polypeptides that are formed once the nascent chain is post-translationally translocated into the ER lumen (Chen, Helenius et al. 1995, Margittai and Sitia 2011). At the same time, however, this environment also induces spontaneous, non-native disulfide bond formation. Remarkably, the temporary occurrence of non-native disulfide bonds is not always problematic but may be necessary and arises in different intermediate states of protein maturation

and folding. Hence, oxidoreductases that assist and enhance oxidative protein folding and prevent the occurrence of erroneous disulfide bonds in the final native structure are of great importance (Braakman and Bulleid 2011, Bulleid 2012). The canonical PDI and its role in oxidative protein folding was discovered more than 50 years ago, when it was shown that PDI is required to catalyze the oxidative reactivation of reduced ribonuclease A (Venetianer and Straub 1963, Givol, Goldberger et al. 1964). To date, more than 20 oxidoreductases are known, all of which exhibit different substrate specificity and redox potentials and thus catalyze oxidoreduction reactions in different ways (Ellgaard and Ruddock 2005, Braakman and Bulleid 2011). Moreover, other ER-resident proteins have been identified that are involved in the PDI-mediated reductive and oxidative protein folding pathway and contribute to its function. One of these proteins is ER oxidoreduction 1 (Ero1), which is essentially required to keep PDI in an oxidized active state in that it transfers disulfide bonds to and accepts electrons from PDI by the help of its flavin cofactor and the oxidizing power of molecular oxygen (Pollard, Travers et al. 1998, Sevier and Kaiser 2008, Benham, van Lith et al. 2013).

PDIs, are not just foldases required to accelerate the formation and shuffling of disulfide bonds. They also actively prevent aggregation of retaining misfolded and unassembled proteins in a process independent of the presence of disulfides in a substrate or the CXXC motif in the active site of PDI. In this way, PDI functions as a true molecular chaperone (Cai, Wang et al. 1994, Quan, Fan et al. 1995, Bottomley, Batten et al. 2001, Hebert and Molinari 2007). In addition, PDIs are heavily involved in ERAD as they can render terminally misfolded proteins retrotranslocation-competent. Based on their reductase activity, PDIs can chaperone the unfolding of ERAD substrates and thus facilitate the dislocation of these through the likely narrow retrotranslocation channel into the cytoplasm for proteasomal degradation. Indeed, it has been shown that the cleavage of disulfide bonds as a prerequisite for efficient retrotranslocation depends on the activity of ERdj5 (Ushioda, Hoseki et al. 2008, Bulleid 2012, Ellgaard, Sevier et al. 2018).

In addition to the quality control system present in the ER lumen, cytosolic chaperones and ubiquitin ligases exist that assist in the folding and quality control of polytypic IMPs if they possess cytosolic domains (Houck and Cyr 2012).

1.3.3 Endoplasmic reticulum-associated protein degradation

ER chaperones and chaperone-like proteins that have evolved in the ER aid folding and scrutinize conformations of their substrate proteins. Despite their activity, misfolding of individual proteins and the incorrect assembly of multi-protein complexes are quite common and bear deleterious consequences for the cell, either due to a loss of function for specific proteins or even a toxic gain of function, e.g., during aggregate or amyloid formation. For this reason, the ER is additionally equipped with two interconnected quality control systems that specifically deal with terminally misfolded or aggregated proteins: A large load of incompletely folded proteins is recognized by sensors of the unfolded protein response (UPR). Following activation, the UPR mainly reduces the ER folding load and causes the transcriptional up-regulation of proteins that increase the protein folding capacity of the ER, such as the aforementioned ER-resident chaperones, which will be discussed in more detail below (Ellgaard and Helenius 2003, Guerriero and Brodsky 2012, Gardner, Pincus et al. 2013). In parallel, if recognized as defective, improperly folded proteins are retrotranslocated back into the cytoplasm, where they are subject to degradation via ERAD by the ubiquitin-proteasome system (Ahner and Brodsky 2004, Vembar and Brodsky 2008). Of particular relevance, misfolding of membrane proteins bears conceptually different scenarios compared to folding abnormalities in soluble proteins. Degradation must occur regardless of whether the misfolded domain is located in the ER lumen, the cytoplasmic side of the ER, or in the ER membrane where entire membrane segments may be destabilized and misintegrated due to point mutations or fail complex assembly caused by lacking binding partners. As a result of the various topologies of substrates, ERAD is classified into three different pathways: ERAD-L refers to the degradation of proteins with a misfolded ER luminal domain, ERAD-M refers to the degradation of membrane proteins with a misfolded domain in the ER membrane, and ERAD-C refers to the degradation

of proteins where the misfolded domain is located on the cytoplasmic side of the ER (Figure 5) (Carvalho, Goder et al. 2006).

Although ERAD is the most common degradation mechanism for removing numerous normal and misfolded ER-localized proteins, alternative pathways exist that are not limited to proteins as substrates and important for proteins that cannot undergo ERAD. Instead of proteasomal degradation, these pathways use the second most prominent degradation system in the cell: the autophagy-lysosome system. Autophagic degradation (ER-phagy) enables the disposal of entire parts of the ER, including its membrane lipids and protein aggregates. This can be achieved by forming of small ER-derived vesicles or large autophagosomes that enclose entire fragments of the ER and deliver internal material like aggregates to the lysosomes. In this way, ER-phagy contributes significantly to controlling the size of the ER (Wilkinson 2019, Chino and Mizushima 2020).

1.3.3.1 Substrate recognition in ERAD

In order to become accessible to the proteasome, misfolded ER proteins have first to be recognized and then be retro-translocated into the cytosol, constituting two critical steps in ERAD. Although these steps equally apply to all ERAD pathways, the key challenge for the different ERAD mediators right from the start is defining the structural basis for recognizing specific substrates and discriminating if a protein is in the process of folding and maturation or determined for ERAD. This is particularly challenging if the substrate is a membrane protein. The reason for this is that there is no uniform structural signature known based on which a substrate is recognized as defective and degraded (Nakatsukasa and Brodsky 2008). For instance, whereas exposed hydrophobic residues inside the ER lumen or on the cytoplasmic side are potentially harmful and recognized by the ERAD-L or ERAD-C machinery, respectively, hydrophobicity is to be expected within the membrane, which is why ERAD-M mediators would not recognize such a signature in transmembrane proteins, but rather recognize exposed polar residues in orphaned TM segments.

Several factors contribute to recognizing ERAD-L substrates and may also act on luminal domains of membrane proteins. Recognition of incompletely folded

proteins in the ER lumen is mainly carried out by chaperones of the Hsp70 family and lectin chaperones (Guerriero and Brodsky 2012). Following binding to hydrophobic patches, the ER-resident Hsp70 chaperone BiP, with the aid of its Hsp40 co-chaperones, can mediate the folding or degradation of its clients. However, besides BiP, PDI might also be implicated in recognizing some ERAD substrates since depletion of PDI prevents their degradation (Wang and Chang 2003). Immature glycoproteins that are intended for ERAD, on the other hand, are recognized by their glycosylation status. Together with an unfolded polypeptide segment, the resulting terminal mannose residues constitute the degradation signal, recognized by other lectins like OS-9 and XTP3-B as mentioned before (Hosokawa, Wada et al. 2008, Hosokawa, Kamiya et al. 2009). Regardless of whether the substrate is a glycoprotein or not and recognized by OS-9 or BiP, respectively, subsequent association with the lectin SEL1L, a cofactor of Hrd1, is required to promote degradation (Christianson, Shaler et al. 2008).

Only a few factors have been identified that can assist in the folding and degradation of terminally misfolded membrane proteins. The ER membrane-associated E3 ubiquitin-ligase Hrd1 and its homolog cell surface glycoprotein of 78 KDa (gp78) are considered essential proteins required to recognize ERAD-M substrates and luminal domains of polytypic membrane proteins. Presumably, recognition and binding of misfolded TM domains is mediated based on interaction with the TM domain of Hrd1 itself, which contains many hydrophilic amino acids, most of which are also conserved in gp78 (Sato, Schulz et al. 2009). Notably, although gp78 on amino acid-sequence level is a homolog to Hrd1, its interaction network and substrates are largely different. Consistently, the degradation of orphaned CD3δ, an ERAD-M substrate and component of the T-cell antigen receptor CD3 complex, depends on gp78, however, it does not require Hrd1 (Fang, Ferrone et al. 2001). Moreover, it has been demonstrated that both enzymes act sequentially, whereby gp78 is localized downstream, and its function is coupled to interaction with BAG6, a cytosolic chaperone (Zhang, Xu et al. 2015). For the process of substrate recognition, Hrd1 forms complexes with distinct cofactors, including SEL1L, OS-9, and Derlin-family proteins (Derlins), subsequently promoting the ubiquitination and degradation of the

substrate protein (Houck and Cyr 2012). While SEL1L and OS-9 represent recognition factors for luminal substrates, Derlin-1 serves as a TM chaperone.

Derlins are multipass membrane proteins with four to six TM helices and represent inactive members of the Rhomboid superfamily of intra-membrane proteases. Mammals, contain three members, Derlin-1, Derlin-2, and Derlin-3, which interact with their clients without cleaving them. By forming complexes with destabilized TM segments, Derlin-1 functions as a recognition factor for misfolded TM domains, causing ER retention and subsequent degradation of selected substrates (Sun, Zhang et al. 2006, Houck and Cyr 2012, Guna and Hegde 2018). Interestingly, the TM segments of Derlin-1 have only low hydrophobicity due to several polar and charged residues that might be involved in substrate recognition processes (Greenblatt, Olzmann et al. 2011). Moreover, it is assumed that through the cooperative interaction of Derlins together with Hrd1 or gp78 and other ER ligases, which all have differing specificity, the range of substrates that are recognized and degraded increases (Houck and Cyr 2012).

In contrast to the aforementioned inactive members, active members of intra-membrane proteases after selective TMD recognition mediate proteolysis of their substrate in the plane of the membrane. In mammals, five different rhomboid proteases are known, most of which are located in the secretory pathway but with differing functions (Bergbold and Lemberg 2013). Interestingly, the intra-membrane rhomboid-like 4 RHBDL4 has been shown to localize to the ER, which, with a few exceptions is free of proteases, as these would counteract the protein folding taking place in the ER (Fleig, Bergbold et al. 2012). Nevertheless, cleavage of other ubiquitinated single spanning and polytypic membrane proteins already in the ER membrane, such as the SST3A paralogue of the OST or orphaned T-cell receptor (TCR-α) subunits, has been demonstrated. Furthermore, by direct interaction with the p97 ATPase, RHBDL4 has been linked to the canonical ERAD pathway. Remarkably, many ERAD substrates of RHBDL4 have positively charged residues within their TM segments contributing to recognition (Fleig, Bergbold et al. 2012, Bergbold and Lemberg 2013, Knopf, Landscheidt et al. 2020).

Recognition processes and quality control in the ER, however, are not free of errors. Some membrane proteins manage to escape the ER and travel to the

Golgi, although they have misfolded or unassembled TMDs. In such cases, sorting factors such as the retention in the ER 1 (RER1) receptor, which is localized to the cis-Golgi, mediate retrieval back to the ER. Albeit most of its substrates are unassembled subunits of protein complexes with exposed polar residues in the TM segments, RER1 is also involved in the ER retention of misfolded membrane proteins (Sato, Sato et al. 2003, Yamasaki, Hara et al. 2014, Briant, Johnson et al. 2017). This could be demonstrated for a mutant variant of peripheral myelin protein of 22 KDa (PMP22), which, as a result of mutation, has a polar residue within the TMD that is exposed to the hydrophobic environment of the lipid bilayer and recognized by RER1 (Hara, Hashimoto et al. 2014). ERAD-C substrates are mostly recognized by the cytosolic Hsp70 / Hsp 40 chaperone system. Recognition is probably due to the exposure of hydrophobic patches and a prolonged engagement of a substrate with the chaperone system, facilitating interaction between the substrate and the ERAD-C specific yeast ubiquitin ligase, Doa10. Consequently, polyubiquitination by Doa10 leads to the proteasomal degradation of the substrate in the cytosol, as will be discussed in more detail below (Nakatsukasa and Brodsky 2008, Sun and Brodsky 2019).

1.3.3.2 Retrotranslocation

In order to become accessible to the cytosolic proteasome, misfolded proteins following recognition have to be retrotranslocated from the ER lumen into the cytosol or extracted from the ER membrane, as in the case for membrane proteins (Figure 5). Recent studies suggest that prior to retrotranslocation, specific ERAD substrates, particularly those containing TM domains, are instead segregated from other ER proteins and exported into specific ER quality control compartments in a process mediated by the integral ER membrane B cell-associated protein of 31 kDa (Bap31). Notably, it is assumed that these specialized ER subdomains represent the site from where substrates destined for retrotranslocation exit the ER into the cytosol (Wakana, Takai et al. 2008). The mechanism of how hydrophobic integral membrane substrates can exit the ER is still ill-defined, with few exceptions (Nakatsukasa and Brodsky 2008).

Irrespective of whether the misfolded domain is located in the lumen or within the ER membrane, the export of both types of ER proteins is hindered by the hydrophobicity of the ER membrane. To overcome this barrier, a proteinaceous retrotranslocation channel, which allows aberrant proteins to move through the ER membrane into the cytosol, is required. Providing a channel that enables polypeptide movement through the ER membrane was first attributed to the Sec61 translocon. Opposed to its canonical function, it could be shown that Sec61 mediates the transfer of non-glycosylated major histocompatibility complex (MHC) class I molecules from the ER to the proteasome based on direct interaction (Wiertz, Tortorella et al. 1996, Zhou and Schekman 1999). Later studies using Sec61 mutants with impaired retrotranslocation function could support this observation. Although ER import was not affected, defects in ERAD and thus substrate export could be observed, indicating that the Sec61 translocon can direct two-way translocation processes (Romisch 2017).

Recently, it could be demonstrated that the yeast Derlin Dfm1 is a crucial component for retrotranslocation. Integral membrane proteins seem to require Dfm1 for extraction from the ER membrane during ERAD. However, in the absence of Dfm1, this activity is taken over by Hrd1 (Neal, Jaeger et al. 2018). Similar activity has been reported for Derlin-1, which explicitly targets movement of MHC class I molecules through the membrane and consequently causes degradation via transmembrane domain interaction (Lilley and Ploegh 2004, Ye, Shibata et al. 2004). Notably, Derlin-1 only has four transmembrane domains making the independent formation of a protein conducting channel unlikely, although oligomerization has been observed (Mehnert, Sommer et al. 2014). Nevertheless, it is assumed that Derlin-1 is essential to initiate translocation by promoting the insertion into the membrane if the substrate is an ERAD-L substrate, but then delivers misfolded substrates for ubiquitylation to Hrd1 as direct interaction of this E3 ligase with the transmembrane segments of Derlin-1 has been shown (Mehnert, Sommer et al. 2014, Romisch 2017). In fact, it has been suggested that the ubiquitin-ligase Hrd1 itself is another promising candidate that promotes the movement of misfolded proteins across the ER membrane. Most of the studies on this behalf have been carried out in yeast but could show in crosslinking experiments that retrotranslocating ERAD-L

substrates associate with Hrd1. Moreover, it could be shown that Hrd1 can promote degradation of ERAD-L substrates even in the absence of the other membrane components that contribute to the formation of the Hrd1 complex, such as the homolog of the cofactor of SEL1L (Carvalho, Stanley et al. 2010). The ability to compensate for the need of its complex partners indicates a central role of Hrd1 in the ERAD dependent translocation of misfolded proteins across the ER membrane. It is most likely by forming a membrane conduit or being part of it since Hrd1 is a multispanning membrane protein consisting of 6 TM segments. Interestingly, the formation of a retrotranslocation pore is regulated by cycles of autoubiquitination what causes the pore to open and deubiquitination of HHrd1 (Vasic, Denkert et al. 2020). Prior substrate ubiquitination, autoubiquitination of several lysine residues in the RING-finger domain of Hrd1 is required to enable retrotranslocation of membrane bound ERAD-L substrates as liposome-based reconstitution experiments have revealed (Baldridge and Rapoport 2016). To counteract the risk of proteasomal degradation that autoubiquitinated Hrd1 is exposed to, deubiquitination of Hrd1 is catalyzed by a deubiquitinating enzyme (DUB) (Peterson, Glaser et al. 2019). Although processes occurring during ERAD-L inside the ER membrane are still under debate, processes occurring inside the ER lumen are better understood. Glycosylated ERAD-L substrates that contain a misfolded domain are jointly recognized by the lectin Yos9 (homolog of OS-9 and XTP3-B), which binds to terminal mannose residues present on trimmed N-linked glycans, and Hrd3 (homolog of SEL1L), which binds to unstructured segment around the glycosylation attachment site. Furthermore, both proteins interact with each other via the luminal domain of Hrd3 and subsequently deliver their substrate to the Hrd1 translocon (Carvalho, Stanley et al. 2010, Stein, Ruggiano et al. 2014).

In fact, whether a retrotranslocation channel really exists or whether other mechanisms enable translocation and which factors might be involved still needs to be further elucidated (Neal, Jaeger et al. 2018). Along the same line, it has been proposed that lipid rearrangements in the ER membrane causing the formation of lipid droplets might contribute to a different possible mechanism that enables exit from the ER (Ploegh 2007).

1.3.3.3 Proteasomal degradation

Once a substrate emerges on the cytoplasmic site, ubiquitin ligases and ubiquitin-conjugating enzymes catalyze polyubiquitination of the respective substrates (Carvalho, Stanley et al. 2010). Although the ER possesses several ubiquitin-protein-ligases (25 in mammals) with different selectivities, they are all subject to the same enzymatic cascade, ultimately leading to substrate ubiquitination (Fenech, Lari et al. 2020).

Ubiquitin is a highly conserved protein and involved many cellular processes, including the degradation of proteins and regulation of their activity or cellular localization. Ubiquitination as a reversible, post-translational method to modify proteins is based on a multi-stage enzymatic cascade where an isopeptide bond is formed between the C-terminal glycine residue of ubiquitin and an ε-amino group of a lysine residue present on the client, which causes monoubiquitination (Figure 5). Notably, the client can be ubiquitin itself. Thus, consecutive addition of further ubiquitin moieties to a lysine residue of the first ubiquitin molecule can lead to the formation of a polyubiquitin chain (Ciechanover, Finley et al. 1984, Finley, Ciechanover et al. 1984). Interestingly, the modification of other residues such as Lys and Thr could also be observed during ERAD (Brodsky and Skach 2011). The ubiquitination reaction is catalyzed by the sequential but coordinated action of three different enzymes. The ubiquitin-activating enzyme (E1) is required for all ubiquitination reactions and initiates ubiquitination in an ATP-dependent reaction. First, the E1 catalyzes the formation of a high-energy thioester bond between a conserved Cys residue in its active center and the C-terminal carboxyl group of ubiquitin. Subsequently, the activated ubiquitin moiety is transferred to a thiol group in the active center of a ubiquitin-conjugating enzyme (E2). Finally, following association of the E2 with the RING domain of the ubiquitin ligase (E3), ubiquitin is transferred from the E2 to the target protein, and the E3 facilitates the conjugation of ubiquitin with the substrate. Therefore, substrate recruitment and the specificity of ubiquitination is mediated by ubiquitin ligases, which explains their frequent occurrence (Pickart 2001, Komander and Rape 2012). Depending on the activity of ubiquitin-conjugating (Ubc) enzymes and which of the seven lysine residues present in ubiquitin is used for ubiquitin linkages, this causes different cellular consequences for the ubiquitylated protein

(Ye and Rape 2009). Polyubiquitin chains linked via Lys 48 (K48) represent the most common type of linkage and are recognized by the 26S proteasome, which leads to proteasomal degradation of the modified substrate, similar to K29-linked chains. Notably, proteomic analysis revealed that also K11-linkages might contribute to the degradation of ERAD substrates (Xu, Duong et al. 2009, Komander and Rape 2012). In contrast, K63-linked polyubiquitin chains mediate proteasome independent functions such as protein-protein interactions or various regulatory functions (Komander and Rape 2012, French, Koehler et al. 2021). In addition to the homotypic type of linkage, mixed or branched polyubiquitin chains exist, significantly increasing the complexity of the unique ubiquitin code (French, Koehler et al. 2021). Beyond that, ubiquitination, as mentioned before, is a reversible process and can be undone by DUBs which further intensifies this effect (Jentsch and Rumpf 2007).

Only two ubiquitin ligases exist in yeast, which belong to the RING finger (RNF) class of E3 ubiquitin ligases and label proteins with polyubiquitin chains determined for proteasomal degradation. Whereas Hrd1 is required for ERAD-M or ERAD-L substrates, proteins with misfolded cytosolic domains use a different ubiquitin ligase, Doa10 (Swanson, Locher et al. 2001, Carvalho, Goder et al. 2006). Ubiquitylation of Doa10 substrates depends on the two E2´s, Ubc6 and Ubc7, which operate sequentially. Thereby, monoubiquitylation of clients mediated by the membrane-embedded Ubc6 is a prerequisite for chain elongation by the cytosolic Ubc7 resulting in K48-linked polyubiquitin chains. Interestingly, besides Lys acceptor sites, Ubc6 also attaches ubiquitin moieties to hydroxylated amino acids present in target proteins and synthesizes particularly K11-linked polyubiquitin chains (Xu, Duong et al. 2009, Weber, Cohen et al. 2016). The processing of Hrd1 substrates mainly depends on the activity of Ubc7 and, to a minor degree, the membrane-bound Ubc1 (Zattas and Hochstrasser 2015). However, both the Hrd1 and Doa10 complex essentially depend on another factor, coupling of ubiquitin conjugation to ER degradation protein 1 (Cue1), required for the activation of Ubc7 and thus efficient chain formation. Following the association with Hrd1, the transmembrane protein Cue1, recruit's ubiquitin-bound Ubc7 to the ER membrane via a distinct binding motif causing its activation and allowing Ubc7 to mediate the formation of K48-linked

polyubiquitin chains. The transfer of ubiquitin moieties is stimulated by a CUE domain present in Cue1, which can bind to the proximal ubiquitin in the elongating chain and thus positions the bound Ubc7 close to the substrate protein (Kostova, Mariano et al. 2009, Zattas and Hochstrasser 2015, von Delbruck, Kniss et al. 2016). Interestingly, unassembled Ubc7 is degraded following autoubiquitination which indicates, that Cue1 is required for the stability of Ubc7 and has a regulatory function in ubiquitylation (Ravid and Hochstrasser 2007).

Although the ERAD machinery is much better characterized in yeast because of the low complexity compared to a mammalian system, several E3 and E2 orthologs have been implicated in ERAD in mammalian cells. Besides the best-studied ER ligases HRD1 and GP78, which are both homologous to Hrd1 as mentioned before, other E3 ligases are known, which reside in the ER membrane or cytosol. Among ER membrane-anchored ligases, these include the TEB4 or MARCH VI ligase, which represent the mammalian homolog of the yeast Doa10 and shares a similar membrane topology, RNF family members such as RMA1 and TRC8 but also Nixin/ZNRF4, which has been demonstrated to be important in the regulation of calnexin turnover as it mediates ubiquitination of CNX and thus its proteasomal degradation (Kostova, Tsai et al. 2007, Neutzner, Neutzner et al. 2011, Claessen, Kundrat et al. 2012). Mammalian E2s include UBE2J1 and UBE2J2 both are homologous to the yeast Ubc6 and post-translationally insert into the ER membrane due to high hydrophobicity in their C-terminus. In contrast, UBE2G1 and UBE2G2 are soluble proteins and correspond to UBC7. Interestingly, their implication in ERAD is very different. While UBE2G2 is required for the activity of various ERAD E3s and thus strongly promotes degradation, UBE2J1 and UBE2J2 are of minor importance. Whether UBE2G1 is involved in ERAD is unclear and still under debate (Kostova, Tsai et al. 2007).

Regardless of which ERAD pathway and E3 ligase is used to conjugate retrotranslocation substrates with degradation signals for the proteasome, after polyubiquitination, all paths converge at the cytosolic valosin-containing protein (VCP)/p97 or its yeast homolog ATPase Cdc48 which is a central component of the ubiquitin-proteasome system (Richly, Rape et al. 2005, Peterson, Glaser et al. 2019). Cdc48 is a highly conserved chaperone-like enzyme with critical regulatory functions in multiple cellular processes that depend on the ability of

Cdc48 to recognize and bind ubiquitinated proteins (Jentsch and Rumpf 2007). Furthermore, Cdc48 can segregate ubiquitinated proteins from unmodified partners and provides the essential pulling force by which ubiquitinated ERAD substrates are extracted from macromolecular complexes or the ER membrane and moved into the cytosol, where they are degraded by the proteasome (Stolz, Hilt et al. 2011). To fulfill this segregase function Cdc48 depends on its ATPase activity, which converts chemical into mechanical energy. Sequential cycles of ATP hydrolysis lead to conformational changes and rotational movement of the homohexameric ring-shaped Cdc48 complex and thus contribute to a pulling force. Consequently, this allows the successive movement of polypeptides through a central pore formed in the center of the ring-shaped structure (Rouiller, DeLaBarre et al. 2002, Pye, Dreveny et al. 2006, Stolz, Hilt et al. 2011).

The function of Cdc48 is strictly regulated by numerous cofactors that interact with Cdc48 via highly conserved domains and binding motifs and thus contribute to the functional diversity. Although Cdc48 can bind ubiquitylated proteins by itself, the recruitment of Cdc48 to the Hrd1 complex is mediated by the heterodimeric substrate recruiting cofactors, ubiquitin fusion degradation protein 1 (Ufd1) and nuclear protein localization protein 4 (Npl4). Both cofactors can recognize polyubiquitin chains with a minimum length of 5 ubiquitin moieties via their ubiquitin-binding domains and enable efficient recruitment of Cdc48 to Hrd1 (Meyer, Wang et al. 2002, Stein, Ruggiano et al. 2014). Additionally, the Ufd1/Npl4 dependent binding of polyubiquitin chains leads to an induced ATPase activity of the Cdc48 complex (Bodnar and Rapoport 2017). However, recruitment of Cdc48 to the ER membrane is not exclusively dependent on Ufd1/Npl4 but can be facilitated by different cofactors such as the transmembrane protein Ubx2 that contains a Ub regulatory X (UBX) motif and binds to the N-terminal domain of Cdc48 (Schuberth and Buchberger 2005). Furthermore, other N-terminal binding Cdc48 ER recruiting cofactors have been reported, which are characterized by the presence of a VCP-interacting (VIM) or VCP-binding (VBM) motif, the latter motif can also be found in Hrd1 (Boeddrich, Gaumer et al. 2006, Morreale, Conforti et al. 2009, Stapf, Cartwright et al. 2011) Remarkably, besides its important segregase activity in ERAD, Cdc48 is also capable of controlling the

degree of ubiquitylation of bound clients by substrate processing cofactors (Jentsch and Rumpf 2007)

In addition to the beforementioned ER-associated ubiquitin ligases, DUBs play an equally important regulatory role in ERAD mediated quality control, although they exhibit opposite activity and hence could inhibit the degradation process. In fact, several ERAD specific DUBs like Ataxin-3 or YOD1/Otu1 have been shown to associate with Cdc48 (Ernst, Mueller et al. 2009, Liu and Ye 2012). Although still under debate, the function of Cdc48 associated DUBs might include trimming of polyubiquitin chains. This can result in an altered affinity of the substrate towards ubiquitin-binding effectors like the Bag6 holdase complex or facilitate the entry of retrotranslocation substrates into the narrow Cdc48 pore or (Wang, Liu et al. 2011, Liu and Ye 2012). In contrast, Ufd2, a polyubiquitylation enzyme, promotes the extension of short ubiquitin chains of Cdc48 bound substrates. Remarkably, the extension of ubiquitin chains can be necessary if the degradation signal is no longer strong enough due to extensive trimming (Richly, Rape et al. 2005, Liu and Ye 2012). Interestingly, the activity of Ufd2 can be inhibited by another factor, Ufd3, which competes with Ufd2 for the same Cdc48 binding interface (Rumpf and Jentsch 2006).

Following retrotranslocation and successful release from the membrane, ubiquitinated proteins are delivered to the 26S proteasome for degradation by the escort factor Rad23 or its homolog Dsk2, which in mammalian cells is represented by four ubiquilins, UBQLN1 to UBQLN4 (Figure 5) (Medicherla, Kostova et al. 2004, Guna and Hegde 2018). The substrate recognition for Rad23 and Dsk2 occurs via a C-terminal ubiquitin-associated domain (UBA) that binds polyubiquitin chains. A ubiquitin-like domain (UBL), located in the N-terminus of both shuttle factors, then mediates binding to the 19S particle, one of the two main subunits of the 26S proteasome. In addition, the proteasome consists of a barrel-shaped 20S core particle covered on one or both ends by the 19S particle. While the 20S subunit has proteolytically active sites for the hydrolysis of ubiquitinated proteins, the 19S particle has regulatory functions required for substrate processing activities. These include recognizing ubiquitinated substrates, their deubiquitination, and finally, the ATP-driven unfolding and translocation of the substrate into the 20S core. Once present in the cylindrical

core, different proteases that have trypsin-, chymotrypsin-, and caspase-like cleavage specificity mediate degradation of the substrate (Bard, Goodall et al. 2018, Greene, Dong et al. 2020).

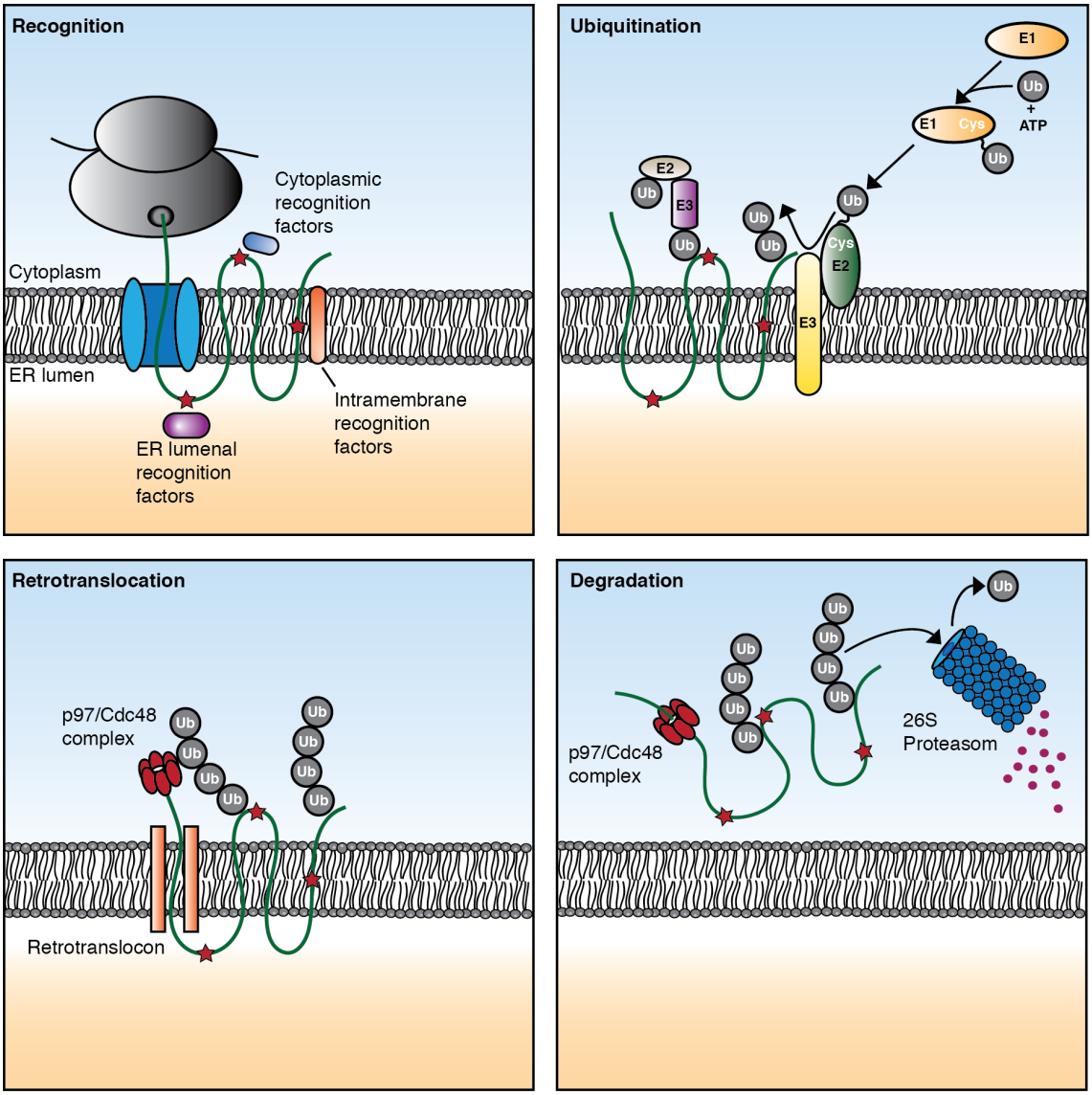


Figure 5: Key steps in ERAD of membrane proteins: *Recognition:* Depending on whether the misfolded region (red star) of a membrane protein is located in the ER lumen, on the cytoplasmic side or within transmembrane domains, the recognition is mediated by different recognition factors. *Ubiquitination:* Following recognition, different ubiquitin ligases are recruited to the substrate and mediate its ubiquitination. The ubiquitination reaction is catalyzed by the sequential but coordinated action of three different enzymes. The ubiquitin-activating enzyme (E1) is required for all ubiquitination reactions and initiates ubiquitination in an ATP-dependent reaction, whereby ubiquitin is transferred to an active site cysteine in a ubiquitin conjugation enzyme (E2). Subsequently, ubiquitin is transferred from the E2 to a lysine residue of the target protein, and a ubiquitin ligase (E3) facilitates the conjugation of ubiquitin with the substrate.

In yeast, ERAD-M or ERAD-L substrates are mainly ubiquitinated by the E3 ubiquitin ligase Hrd1, while proteins with misfolded cytosolic domains use a different ubiquitin ligase, Doa10. *Retrotranslocation:* To become accessible to the cytosolic proteasome, misfolded proteins have to be retrotranslocated from the ER lumen into the cytosol or extracted from the ER membrane, in the case for membrane proteins. To overcome the hydrophobicity of the ER membrane, a proteinaceous retrotranslocation channel is formed which allows aberrant proteins to move through the ER membrane. In addition, the p97 / Cdc48 complex converts chemical into mechanical energy through ATP hydrolysis, which allows the segregation of substrates from the membrane. *Degradation:* Following retrotranslocation and successful release from the membrane, ubiquitinated proteins are delivered to the 26S proteasome for degradation (Figure based on Guerriero and Brodsky 2012).

1.4 ER stress and the unfolded protein response

Degradation of aberrant secretory pathway proteins is mainly carried out by ERAD or autophagy. However, failure of these cellular degradative processes can lead to the accumulation of misfolded proteins in the ER and thus cause an imbalance between the ER folding load and capacity. Furthermore, this disruption in protein folding homeostasis leads to cellular conditions termed ER stress. To counter threats emanating from malfolded ER proteins and ultimately to restore ER proteostasis, cells have evolved highly conserved cellular stress response mechanisms, referred to as the unfolded protein response (UPR) (Gardner, Pincus et al. 2013). The first time, such a response mechanism and thus the prototype of the UPR was described when it could be shown that accumulating, misfolded proteins in the ER are the trigger for the markedly enhanced expression of BiP and Grp94 (Kozutsumi, Segal et al. 1988, Mori 2015).

In order to maintain homeostasis, the UPR monitors conditions within the ER and transmits this information to downstream processes that mainly affect the up-regulation of specific gene expression programs. UPR activation mediates an overall increase of ER folding capacity through an enhanced synthesis of pro-folding chaperones and folding enzymes and an ER expansion by inducing proteins involved in lipid biosynthesis. In addition, the expression of the ERAD machinery is upregulated to deal with the accumulation of misfolded proteins. At the same time, the UPR mediates the reduction of the ER protein folding load through general translational repression, specific mRNA degradation, and ER

clearance by increased protein degradation (Guerriero and Brodsky 2012). Allowing the cell to deal with ER stress conditions, the UPR is considered to be cytoprotective. However, unresolved stress conditions in the ER indicate that homeostasis cannot be restored, thus inducing apoptosis (Gardner, Pincus et al. 2013).

In general, the mammalian UPR consist of three different signaling pathways, which are characterized by the ER-resident TM proteins inositol requiring enzyme 1 (IRE1), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6) (Walter and Ron 2011, Gardner, Pincus et al. 2013). Each pathway possesses a unique mechanism of ER stress sensing in the ER lumen or membrane and signal transduction in the cytosol that collectively comprises the UPR.

Under basal conditions, ATF6, which can occur as a monomer, dimer, and oligomer, is maintained in an inactive state through the binding of BiP to its luminal domain. Upon stress conditions in the ER, ATF6 is released from BiP, and its disulfide bonds are remodeled. In this reduced, monomeric state ATF6 is allowed to relocate to the Golgi apparatus where it is proteolytically processed by the sequential actions of two membrane-embedded proteases, namely site-1 and site-2 proteases (S1P and S2P). Remarkably, due to the deoligomerization of ATF6 in response to ER stress, this branch of the UPR differs significantly from the other pathways in which oligomerization occurs. Removal of the luminal and transmembrane domains allows the amino-terminal transcription factor domain of ATF6 to translocate into the nucleus. Subsequent binding to ER stress response elements (ERSEs) initiates activation of genes required for UPR (Maiuolo, Bulotta et al. 2011, Gardner, Pincus et al. 2013).

In contrast, the ER-resident kinase PERK, in response to ER stress oligomerizes which in turn causes autophosphorylation and thus inactivation of the eukaryotic translation initiation factor 2 α (eIF2α). This reduces the overall cellular protein synthesis, and thus the amount of proteins entering the ER is reduced (Harding, Zhang et al. 1999).

IRE1 is the most conserved and well-characterized signaling component and present in all eukaryotic cells. It belongs to the family of type I TM proteins characterized by an amino-terminal ER lumenal domain. In the absence of ER

stress, IRE1 resides as a monomer within the membrane, and its lumenal domain, which functions as the stress-sensing domain, is stabilized by interaction with BiP (Okamura, Kimata et al. 2000). Moreover, IRE1 contains a carboxy - terminal cytoplasmic ribonuclease (RNase) domain which undergoes trans-autophosphorylation following assembly of IRE1 into higher-order oligomers upon ER stress and dissociation of BiP (Gardner, Pincus et al. 2013). This results in the activation of its endonuclease activity and initiates the processing of distinct mRNA molecules that encode for a transcription factor, termed XBP1 in mammals. Following translation into its active protein product, XBP1 then relocates into the nucleus and induces the activation of ERSEs that encode for proteins of the ER-folding machinery and lipid biosynthesis. Notably, many of the up-regulated UPR target genes also encode for specific factors involved in the ERAD machinery (Walter and Ron 2011, Guerriero and Brodsky 2012, Gardner, Pincus et al. 2013). However, despite them being the most abundant proteins produced in the ER, how membrane protein misfolding is sensed by the UPR or ERAD remains mostly unknown.

1.5 Transmembrane domain chaperones and intra-membrane quality control factors

Intra-membrane chaperones and quality control factors that can efficiently recognize and monitor the assembly status of TMDs directly within the lipid bilayer to support folding and detect misfolding are a prerequisite for protein homeostasis of any eukaryotic cell. However, chaperoning of TM segments of multi-spanning membrane proteins in the plane of the membrane and underlying molecular mechanisms are poorly characterized so far.

Recently, the conserved, multi-subunit ER membrane protein complex (EMC) and its function in membrane protein topogenesis has come into focus. EMC functions as a membrane protein chaperone and TM domain insertase (Guna, Volkmar et al. 2018). Its chaperoning function was first revealed in a yeast screen to identify genes required for protein folding in the ER. Interestingly, it could be observed that in the absence of EMC, misfolded membrane proteins accumulate (Jonikas, Collins et al. 2009). Later, it could be demonstrated that EMC integrates tail-anchored proteins with only moderately hydrophobic TMDs post-

translationally and TMDs functioning as signal anchors co-translationally, as shown for G protein-coupled receptors (GPCRs) (Chitwood, Juszkiewicz et al. 2018, Guna, Volkmar et al. 2018, Tian, Wu et al. 2019). During biogenesis of these multi-pass membrane proteins, the EMC complex is required to initiate accurate insertion of the first TM segment as its depletion can lead to a misorientation of the first TM segment. After inserting the first TM segment, however, insertion of the following TM segments is mediated through cooperation with the Sec61 translocon (Chitwood, Juszkiewicz et al. 2018). The specific insertion of mildly hydrophobic TM segments by EMC is not limited to TA proteins but also takes place in the biogenesis of multipass transmembrane proteins that contain destabilizing features in their TMDs. In fact, it could be demonstrated that EMC preferably engages TM segments with uncommon transmembrane amino acids such as charged or bulky residues, which usually can be found in transporter proteins (Shurtleff, Itzhak et al. 2018).

Interestingly, similar to SRP54 or Get3, one subunit of the EMC, which at the same time is most important for interaction with TA proteins, has an unstructured motif rich in methionine residues and resides just below the lipid-exposed hydrophilic insertase vestibule formed by two other subunits. Since mutation of the methionine residues affects the biogenesis of co- and post-translational EMC substrates, it is assumed that these residues contribute to the recognition and guidance of substrates towards the membrane.

Furthermore, following substrate engagement, the methionine enriched patch might assist in orienting the substrate correctly before it is released through the vestibule into the lipid bilayer (O'Donnell, Phillips et al. 2020, Pleiner, Tomaleri et al. 2020, Bai and Li 2021). Remarkably, because substrate translocation into the lipid bilayer is an energetically unfavorable process, the EMC causes local thinning of the membrane and uses the positive charge in the hydrophilic vestibule to decrease the energetic barrier for insertion into the membrane (Pleiner, Tomaleri et al. 2020). As mentioned before, EMC and Sec61 cooperatively function in the co-translational insertion of membrane proteins. In addition to functional studies, this has also been observed in structural studies. However, due to the observation that various secretory proteins require both EMC and Sec61 cofactors such as TRAP or Sec62 for efficient biosynthesis, it is

assumed that EMC has a rather regulatory function during the co-translational translocation mediated by Sec61 (O'Keefe, Pool et al. 2021). A different, novel protein complex previously shown to cooperate with Sec61 and function as an intra-membrane chaperone during the co-translational biogenesis of membrane proteins is the PAT complex.

The heterodimeric PAT complex is characterized by two membrane protein subunits, coiled-coil domain-containing protein 48 (CCDC47) and Asterix. Both PAT complex subunits are required for efficient biogenesis of numerous multi-spanning membrane proteins like GPCRs, however, not for TA-proteins. This was demonstrated by depletion of either subunit of the complex by siRNA, which resulted in the loss of the other and ultimately causes reduced biogenesis (Chitwood and Hegde 2020). Initially, Asterix was identified as a protein associated with the ER translocon of 10 kDa (PAT10) that engages the first TMD of the multi-span transmembrane domain protein opsin after its lateral exit from the Sec61 translocon (Meacock, Lecomte et al. 2002, O'Keefe, Pool et al. 2021).

In addition, it could be demonstrated that the PAT complex preferentially engages with nascent TM segments containing unshielded hydrophilic amino acid residues. Remarkably, chaperoning of a substrate by the PAT complex persists throughout biogenesis even after the translation has ended. However, once TMDs are assembled correctly and hydrophilic residues are shielded in the completely folded protein by the native structure, the PAT complex disengages. Based on the finding that various substrates have different dependencies for either the EMC or the PAT complex, it is assumed that both membrane chaperones facilitate membrane protein biogenesis differently (Meacock, Lecomte et al. 2002, Chitwood and Hegde 2020).

Importantly, although insertases and chaperones are indispensable for maintaining protein homeostasis, their activity is mainly restricted to the biogenesis of membrane proteins. However, the subsequent targeting of proteins to the appropriate membrane can also be error-prone, resulting in the mislocalization of otherwise correctly folded membrane proteins. Given that many mislocalized proteins bear deleterious consequences for the cell as they cause cellular stress and organelle dysfunctions, quality control factors with mechanistically opposing activities to the EMC are required to remove proteins

from the wrong membrane (Gamerdinger, Hanebuth et al. 2015, Costa, Subramanian et al. 2018). Recently, it could be shown that the P5A-ATPase transporter ATP13A1 has such a translocation activity and thus enables quality control for topogenesis and certain mistargeted proteins (McKenna, Sim et al. 2020).

The membrane protein P5A ATPase belongs to the superfamily of P-type ATPases and can be found in all eukaryotic cells. P-type ATPases represent a major class of active transporters that use conformational changes induced by alternating phosphorylation and dephosphorylation events to facilitate the transport of substrates across membranes (McKenna, Sim et al. 2020). Although the P5A ATPase yeast homolog Spf1 is known to be involved in the mislocalization of mitochondrial TA proteins to the ER membrane by extraction, no direct interaction between Spf1 and its substrate has been observed (Krumpe, Frumkin et al. 2012). However, later studies on the P5A ATPase could demonstrate that the ATPase directly interacts with TA proteins via their TM segment. Moreover, it could be revealed that P5A ATPase removes moderately hydrophobic transmembrane helices with adjacent luminal hydrophilic segments, particularly those which are inserted in the wrong orientation, from the ER membrane in an ATP dependent manner. Even though a detailed mechanism for mislocalization is not yet known, cryo-EM structures of Spf1 suggest that substrate extraction occurs via a large substrate-binding pocket formed by distinct TM segments. The comparatively large and V-shaped pocket is equipped with different hydrophilic and hydrophobic side chains and is alternately opened to the cytosol and ER lumen and laterally into the plane of the membrane. This allows the P5 ATPase to flip the luminal segment on the other side of the membrane while the TM segments largely remain in the membrane. Interestingly, although the P5 ATPase and EMC share the same class of substrates, they show different activity at the ER (McKenna, Sim et al. 2020).

Accordingly, it is not yet known how the activity of different TM insertases or translocases is coordinated with one another and together with membrane-bound proteins mediating different PTMs like N-glycosylation (O'Keefe, Pool et al. 2021). Interestingly, besides the important role of CNX in glycan-dependent protein folding as described above, several recently published studies suggest that CNX

also has a function as an intra-membrane chaperone. However, a detailed mechanism for intra-membrane client recognition and chaperoning by CNX has not yet been discovered.

1.6 Calnexin

One of the first chaperones discovered to be involved in folding membrane proteins was CNX (Anderson and Cresswell 1994, Hammond, Braakman et al. 1994, Jackson, Cohen-Doyle et al. 1994). As briefly described above, CNX is a type-I integral ER membrane chaperone that plays a crucial role in protein folding and transiently interacts with numerous newly synthesized glycoproteins that pass through the ER. Its participation in protein folding and assembly was first discovered in 1991 when CNX was shown to associate with partially assembled MHC class I molecules, as well as T- and B-cell receptors (Degen and Williams 1991, Hochstenbach, David et al. 1992). As part of the ER-mediated quality control system, CNX assists folding processes by recruiting distinct co-chaperones and preventing incompletely folded substrates from leaving the ER (Danilczyk and Williams 2001). Among plants, fungi, and animals, CNX and its male germ cell-specific variant Calmegin (which has a similar structure to CNX apart from a slightly longer cytosolic domain) are highly conserved and characterized by one unique feature: A lectin-like-domain that shows a marked preference for N-linked monoglucosylated oligosaccharides on clients (Hebert, Foellmer et al. 1995). Lectin activity for CNX was first suggested after observing that treatment of cells with glycosylation or oligosaccharide-processing inhibitors resulted in a decreased interaction of the chaperone with most newly synthesized glycoproteins (Ou, Cameron et al. 1993, Hammond, Braakman et al. 1994). Later, through the practical use of glucosidase inhibitors, the recognition motif for CNX could be defined as the processed $Glc_1Man_9GlcNAc_2$ oligosaccharide. In addition, based on x-ray crystallographic analysis and mapping studies, the glycan-binding site was assigned to a cleft located on the surface of the ER-localized globular lectin domain of CNX (Schrag, Bergeron et al. 2001, Leach, Cohen-Doyle et al. 2002, Williams 2006).

1.6.1 CNX structure and binding sites

The CNX luminal part consists of two domains, a ß-sandwich domain, and an extended arm domain. The ß-sandwich of the globular lectin domain is mainly formed by two large ß-sheets and accommodates the lectin binding site (Williams 2006). Within this domain, six amino acid residues enable lectin activity by coordinating the terminal glucose residue (Figure 6). Notably, depletion of only one of these residues results in a loss of interaction with monoglucosylated oligosaccharides and causes aggregation of the respective substrates (Leach and Williams 2004). However, although the lectin domain specifically binds monoglucosylated oligosaccharides, three mannose residues located below the terminal glucose residue contribute to the recognition process (Spiro, Zhu et al. 1996, Vassilakos, Michalak et al. 1998). In addition to the lectin binding site, the globular domain contains Zn^{2+} and various high and low-affinity Ca^{2+} binding sites. Calcium-binding sites are important for lectin activity and structure but also contribute to the ER-mediated Ca^{2+} storage. Furthermore, binding of ATP by CNX has been observed, which presumably affects conformational changes of the chaperone, however, ATPase activity is unknown.

In contrast, the arm domain of CNX consists of two sequentially repeated ß-strand sequence motifs, which are Pro-rich and form a long, curved hairpin (Williams 2006). With the most distal part of the hairpin, CNX recruits co-chaperones such as disulfide-isomerase ERp57, cyclophilin B, or ERp29, which accelerate slow folding reactions, including disulfide bond formation and isomerization or mediate general chaperone activity (Figure 6) (Kozlov and Gehring 2020). Interestingly, due to an overall sequence identity of 39 %, it is assumed that the globular lectin domain of CNX´s soluble homolog, CRT, possesses a highly similar structure and lectin activity (Williams 2006, Kozlov, Pocanschi et al. 2010).

Unlike the luminal domain, the significantly shorter C-terminal tail of CNX is not necessary for classic lectin-dependent functions. However, cytoplasmic signaling events caused by different posttranslational modifications occurring at the C-tail, including palmitoylation or phosphorylation have been linked to effect different ER luminal events (Figure 6). For instance, phosphorylation plays an

important role in regulating the calcium concentration in the ER lumen (Roderick, Lechleiter et al. 2000, Chevet, Smirle et al. 2010).

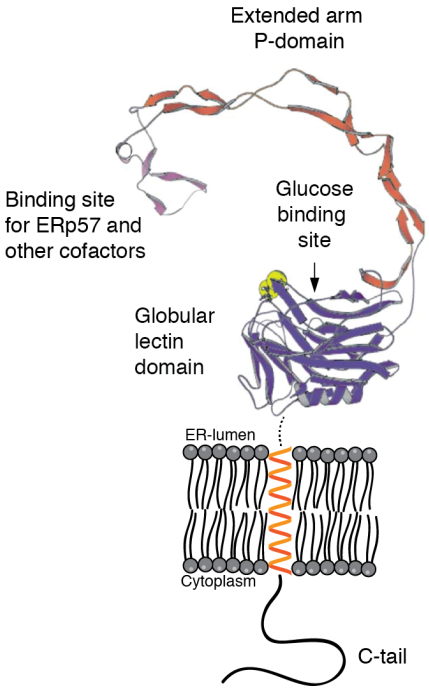


Figure 6: A structural model of calnexin: Crystal structure analysis reveals a highly asymmetric structure of the ER luminal domain, which consists of a globular lectin domain containing the oligosaccharide-binding site and an elongated arm domain, which is important for the interaction with certain co-chaperones and cofactors. In addition, as a type I integral membrane protein, calnexin has a single transmembrane helix, and its short C-terminal tail undergoes various post-translational modifications that affect ER lumen events and is required for different interaction processes.

Calcium ions function as ubiquitous second messenger and are involved in a broad spectrum of physiological and pathological processes. Amongst others, conformational changes of proteins and protein folding per se depends on the presence of calcium ions (Ca^{2+}), and elevated levels of cytoplasmic Ca^{2+} control a multiplicity of cellular signaling (Clapham 2007). In addition, mitochondrial respiration or gene transcription control mainly relies on spatial and temporal changes of intracellular Ca^{2+} levels (Dolmetsch, Xu et al. 1998, Robb-Gaspers, Burnett et al. 1998). Two opposing factors essentially mediate these repetitive changes in concentration: Whereas the inositol 1,4,5-trisphosphate receptor channel (IP3R) is responsible for Ca^{2+} release from the ER, the sarco

endoplasmic reticulum calcium ATPase (SERCA)-type Ca^{2+} ATPase causes the opposite effect and mediates transport of calcium ions from the cytosol into the internal ER store (Dawson 1997, MacLennan, Rice et al. 1997). As a recently published study has demonstrated, CNX fulfills a crucial regulatory function in this Ca^{2+} oscillation process, which is dependent on the phosphorylation status of CNX´s cytosolic domain. Three different consensus sites within its cytosolic domain allow phosphorylation, and several kinases have been identified in the phosphorylation of CNX (Wong, Ward et al. 1998). However, only phosphorylation of Ser562 mediated by the proline-directed kinase (PDK) appears to exhibit significant functional and regulatory consequences on Ca^{2+} levels inside the ER. Phosphorylation of Ser562 causes enhanced interaction of CNX with the C-terminal tail of SERCA 2b, the most prominent isoform of the SERCA family, resulting in an inhibited Ca^{2+} uptake in the ER. In contrast, the IP3 mediated release of Ca^{2+} causes dephosphorylation of the same site and most likely also leads to reduced interaction with SERCA 2b, as experiments analyzing the effect of phosphatase treatment have revealed. Consequently, the dephosphorylation-dependent dissociation of CNX from SERCA controls the uptake of Ca^{2+} in the ER and influences the Ca^{2+} sensitive protein folding machinery inside this organelle. Thus, CNX is regulated by a phosphorylation switch located on the very C-terminal end of the protein, which is highly sensitive towards Ca^{2+} and influences ER luminal binding events (Roderick, Lechleiter et al. 2000).

Of note, the same amino acid residue phosphorylation was furthermore identified to exhibit a role in ER-mediated quality control. Following ER stress, this site undergoes enhanced phosphorylation, leading to a prolonged interaction of CNX with its misfolded substrate and subsequent degradation (Cameron, Chevet et al. 2009). Moreover, phosphorylation is a prerequisite for CNX recruitment to translocon-associated ribosomes (RTC, ribosome translocon complex). In this case, however, phosphorylation depends on the synergistic action of a different member of the PDK kinase family, namely extracellular-signal-regulated kinase 1 (ERK-1) together with casein kinase 2 (CK2). Therefore, it is assumed that the multiplied presence of CNX in the vicinity of the translocon reinforces protein synthesis through increased glycoprotein folding

mediated by CNX (Chevet, Wong et al. 1999). Interestingly, this would imply an effect opposite to that caused by various members of the UPR response, which mediates a reduction in protein synthesis following the induction of ER stress as described in a previous section.

However, the enhanced association of CNX with the RTC is not only restricted to phosphorylation events occurring in CNX C-terminal tail but has also been observed as a consequence of other post-translational modifications. For instance, upon palmitoylation of two membrane-proximal cysteine residues, located in CNX C-terminal tail, by the transmembrane palmitoyltransferase DHHC6, CNX localizes to the rough ER. At the rough ER, CNX interacts with components of the RTC and contributes to the formation of a CNX-RTC supercomplex. Following the successful assembly, CNX then mediates recruitment of actin which significantly contributes to the stability of the complex. Importantly, CNX must be both part of the RTC complex and palmitoylated to capture nascent glycoproteins when they emerge from the translocon co-translationally (Lakkaraju, Abrami et al. 2012).

Interestingly, palmitoylation modification of identical juxtamembrane cysteine residues can affect CNX's cellular localization differently, as observed in other studies. It could be shown that following palmitoylation, CNX is targeted not only to the rough but also to a different region of the ER, namely the mitochondria-associated membrane (MAM), which fulfills a critical role in lipid and calcium metabolism. However, whether and how localization of CNX to different ER domains based on its palmitoylation status is regulated is not yet known (Lynes, Bui et al. 2012).

Noteworthy, palmitoylation not only seems to regulate the overall chaperone activity of CNX or influences its cellular localization but appears to exhibit a direct effect on the orientation of the cytosolic tail with respect to the CNX TMD, as molecular dynamics simulations have revealed. Therefore, it seems conceivable that palmitoylation can significantly change the conformation of CNX, which as a consequence, would have an impact on the ability of CNX to interact with distinct substrates in the cytoplasm as well as within the membrane (Lakkaraju, Abrami et al. 2012).

In addition to post-translational modifications, which affect the cytoplasmic domain of CNX and thus exhibit a decisive influence on the function and localization of calnexin as described above, other modifications of the CNX C-terminal tail have been reported. For instance, it has been shown that following both ER stress-inducing and non-stress-inducing apoptotic stimuli, CNX is proteolytically cleaved, most likely by either caspase-3 or caspase-7, which recognize a distinct DXXD motif in the carboxy-terminal region of CNX. It is speculated that the resulting cleavage product can inhibit or a least reduce ER-stress-induced apoptosis (Takizawa, Tatematsu et al. 2004).

To date, few studies have looked closer at the structure and properties of the TMD of CNX. Its TMD consists of 21 amino acids that form an α-helix, and the membrane-embedded region ranges from Trp482 to Cys502. In addition to primarily non-polar amino acid residues, polar residues including Tyr487 or Thr490 are present in the TM domain region, but no charged residues can be found. As a result, the TMD is mostly hydrophobic which leads to stable membrane integration (ΔG_{app} = -2.87). Notably, almost centrally located, the CNX TMD contains a Proline residue. Although proline residues in the center of transmembrane domains of integral membrane proteins are not uncommon, it renders their occurrence interesting because proline residues exhibit helix breaking properties, and their functional role has not been fully elucidated (Chang, Cheng et al. 1999). What makes the appearance of the proline residue in the TM region of CNX even more interesting is that this residue is also conserved in Calmegin, the homologous member of the calnexin family. Conservation of this residue and the TMD kink caused by it suggests that this residue might play an important functional role. Indeed, it has been shown that interaction of CNX with the ribosome-translocon complex depends essentially on this Pro residue (Lakkaraju, Abrami et al. 2012). In line with this, several recently published studies indicate that the CNX TM domain is more than just a simple membrane anchor and contributes an essential function in intra-membrane client recognition and chaperoning. So far, however, without revealing many details about the underlying mechanism or potential substrates and biological implications.

1.6.2 Glycan independent function of CNX and the importance of the transmembrane domain

In addition to the key role of CNX in the folding of ER glycoproteins, a role of CNX in the quality control of membrane proteins with incorrectly folded or assembled TM domains has been described. Interestingly, in many of these studies, binding of CNX to multi-membrane-spanning proteins such as Cx32, the inositol triphosphate receptor or CFTR occurred in a glycan-independent manner (Pind, Riordan et al. 1994, Joseph, Boehning et al. 1999, Fontanini, Chies et al. 2005, Coelho, Stahl et al. 2019). This consequently raises the question of how substrate specificity is mediated if the glycosylation status of a client not solely determines recognition. Remarkably, although CNX and CRT have almost identical lectin domains, genetic deletion causes different effects. Whereas knockout cell lines of either protein are viable, CRT deletion in mice leads to failures in heart development and prenatal lethality. In contrast, deletion of CNX strongly affects nerve fibers and causes early postnatal death (Denzel, Molinari et al. 2002). These and other findings, which could show different substrate preferences for CNX and CRT indicate that CNX´s topology might dictate substrate selection. In particular focus: the TM domain of CNX, which is the major discriminating feature between both ER chaperones. By anchoring Crt in the membrane, through fusion with the TM domain of CNX, one can change the spectrum of proteins associated with Crt similar to that of CNX, indicating that CNX´s TM domain might contribute to client selection (Wada, Imai et al. 1995, Danilczyk, Cohen-Doyle et al. 2000). Nonetheless, a simple explanation for this observation could also be the different localization of CNX and CRT in the membrane versus ER-lumen. For instance, access to the Sec61 translocon or to glycans that reside near the membrane could change as a result. However, this cannot account for many observations concerning the client binding of CNX. Only shortly after CNX was discovered, it could be shown that the observed association of the chaperone with nascent MHC class I molecules is based on direct transmembrane interactions (Margolese, Waneck et al. 1993). Later studies on the proteolipid protein (PLP) showed for the first time that CNX binds to isolated TM domains. Furthermore, it was shown that CNX directly interacts with a misfolded or unassembled TM domain of PLP and thus prevents its

degradation (Swanton, High et al. 2003). Similar findings for the quality control function of CNX during transmembrane domain assembly were obtained from studies on the tetraspin CD82. Even though CD82 is a multi-span glycoprotein, CNX selectively prevents ER exit of truncated CD82 lacking the first TM domain; however, it does not affect full-length CD82. Interestingly, simultaneous co-expression of the first TM segment with the truncated CD82 variant can partially reverse retention. This indicates that CNX can distinguish the native and unassembled status of a membrane protein by directly monitoring the assembly status within the lipid bilayer (Cannon and Cresswell 2001). Further evidence for this activity is based on studies of PMP22, where it was shown that CNX interacts with the first TM segment of PMP22 in a glycan-independent manner (Fontanini, Chies et al. 2005). Taken together, CNX recognizes and binds to truncated IMP substrates that lack one or more TM segments – presumably because they are recognized as misfolded and/or unassembled (Cannon and Cresswell 2001, Swanton, High et al. 2003, Fontanini, Chies et al. 2005, Wanamaker and Green 2005, Coelho, Stahl et al. 2019). Remarkably, however, the exact details of what renders a TM domain as “misfolded” and motifs or features that CNX recognizes are not known.

2 Aim of studies

Membrane proteins make up one-third of the human proteome, and their biosynthesis is based on a series of precisely coordinated events. These include the translocation and integration of specific TMD structures into the lipid bilayer and folding domain structures on both the lumenal and cytoplasmic sides of the ER. Moreover, biosynthesis for multispanning or oligomeric IMPs involves the correct assembly of TMDs within the bilayer of the ER. Due to these multiple topologically demanding folding events, integral membrane proteins represent a particular challenge to the ER folding machinery and quality control system. Failures of these processes can have deleterious consequences for the cell and the entire organism. Despite this, knowledge about how molecular chaperones guide and control the structure formation of membrane proteins in the lipid bilayer is very limited.

In addition, most studies on the canonical degradation and quality control pathways have focused on proteins where the misfolded lesion is located either inside the ER lumen or in the cytosol. However, proteins located within the ER membrane, containing aberrant TM domains cannot be recognized by the very same cytosolic or luminal factors. Considering that incorrect folding and assembly of IMPs and destabilizing mutations that introduce charges within TMDs of IMPs are linked to various human diseases, only very little is known about factors or quality control mechanisms that interact with unstable TM helices.

Therefore, this study aims to mechanistically investigate novel intra-membrane chaperones and quality control mechanisms that, within the lipid bilayer, monitor membrane protein folding, the assembly status of TMDs, and folding defects in those. At the same time, we aim to define sequence motifs that constitute a “misfolded” TM domain and are thus recognizes by intra-membrane chaperones.

Since natural TM proteins, as outlined above, generally possess complex sequences and structures and may engage multiple topically and functionally distinct layers of quality control, which precludes an unbiased analysis, the initial objective of this study is to establish an artificial minimal consensus membrane (CoMem) reporter system that allows for the systematic analysis of intra-membrane recognition processes. Then, to define principles of intra-membrane

client recognition, we use Calnexin (CNX), one of the most abundant ER chaperones, and putative intra-membrane chaperone as a model. In addition to its canonical function, CNX is known to directly interact with TM helices of multi-membrane-spanning proteins, although an underlying mechanism is not known. By combining experimental and computational approaches, we aim to systematically dissect the intra-membrane recognition process of misfolded membrane clients by the transmembrane domain of CNX. In detail, we aim to uncover a structural understanding of client recognition by CNX and the biological implications of this recognition process.

Hence, this study aims to provide a comprehensive understanding of intra-membrane substrate recognition by molecular chaperones as a basis for quality control of IMPs in the ER, which in the future could contribute to a better understanding of numerous membrane protein-misfolding diseases.

3 Results

3.1 Calnexin interacts with transmembrane domains of non-glycosylated clients

Membrane proteins are a major class of proteins produced in the ER. Despite this, principles of their molecular chaperoning remain ill-defined. CNX is one of the most abundant ER chaperones and several studies indicate a role for its TMD in substrate recognition (Cannon and Cresswell 2001, Swanton, High et al. 2003, Fontanini, Chies et al. 2005, Wanamaker and Green 2005, Korkhov, Milan-Lobo et al. 2008, Li, Su et al. 2010, Coelho, Stahl et al. 2019). Despite the number of reports on intra-membrane client recognition by CNX, this process remains very incompletely understood in terms of mechanisms, range of substrates, and biological implications. To address this gap in knowledge and provide insights intro principles of intra-membrane chaperoning processes, we used Connexin 32 (Cx32) as a first model client protein. Cx32 is a four-helix integral membrane protein (Figure 7A) that forms homo-hexameric connexons. Two of these connexons embedded in different membranes can dock onto each other to form a gap junction channel (Pantano, Zonta et al. 2008, Maeda, Nakagawa et al. 2009). Mutations in Cx32 cause X-linked Charcot-Marie-Tooth disease (CMTX), one of the most common genetic disorders of the peripheral nervous system (Scherer and Kleopa 2012). A recent study from us has revealed interaction of Cx32 with CNX in mass spectrometry experiments (Coelho, Stahl et al. 2019). Cx32 is not glycosylated (Figure 7B) and possesses only very short ER-lumenal loops (Figure 7A). This renders a potential Cx32:CNX interaction a possible starting point towards defining intra-membrane client binding by CNX as it can neither rely on recognition of sugar moieties nor on binding of large ER-lumenal domains by the CNX lectin domain.

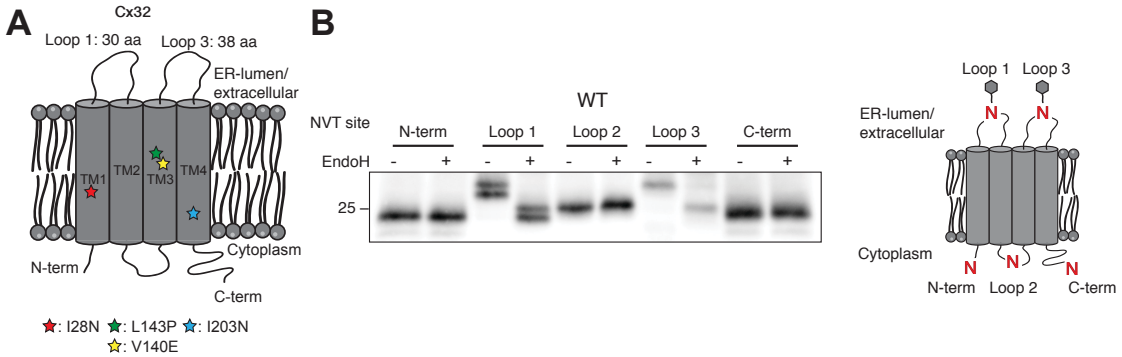
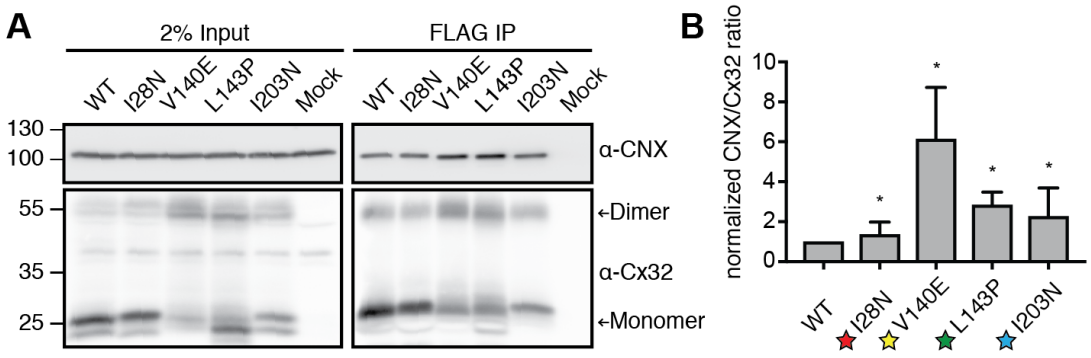


Figure 7: Topology analysis of Cx32. (A) Schematic of the Cx32 structure and its orientation within the membrane. Positions of mutations analyzed in this study are indicated with stars. The overall topology and loop lengths were obtained from the UniProtKB server (human GJB1 gene). **(B)** EndoH digest of Cx32 WT used in this study to verify the proper orientation within the membrane. Membrane integration, and reporter site glycosylation are shown (N: NVT reporter sites for glycosylation, which were individually introduced. Only the most N-terminal site (located in the cytoplasm) is endogenously present in Cx32).

To investigate the nature of the Cx32:CNX interaction in more detail, we expressed FLAG-tagged Cx32 in human HEK293T cells and analyzed their interactions with endogenous CNX in co-immunoprecipitation experiments. These confirmed interaction of Cx32 with CNX (Figure 8A) and revealed interaction of CNX with monomeric and dimeric Cx32 (Figure 8C). If CNX indeed acted as a chaperone on Cx32, one would expect a preferential interaction with misfolded variants of the client. We thus proceeded to study four disease-causing mutants of Cx32 (Bone, Deschenes et al. 1997, Rouger, LeGuern et al. 1997, Kleopa, Zamba-Papanicolaou et al. 2006). All of these contain mutations in their TMDs (Figure 7A) but are properly integrated into the lipid bilayer (Figure 8D).

Strikingly, all of the mutants showed a statistically significant increase in interaction with CNX, which was up to approximately 6-fold stronger than the interaction with wild type Cx32 (Cx32-WT) (Figures 8A and 8B). In contrast to Cx32-WT, all mutants were retained in the ER, arguing for misfolding and recognition by the ER quality control system (Figure 8E). Taken together, these data indicate that CNX can directly recognize misfolded Cx32, a non-glycosylated client, possibly in the membrane where the mutations are located



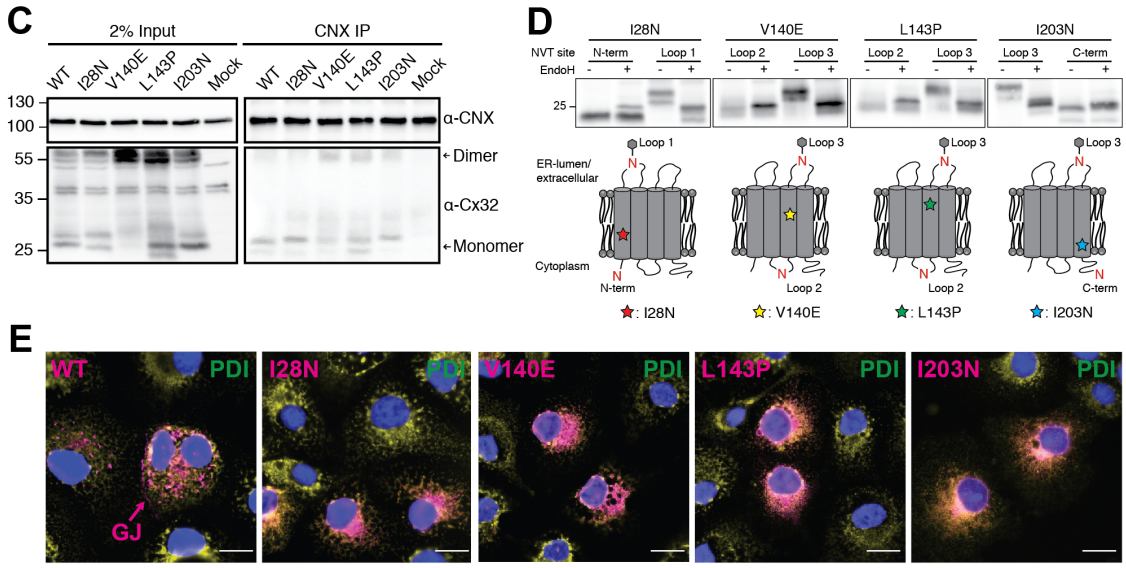
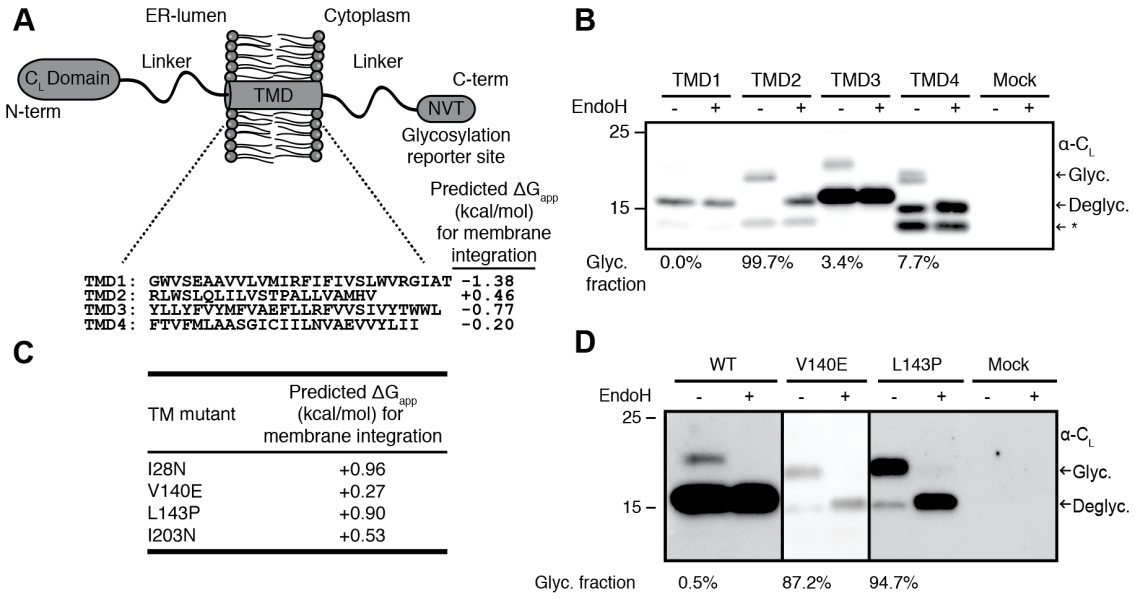


Figure 8: Calnexin binds to non-glycosylated membrane proteins and their individual transmembrane domains. (A) Co-immunoprecipitations of endogenous CNX with FLAG-tagged Cx32 WT and its mutants. Constructs were expressed in HEK293T cells. One representative immunoblot is shown. Monomers and dimers of Cx32 are indicated with arrows. **(B)** Quantification and statistical analysis of (A). All samples were normalized to WT (mean ± SEM, N ≥ 4, *P value < 0.05, two-tailed Student's t tests). **(C)** HEK293T cells were transfected with Cx32 WT or the indicated mutants. Immunoprecipitations of endogenous CNX reveal interactions with monomeric and dimeric Cx32 species. **(D)** EndoH digest of Cx32 mutants used in this study to verify their proper orientation within the membrane. Schematics of mutations studied, membrane integration, and reporter site glycosylation are shown (N: NVT reporter sites for glycosylation, which were individually introduced. Only the most N-terminal site (located in the cytoplasm) is endogenously present in Cx32). **(E)** Immunofluorescence images demonstrating localization of Cx32 and its mutants. COS-7 cells were transiently transfected with the indicated FLAG-tagged Cx32 constructs and immunofluorescence microscopy was performed using anti PDI (green) as an ER marker. Detection of Cx32 was performed using anti FLAG antibodies and subsequent staining of primary antibodies with labeled secondary antibodies (magenta). Nuclei were stained with DAPI (blue). All three channels are overlayed. Images are representative of cells from at least three different biological replicates. GJ denotes gap junctions observed in the cells transfected with WT Cx32. Scale bars correspond to 20 μm.

To further test this hypothesis, we individually fused each of the four Cx32 TMD domains to an antibody light chain constant domain (C_L) (Figure 9A). A related system was recently established to assess chaperone:client interactions for soluble proteins in a systematic manner (Feige and Hendershot 2013, Behnke,

Mann et al. 2016). By using a glycosylation reporter site downstream of the TMD segment we could verify that the majority of each TMD segment was integrated into the ER membrane (Figure 9B). The only exception was TMD segment 2 (Figure 9B), which did not integrate properly into the membrane, in agreement with a recent study (Coelho, Stahl et al. 2019) and the predicted membrane integration potentials (Figure 9A). Using this system, we probed which of the TM segments from Cx32 was bound by CNX. As a small portion of the TMD3 and TMD4 constructs could enter the ER, we replaced the sugar-accepting Asn residue downstream of each TMD segment by a Gln residue, since our interest was in glycan-independent interaction with CNX. TMD segment 2 was excluded from these analyses since it failed to integrate into the membrane. Introducing mutations into the individual TMD segments also compromised their proper membrane integration (Figures 9 C and D). We thus focused on analyzing interactions of CNX with wild type TMD segments 1, 3 and 4 of Cx32. Interaction was observed for all three TMD segments and among those, by far the strongest interaction was observed with TMD1 (Figures 9E and 9F). Taken together, our data clearly show that the chaperone CNX binds to Cx32 in a glycan-independent manner. Binding increases for Cx32 variants misfolded in the membrane and CNX can bind individual Cx32 TMD segments.



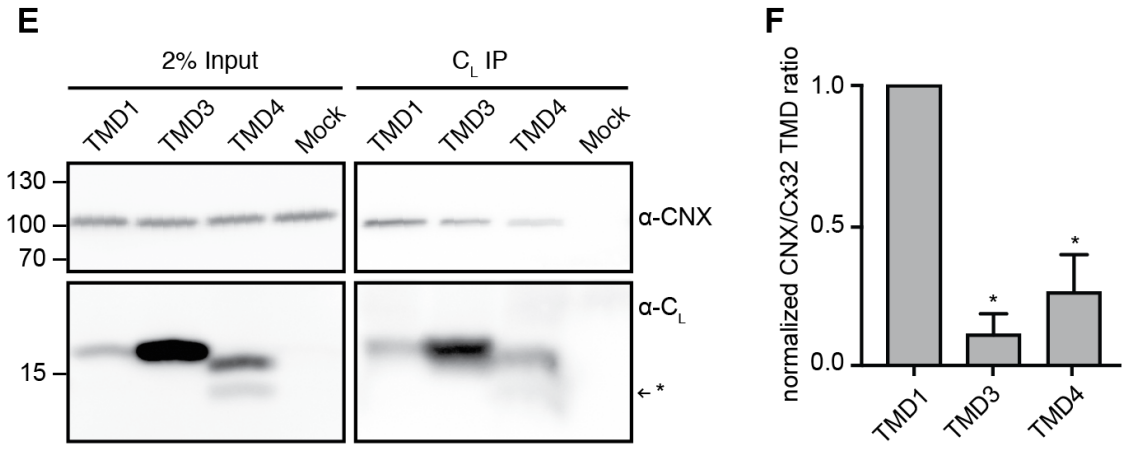


Figure 9: Calnexin binds to individual transmembrane domains of a non-glycosylated client. (A) Schematic of the C_L-TMD reporter construct to assess CNX interactions with individual Cx32 TMD segments. The four WT sequences of the Cx32-TMDs are shown. Predicted membrane integration energies are given next to them using DGPred (Hessa, Meindl-Beinker et al. 2007). Sequences of TMD1 and TMD3 were inverted in the respective constructs to reflect the correct orientation within Cx32 (see Figure 7A). **(B)** EndoH digest of the four C_L-TMD reporters to assess their integration and orientation in the membrane. The different detected species of C_L-TMD constructs are indicated with arrows. Percentages below the blot indicate the C-terminally glycosylated fraction, giving an estimate of the fraction that fails to integrate into the ER membrane. The asterisk (*) denotes a fraction of cleaved species. Cleavage was also previously observed for similar reporter constructs containing the C_L domain, which occurred post-lysis (Behnke, Mann et al. 2016). **(C)** Predicted ΔG_{app} values for membrane integration for the isolated TMDs of investigated Cx32 mutants. **(D)** EndoH digest of the C_L-TMD3 domain reporter and its indicated mutants to assess their membrane integration. Percentages below the blot indicate the C-terminally glycosylated fraction, giving an estimate of the fraction that fails to integrate into the ER membrane. **(E)** Co-immunoprecipitations of endogenous CNX with the different C_L-TMD constructs (NVT glycosylation site mutated to QVT). One representative immunoblot is shown. The asterisk (*) denotes a fraction of cleaved species. **(F)** Quantification and statistical analysis of (F). All samples were normalized to the construct containing TMD1 (mean ± SEM, N ≥ 3, *P value < 0.05, two-tailed Student's t tests).

3.2 The transmembrane domain of Calnexin binds clients in the membrane

Our studies on Cx32 show that CNX can bind individual TM segments of its clients. Based on these findings, we next aimed at defining which structural elements of CNX were necessary for binding. Toward this end, we designed a

minimal CNX (minCNX) construct that only contained the CNX TMD and a few additional C-terminal residues. The ER-lumenal domain of CNX was replaced by the fluorescent protein mScarlet-I (Figure 10A). To maintain ER retention of the construct, an endogenous cytosolic di-lysine motif was left in place (Figures 10A and 23A) (Jackson, Nilsson et al. 1990). ER-localization was confirmed by fluorescence microscopy (Figure 10B). Using this construct, we could show that the CNX TMD segment was necessary and sufficient for binding to full-length Cx32 (Figure 10C). Further extending this finding, we could show that an indvidual TMD of Cx32 bound to the minCNX construct as well (Figure 10D). In either case, an ER-retained control construct lacking the CNX TMD region (minCNX$^{\Delta TMD}$, Figure 22B) did not bind to the different clients (Figures 10C and 10D). Thus, binding occurred independently of the CNX lectin domain or its C-terminal tail. Accordingly, the CNX TMD contains the relevant features for client recognition in the membrane, and this can be recapitulated with single TMD regions derived from a client protein.

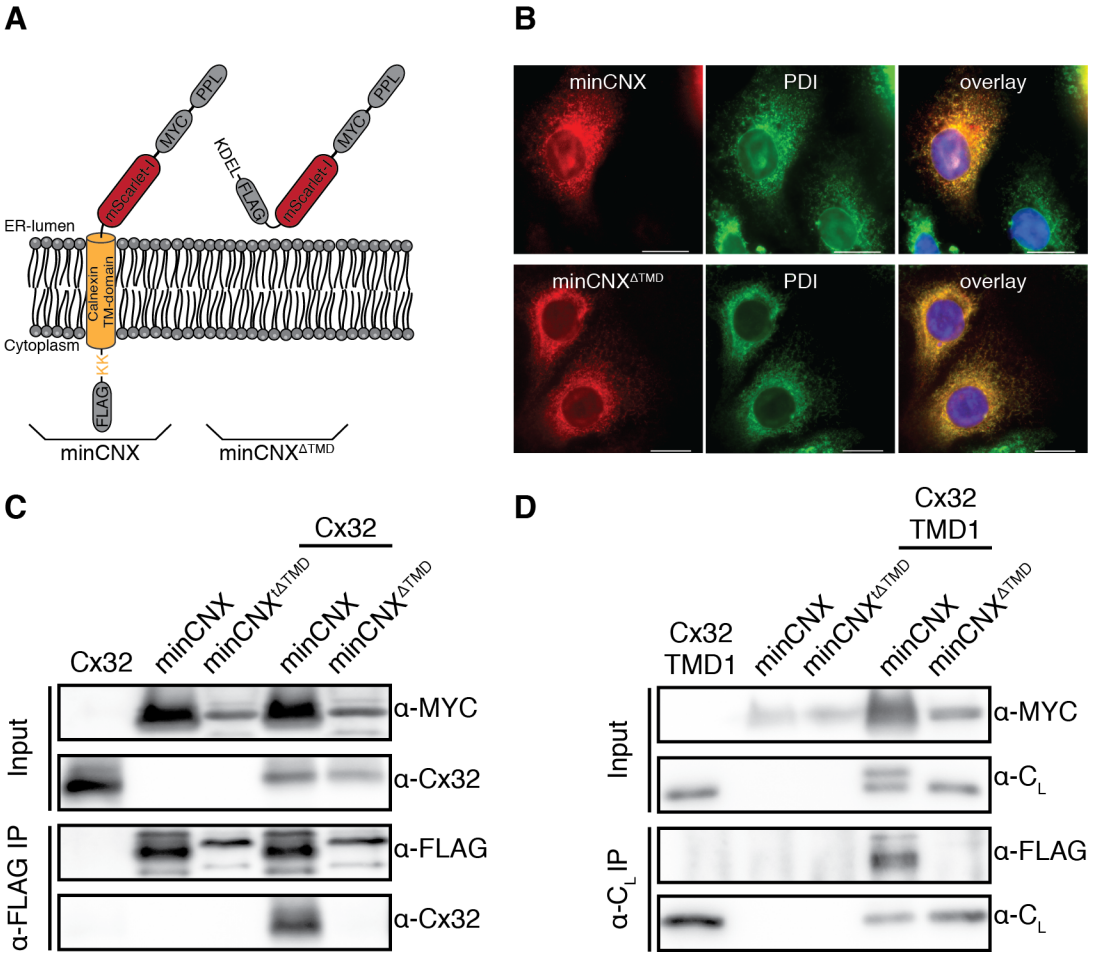


Figure 10: Design and validation of a minimal Calnexin construct. (A) Schematic of the minimal CNX construct (minCNX). This consists of a preprolactin (PPL) ER import sequence, followed by an N-terminal MYC tag, the monomeric red fluorescent protein mScarlet-I, the TMD segment of CNX including an endogenous double lysine motif downstream of the TMD region and a C-terminal FLAG-tag. Flexible linker regions connects the individual components. Parts derived from CNX are shown in orange. In contrast, a control construct, minCNX$^{\Delta TMD}$, is lacking the entire CNX TMD segment and only consists of the PPL ER import sequence as well as the fluorescent reporter mScarlet-I flanked by a MYC and FLAG-tag. A C-terminal KDEL sequence was included for ER retention. **(B)** COS-7 cells were transiently transfected with the indicated constructs and immunofluorescence microscopy was performed using anti PDI (green) as an ER marker. Detection of minCNX constructs was carried out by mScarlet-I fluorescence (red). Nuclei were stained with DAPI (blue). Images are representative of cells from at least three different biological replicates. Scale bars correspond to 20 μm. **(C)** Interaction of wild type Cx32 with the minCNX system. Representative blots from co-immunoprecipitation experiments from HEK293T cells transfected with the indicated Cx32 WT and minCNX constructs including relevant controls are shown. **(D)** The CNX TMD is sufficient for binding to another single TMD region, Cx32-TMD1. MinCNX constructs and a control variant devoid of the CNX TMD region (minCNX$^{\Delta TMD}$, see (A)) were co-transfected with Cx32-TMD1 into HEK293T cells. Interaction between the constructs was analyzed by co-immunoprecipitation experiments followed by immunoblotting.

3.3 Development of a tool to systematically assess intra-membrane recognition processes

Our data show that CNX directly binds to individual TM segments of its clients and that the CNX TMD is sufficient for binding to occur. This established a minimal system for client recognition by a chaperone in the membrane. Based on these findings, we next aimed to define which intra-membrane features of its clients CNX recognizes. This would be a major step forward in our understanding of intra-membrane chaperones but is very difficult to accomplish by using (parts of) natural proteins, as these generally will possess complex sequences and structures which precludes unbiased analyses. We thus decided to design a client protein that allows for the systematic analysis of intra-membrane recognition processes. Toward this end, we performed a multiple sequence alignment of 200 randomly selected human single pass plasma membrane proteins from the membranome database (Lomize, Lomize et al. 2017, Lomize,

Hage et al. 2018). This gave rise to an average TMD of a transport-competent protein, which thus can be expected to lack major chaperone recognition sites. We termed this protein *minimal consensus membrane protein* (CoMem). The design of CoMem included a superfolder GFP (sfGFP) moiety for microscopic localization studies, a C-terminal HA-epitope tag, and two consensus glycosylation sites to analyze its topology and intracellular transport (Figure 11A). In agreement with our assumption that, on average, TM segments from single-pass cell surface TMD proteins are close to optimal, the most frequent amino acid at most positions turned out to be Leu (Figure 11A). TM Leu-zippers, however, have a strong self-assembly propensity that would compromise our analyses (Gurezka, Laage et al. 1999). We thus proceeded with the second most frequently occurring amino acids, which were also entirely free of unfavorable residues for a TM sequence (Hessa, Meindl-Beinker et al. 2007). The multiple sequence alignment resulted in a TMD of 26 amino acid in length in CoMem, which is in very good agreement with recent studies on average TMD lengths in the plasma membrane (Sharpe, Stevens et al. 2010, Singh and Mittal 2016). Interestingly, it was flanked by an N-terminal Pro-residue, breaking the helical TMD structure (Cordes, Bright et al. 2002), and a C-terminal Lys residue (Figure 11A and Figure 22C). A C-terminal Lys will induce a type I orientation (von Heijne and Gavel 1988), placing the Lys residue in the cytoplasm, which reflects the nature of our sequence set (76% of the proteins were type I). Individually mutating the first or second consensus glycosylation site in CoMem showed that it was exclusively modified at the first site, which confirms the predicted topology (Figure 11B). Enzymatic deglycosylation with EndoH (which only removes N-linked sugars not further modified in the Golgi) and with PNGaseF (which removes all N-linked sugars) further revealed that CoMem glycosylation was EndoH-resistant, arguing that it was able to traverse the Golgi as expected (Figure 11B). Localization to the plasma membrane was confirmed by microscopic studies (Figure 11C). Taken together, CoMem now provided us with an ideal tool to systematically dissect intra-membrane substrate recognition by CNX.

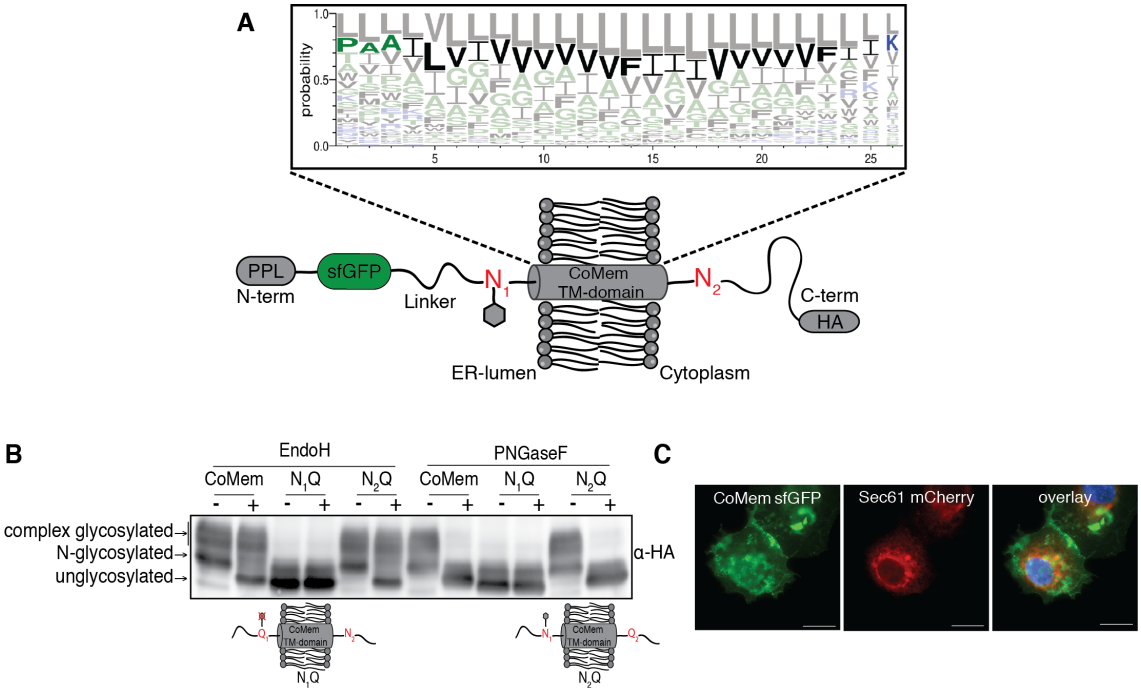


Figure 11: Design and validation of CoMem, a tool to query TM-domain recognition. (A) Schematic of the the minimal consensus membrane protein *CoMem* which contains a preprolactin (PPL) ER import sequence, a superfolder GFP (sfGFP), a TMD flanked by two individual NVT glycosylation motifs (N_1 and N_2) and a C-terminal HA-tag. Individual construct components are connected by flexible linker regions. As illustrated, in the predicted topology of CoMem, only the first NVT glycosylation site (N_1) is accessible to the ER glycosylation machinery (grey hexagon). The TMD of CoMem was designed on the basis of a multiple sequence alignment of 200 predicted human single pass TMD sequences as illustrated in the sequence logo. Hydrophilic amino acid residues are depicted in blue, neutral ones in green and hydrophobic residues in black. The predicted amino acid sequence with the second highest score was selected as the TMD consensus sequence for CoMem (bold). **(B)** HEK293T cells were transiently transfected with plasmids expressing CoMem or the indicated variants, where either the first (N_1) or the second (N_2) NVT glycosylation motif was altered (N to Q mutation), to prevent glycosylation of that specific site. Lysates were treated with or without EndoH or PNGaseF as indicated and analyzed by immunoblotting. N-glycosylation occurs in the ER, complex glycosylation in the Golgi. **(C)** COS-7 cells were transfected with the indicated constructs and fluorescence microscopy was performed using Sec61 mCherry (red) as an ER marker. CoMem, detected by sfGFP fluorescence (green), is not retained in the ER but localized to the plasma membrane. Nuclei were stained with DAPI (blue). Images are representative of cells from at least three different biological replicates. Scale bars correspond to 20 μm.

3.4 Defining intra-membrane recognition motifs for Calnexin

To dissect features recognized by CNX in the membrane, we first replaced the central Val residue at position 13 in CoMem by all other 19 amino acids. Of note, for all of these substitutions, CoMem was still predicted to be stably integrated into the membrane (Figure 12A) which was experimentally confirmed for several constructs and different locations of polar amino acids (Figure 12B). Using this CoMem panel, we first established suitable conditions for co-immunoprecipitaion experiments (Figure 12C) and then investigated interactions with CNX.

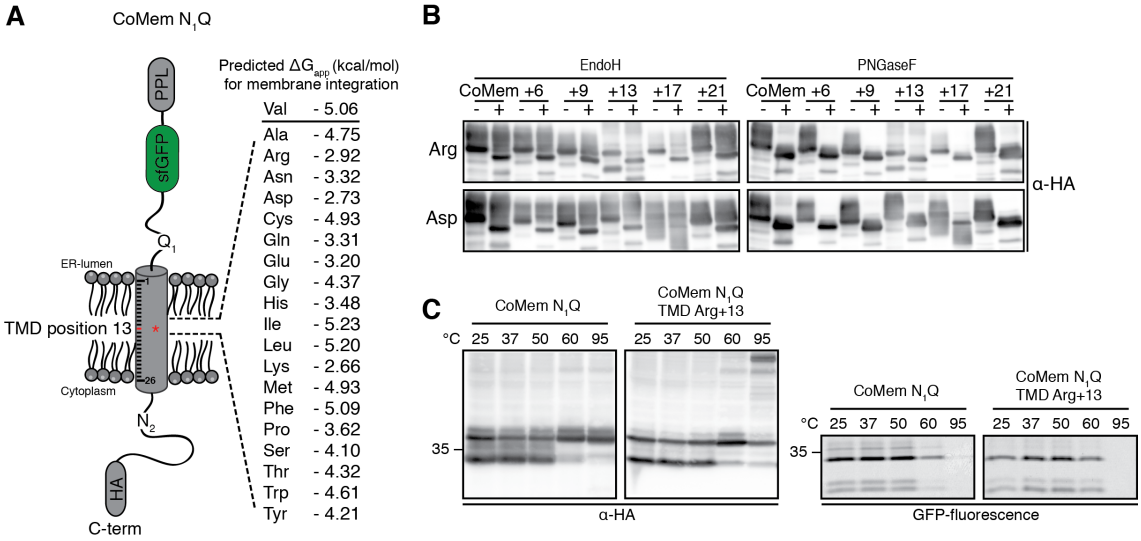


Figure 12: Defining experimental conditions to assess intra-membrane recognition motifs for Calnexin, part I. (A) Schematic of CoMem N_1Q where a central Val residue at position 13 within its TMD was replaced by all other 19 amino acids. Free energies for TMD insertion were predicted according to (Hessa, Meindl-Beinker et al. 2007). **(B)** CoMem and CoMem mutant variants containing amino acid replacements against arginine and asparagine at the indicated positions of the TMD region were transfected into HEK293T cells. Cell lysates were treated with or without EndoH or PNGaseF as indicated and analyzed by immunoblotting. Comparison of the glycosylation pattern with respect to CoMem indicates no misintegration for the different CoMem TMD mutants. **(C)** CoMem N_1Q and CoMem N_1Q Arg+13 were transfected into HEK293T cells. Cell lysates were split and exposed to different denaturing temperatures in Lämmli buffer as indicated. Analysis by immunoblotting and fluorescent imaging of sfGFP revealed some aggregation at high temperatures. The two lower species correspond to CoMem N_1Q with an unfolded (upper) or a folded (lower, more compact) sfGFP moiety, as can be seen in the fluorescence-imaged gel on the right to detect sfGFP fluorescence.

Although CoMem containing an ER-lumenal glycosylation site bound stronger to CNX, significant binding was also observed without this site, which shows that glycosylation of CoMem increases binding to CNX but is not required for it to occur (Figure 13A). No binding of sfGFP (Figure 22 D) to CNX was observed (Figure 13B), neither did Cx32 bind to CoMem (Figure 13C), showing the specificity of the CoMem:CNX interactions. Together, this further corroborated binding of CNX to TM regions in the membrane. For all subsequent experiments, CoMem lacking its ER-lumenal glycosylation site was used to specifically investigate glycan-independent binding to CNX. Importantly, CNX bound to CoMem although it was mostly an ideal TMD segment. This allowed to assess features increasing and decreasing binding to CNX.

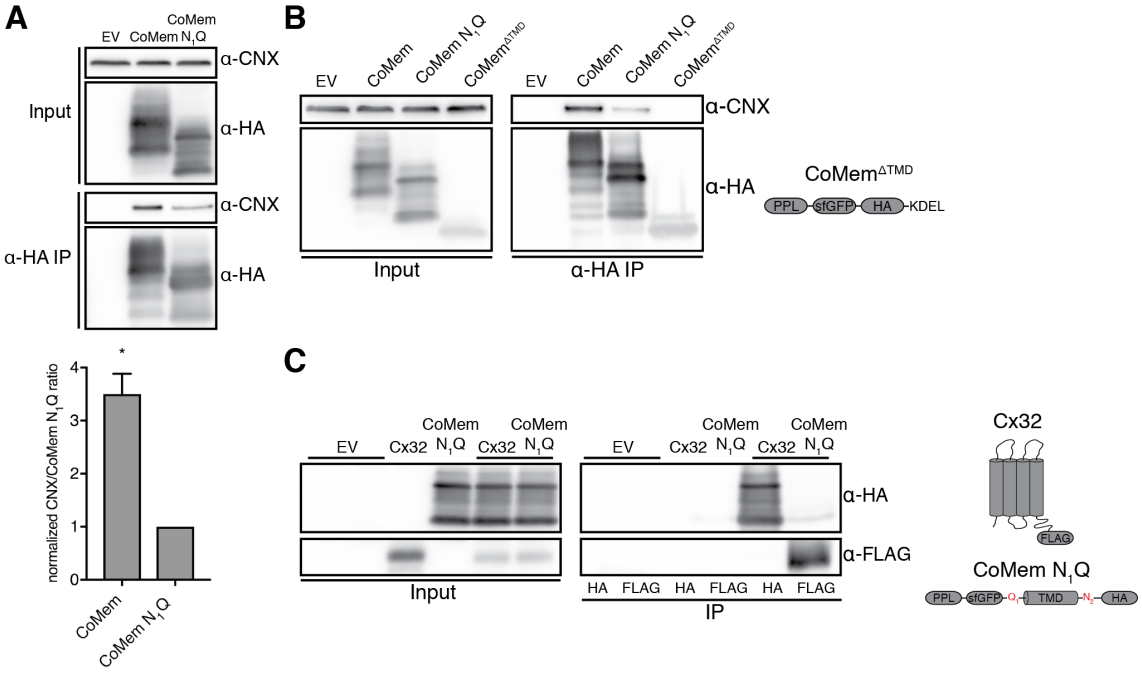
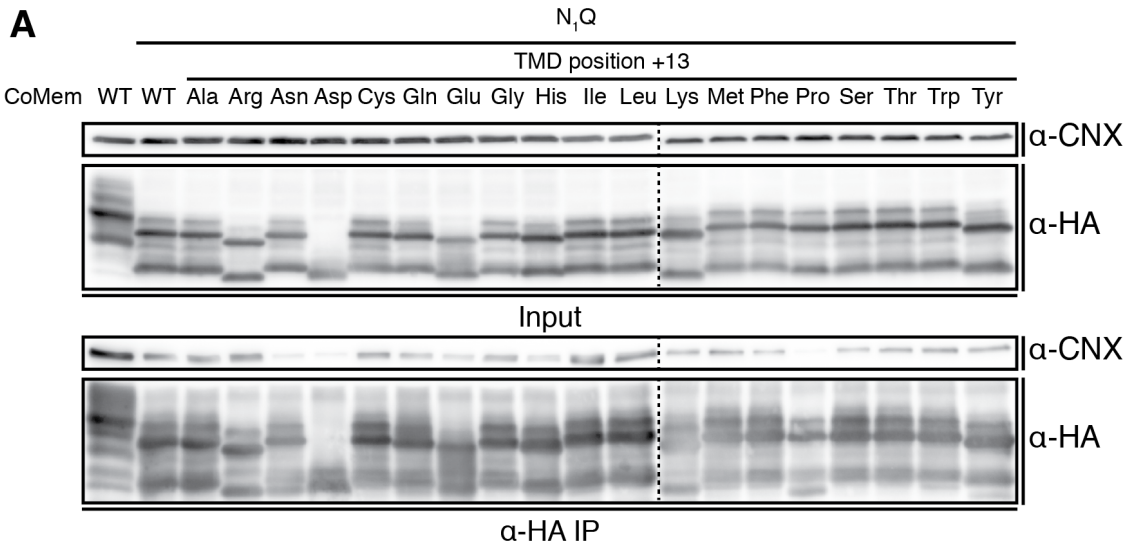


Figure 13: Defining experimental conditions to assess intra-membrane recognition motifs for Calnexin, part II. (A) Representative blots from co-immunoprecipitation experiments from HEK293T cells transfected with the indicated CoMem constructs. Interaction of CoMem with endogenous CNX was significantly decreased but still present for CoMem lacking its ER-lumenal glycosylation site (mean ± SEM, N ≥ 3, *P value < 0.05, two-tailed Student's t tests). Individual values were normalized to CoMem N_1Q (Val) values that were set to 1 **(B)** CNX does not bind to $CoMem^{\Delta TMD}$. HEK293T cells were transiently transfected with CoMem, CoMem N_1Q or $CoMem^{\Delta TMD}$ as indicated. Cell lysates and HA-immunoprecipitates were analyzed for HA-tagged CoMem variants and co-immunoprecipitating endogenous CNX. **(C)** Cx32 does not interact with

CoMem. HEK293T cells were transiently transfected with either HA-tagged CoMem N_1Q constructs, FLAG-tagged Cx32 constructs or both together. HA-Immonoprecipitates were analyzed for CoMem N_1Q and co-immunoprecipitating Cx32 and vice-versa.

Using this approach revealed a highly distinct binding pattern for CNX to the 20 CoMem variants with the membrane-central amino acid exchanged. For some, e.g. Arg, binding was significantly increased, whereas for others, e.g. Pro, binding was decreased (Figure 14A and B). Arg introduces an unfavorable polar residue into the membrane, whereas Pro acts as a TMD helix breaker. Based on these findings, we further continued to investigate features recognized by CNX. We selected one amino acid, where increased binding to CNX was observed if it was present in a central location in the CoMem TMD segment (Arg), and one that decreased binding (Pro) (Figure 14B). For these, we moved the mutation site through the entire CoMem TMD segment to analyze a possible positional dependency for effects on CNX binding (Figure 14C). In each case, the central position 13 (out of 26 amino acids) turned out to show the strongest effects, but replacements at other positions also influenced binding, with slightly different positional dependencies for the two selected amino acids (Figure 14C). Taken together, these data show that CNX can differently recognize clients with well-defined lesions in the membrane.



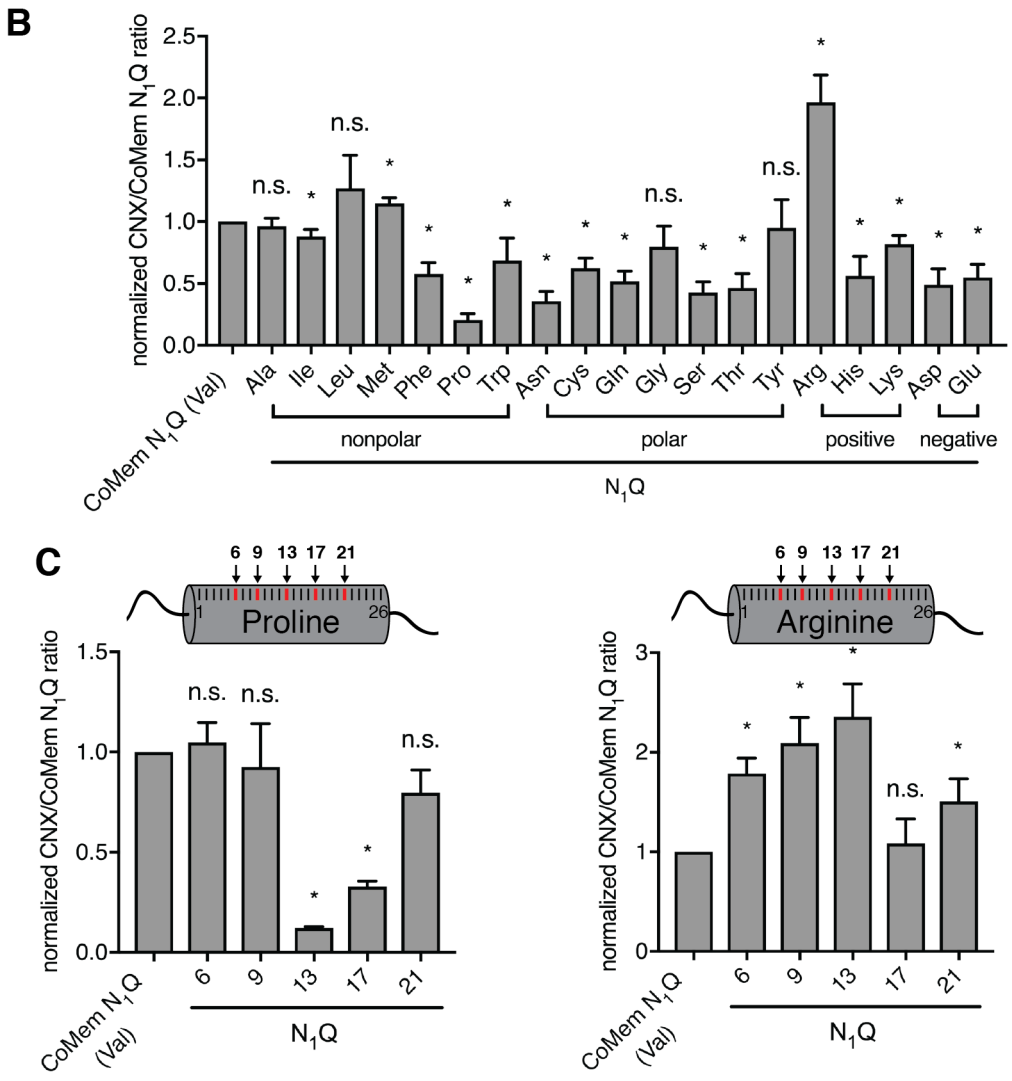


Figure 14: Defining intra-membrane recognition motifs for Calnexin. (A) Representative blots of co-immunoprecipitation experiments between CoMem and CNX from HEK293T cells transfected with the indicated CoMem constructs, each of which contained one of the 20 amino acids on position 13 within the TMD region **(B)** Interaction of CoMem N_1Q variants as described in (12A) with endogenous CNX (mean ± SEM, N ≥ 5, *P value < 0.05, two-tailed Student's t tests). **(C)** same as in (B) only that selected amino acids which have shown the most significant impact on CNX interaction (Proline and Arginine) where shifted through the CoMem N_1Q TMD segment to five different positions as shown in the illustration, revealing positional binding dependencies. (mean ± SEM, N ≥ 4, *P value < 0.05, two-tailed Student's t tests). Individual values in (B) and (C) were normalized to CoMem N_1Q (Val) values that were set to 1.

3.5 A structural understanding of intra-membrane Calnexin:client recognition

Having defined client-intrinsic binding patterns for CNX in the membrane, we next proceeded to analyze the features within the CNX TMD segment that allow client binding to occur. Towards this end, we performed molecular dynamics simulations on either the CNX TMD region together with the CoMem TMD segment or with the first TMD of Cx32 (Cx32-TMD1), which our data have shown to be interacting systems in cells (Figures 10 and 13). In agreement with these experimental findings, molecular dynamics simulations revealed interactions for both systems (Figure 15A). Of note, for CoMem, as well as for Cx32-TMD1, similar regions in the CNX TMD region were involved in the binding process. These regions involved a Tyr, a Thr and a Leu on the same face of the CNX TMD region (Figures 15A - C).

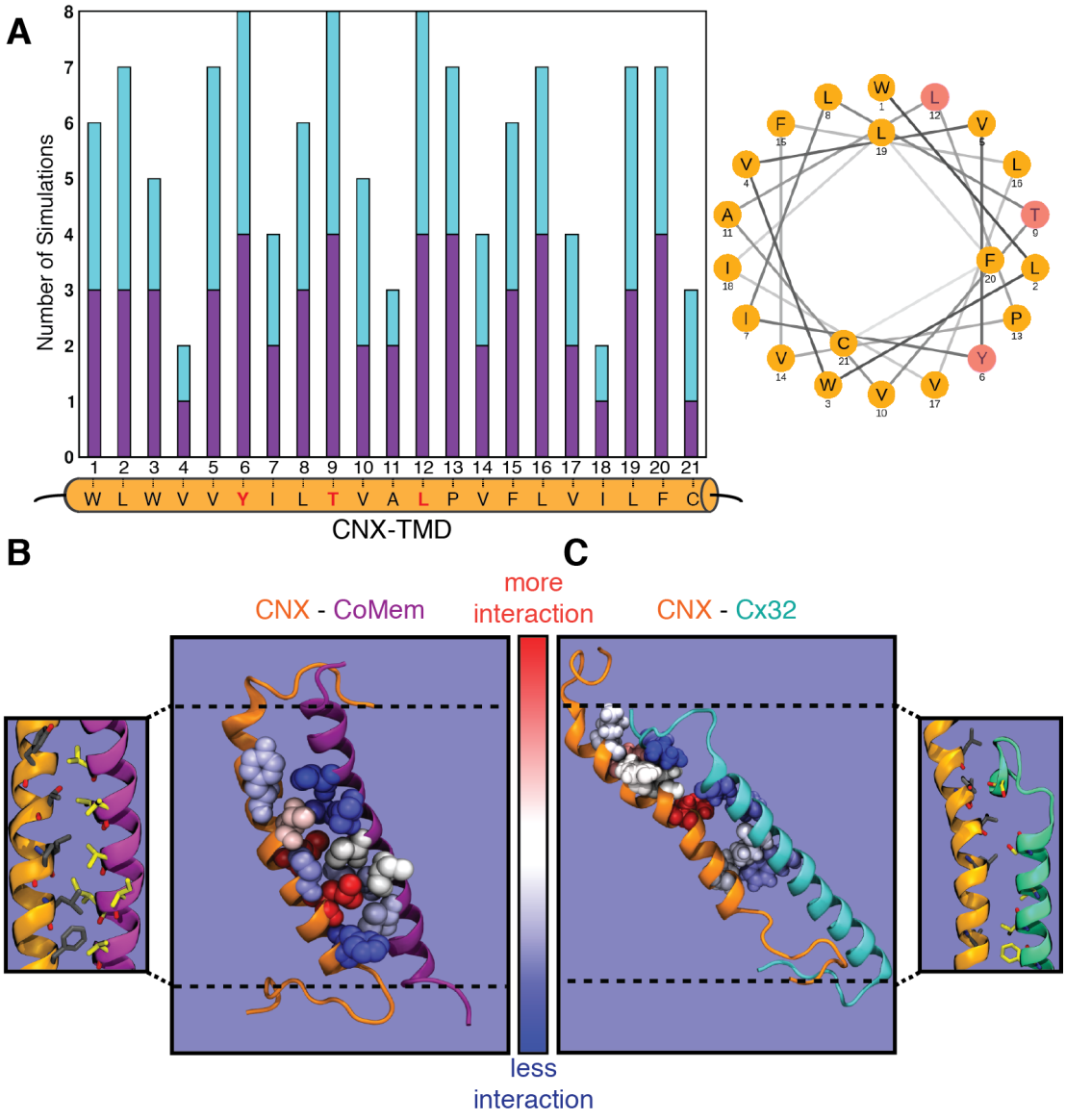


Figure 15: A structural analysis of intra-membrane Calnexin:client recognition. (A) Meta-analysis of MD simulations (N=8) where complex formation between CNX and Cx32-TMD1 or CNX and CoMem was observed. On the x-axis the CNX TMD residues are shown. The height of the bars corresponds with the number of simulations in which a specific CNX TMD residue interacted with the substrate helix CX32-TMD1 (turquoise) or CoMem (violet). Only CNX TMD residues Y17, T20 and L23 interacted with CX32-TMD1 or CoMem in all sampled complexes. **(B)** Depiction of residues exhibiting most interactions with the partner helix in simulations leading to complexes between CNX and CoMem. **(C)** Same as (B) but for the CNX-Cx32 simulations. CNX is depicted in orange, while CoMem is shown in purple and Cx32 in turquoise. Residues exhibiting most interactions with the partner helix are colored red, while residues in dark blue formed the least interactions. The dashed line indicates the membrane embedded region. Only residues with interaction frequencies of at least 30% of the top interacting residues are shown. For CNX residue statistics, only simulations in which complex formation was observed have been considered. The CoMem and Cx32 residues depicted here have been selected and colored with respect to how often they form interactions with the highlighted CNX residues (also in simulations in which stable complexes are formed) (Data obtained from Manuel Hitzenberger, Zaccharias Lab).

To assess our computational predictions experimentally, we mutated these predicted interaction sites to presumably inert Val residues in a CNX construct that we overexpressed in mammalian cells. This construct was furnished with a V5 epitope tag for specific immunoprecipitation and overexpression was only slightly higher than the endogenous CNX level. Strikingly, whereas CoMem and Cx32-TMD1 co-immunoprecipitated with V5-tagged CNX as expected, mutation of the YTL-motif, and even of only the Y and T residues to Val, significantly reduced interactions between CNX and CoMem (Figure 16A). This was even more pronounced for Cx32-TMD1 (Figure 16B), and when the YTL-motif was exchanged against Ala instead of Val, a similar reduction in binding was observed (Figure 16C).

This argues that mutating the interaction site *per se*, but not the choice of the residues mutated to, accounts for the effects. Together, these findings confirmed our simulation data and further corroborated the conserved nature of the interactions. Of note, when CoMem with its ER-lumenal glycosylation site was used, mutations in the CNX TMD region did not significantly affect binding, showing that for this glycosylated simple client, binding via the lectin domain is

dominant (Figure 16D). The results obtained with these simplified clients also applied when we investigated CNX interactions with its full-length client Cx32 (Figure 16E) as well as the Cx32 V140E mutant (Figure 16F).

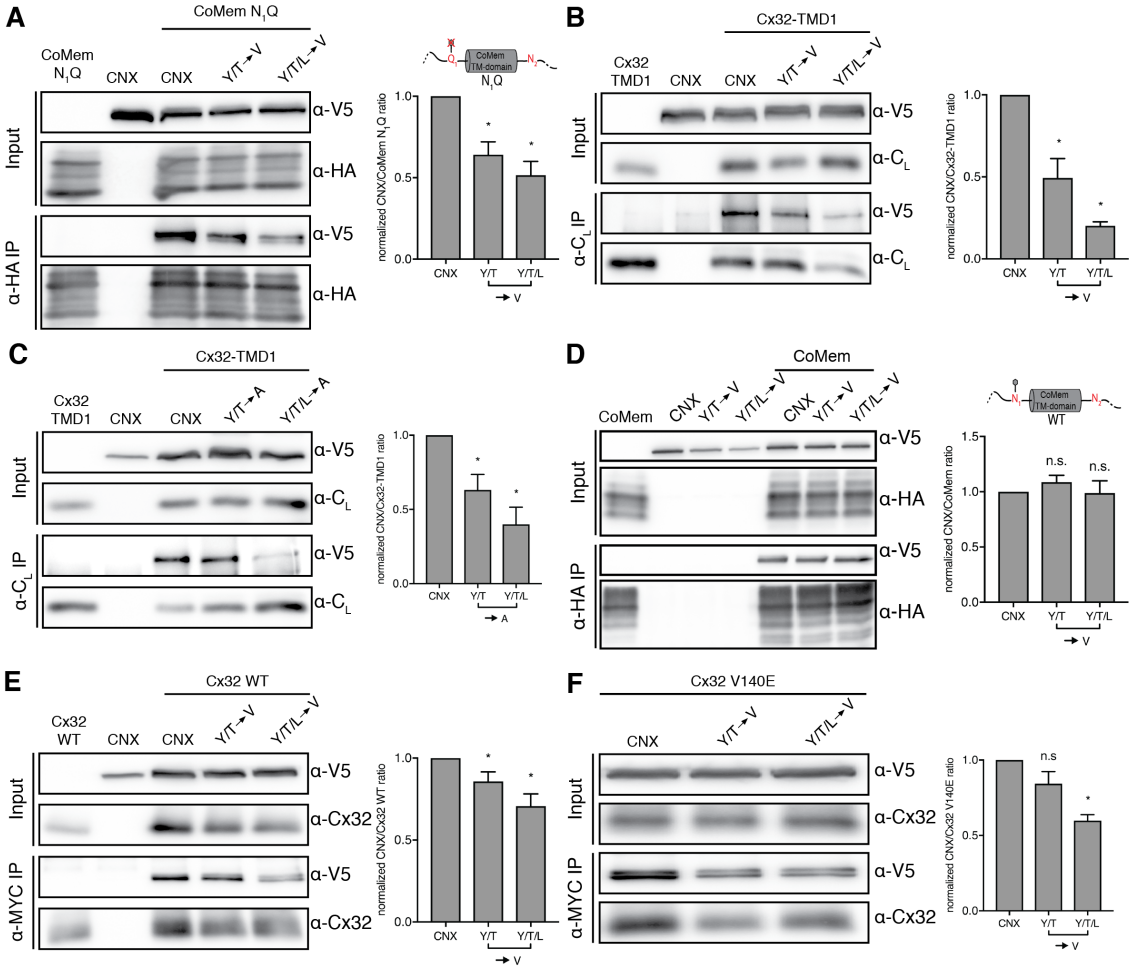


Figure 16: A structural analysis of intra-membrane Calnexin:client recognition. (A) Interaction of non-glycosylated CoMem N_1Q constructs with V5-tagged CNX. CNX variants where amino acids important for interaction as revealed in (15A) were replaced by valine residues. HEK293T cells were transiently transfected with the indicated constructs and cell lysates and HA-immunoprecipiates were analyzed for HA-tagged CoMem N_1Q and co-immunoprecipitating CNX mutants. One representative blot is shown. The graph shows results from nine independent experiments (mean ± SEM, N ≥ 9, *P value < 0.05, two-tailed Student's t tests). **(B)** The same as in (A) for Cx32-TMD1 (mean ± SEM, N ≥ 6, *P value < 0.05, two-tailed Student's t tests). **(C)** Interaction of Cx32-TMD1 is equally reduced for CNX mutants where amino acids important for interaction as shown in (15A) were exchanged by alanine instead of valine residues. The graph shows results from five independent experiments (mean ± SEM, N ≥ 5, *P value < 0.05, two-tailed Student's t tests). **(D)** Mutations in the CNX TMD region do not affect binding of glycosylated CoMem. The graph shows

results from three independent experiments (mean ± SEM, N ≥ 3, n.s.: not significant, two-tailed Student's t tests). **(E)** The same as in (B) for Cx32 full-length (mean ± SEM, N ≥ 4, *P value < 0.05, two-tailed Student's t tests). **(F)** Interaction of Cx32 V140E full-length with CNX behaves similar compared to Cx32 WT. The graph shows results from six independent experiments (mean ± SEM, N ≥ 6, n.s.: not significant, *P value < 0.05, two-tailed Student's t tests).

To further verify the specificity of the binding motif we have identified within the CNX TM region, we selected three non-Val residues that had the lowest overall predicted client interactions from our simulations (an Ile (TMD position 7), an Ala (TMD position 11) and another Ile (TMD position 18), Figure 15A). Replacing either the first two or all three of these three residues by Val did not affect binding of CNX to its client Cx32-TMD1, further corroborating our molecular interpretation of CNX client binding in the membrane (Figure 17A). In contrast, when we shifted the identified YTL-motif N-terminally within the CNX TMD, this also significantly reduced binding to Cx32-TMD1 (Figure 17B), revealing positional specificity in the recognition process. Taken together, our combined computational and experimental approach revealed a molecular recognition motif within the CNX TMD that allows CNX to bind clients in the lipid bilayer.

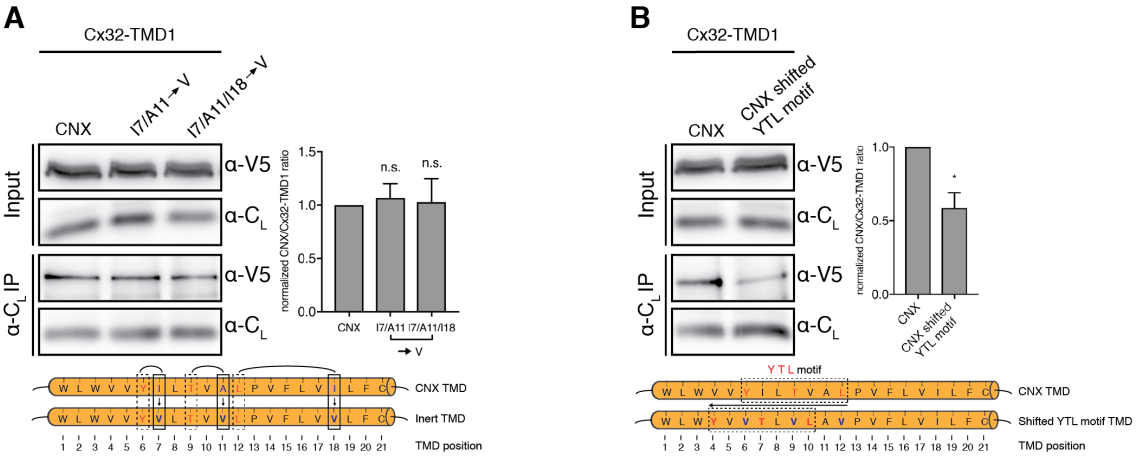
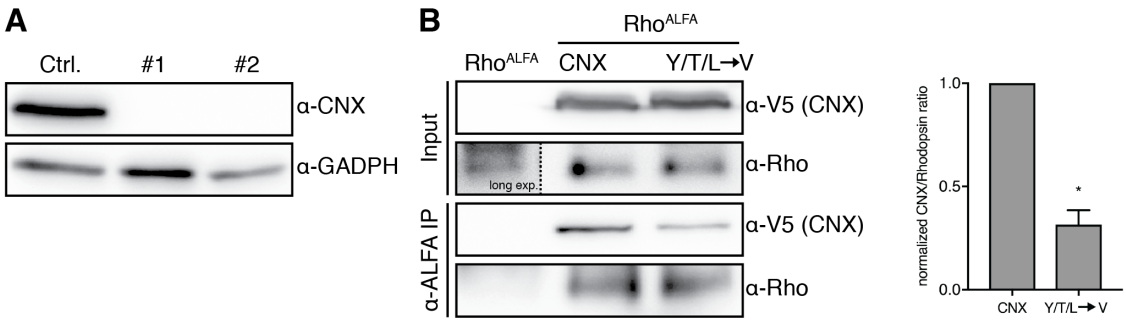


Figure 17: A structural analysis of intra-membrane Calnexin:client recognition. (A) Interaction of Cx32-TMD1 remains unchanged for CNX mutants where amino acids not predicted to be important for interaction as shown in (A) were exchanged by valine residues. HEK293T cells were transiently transfected with the indicated constructs and a representative blot is shown. The graph shows results from nine independent experiments (mean ± SEM, N ≥ 9, n.s.: not significant, two-tailed Student's t tests). **(B)** Shifting of the residues Y, T and L, most important for CNX substrate interaction, and replacing these against Valine

residues, results in a reduced interaction with Cx32-TMD1. HEK293T cells were transiently transfected with the indicated constructs and a representative blot is shown. The graph shows results from nine independent experiments (mean ± SEM, N ≥ 9, *P value < 0.05, two-tailed Student's t tests)

3.6 Biological functions of intra-membrane client recognition by Calnexin

Our comprehensive analyses provided us with detailed molecular insights into CNX:client recognition in the membrane. Using these insights, we were able to generate a CNX version that was compromised in client binding within the membrane while still having a functional lectin domain (Figures 15-17). To define functional consequences of ablating intra-membrane client binding we used Rhodopsin (Rho) as a model protein as it has been described as a CNX substrate. Furthermore, CNX appears to be involved in the quality control of Rho, however, this role is differently pronounced in different organisms (Rosenbaum, Hardie et al. 2006, Kosmaoglou and Cheetham 2008, Noorwez, Sama et al. 2009). We first established CNX knockout (k/o) cell lines (Figure 18A). In these cell lines, we analyzed interaction of Rho with either reconstituted wt CNX or the mutant deficient in the intra-membrane YTL-motif. In agreement with our data on a panel of other model proteins (Figures 15-17), Rho binding to the CNX mutant was significantly reduced (Figure 18B). Strikingly, Rho degradation was significantly accelerated in CNX k/o cells complemented with the YTL-motif mutant in comparison to wt CNX (Figure 18C), revealing a protective role of intra-membrane substrate binding by CNX for a labile client. This protective role of intra-membrane client binding appeared to work synergistically with lectin-based client binding as the complete absence of CNX had an even stronger effect on Rho degradation (Figure 18C).



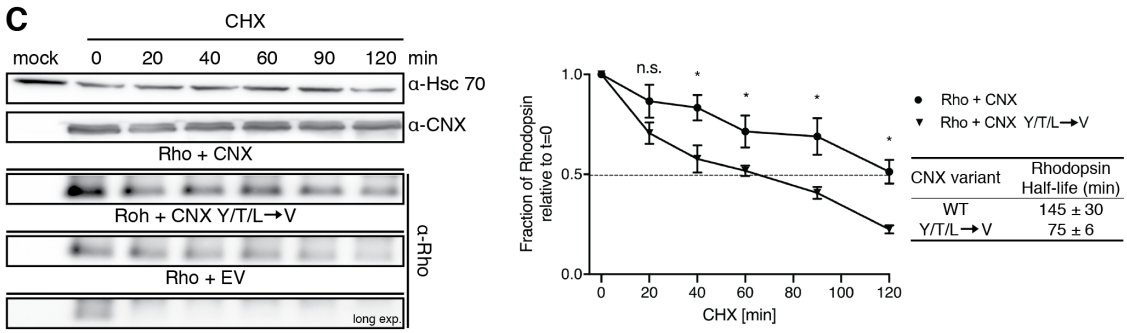


Figure 18: Biological functions of intra-membrane client recognition by Calnexin. (A) Immunoblot analysis of overexpressed CNX in CNX knockout cell lines. Generation of CNX knockout HEK293T cells was performed as described in the method section. **(B)** The presence of CNX is essentially required for the detection of Rhodopsin and interaction of the binding deficient CNX YTL TMD variant to Rhodopsin is significantly reduced compared to CNX WT. CNX knockout HEK293T cells were transiently transfected with either N-terminal ALFA-tagged Rhodopsin alone or in combination with the indicated V5-tagged CNX constructs. Cell lysates and ALFA-immunoprecipiates were analyzed for Rhodopsin and co-immunoprecipitating CNX. One representative blot is shown. The graph shows results from 4 independent experiments (mean ± SEM, N ≥ 4, *P value < 0.05, two-tailed Student's t tests). **(C)** Reduced interaction of CNX with Rhodopsin accelerates its degradation. CNX knockout HEK293T cells transfected with the indicated constructs were incubated with cycloheximide and lysates were collected at different timepoints. Overexpression of the binding deficient CNX YTL TMD mutant significantly accelerates Rhodopsin degradation. Quantifications are shown beside the immunoblots (mean ± SEM, N ≥ 8, *P value < 0.05, two-tailed Student's t tests).

Taken together, our data revealed several membrane protein clients whose binding to CNX depend on recognition in the membrane. To extend these findings and to define more relevant TM clients, especially non-glycosylated ones, we decided to perform a global analysis on the interactome of CNX. As a prerequisite for an unbiased global client analyses neither the CNX knockout nor expression of CNX in the knockout background caused detectable ER stress (Figure 19A). For the interactome analysis, like for our Rho interaction studies (Figure 18B), we relied on a CNX construct fused to a C-terminal ALFA-tag (Gotzke, Kilisch et al. 2019), which showed normal ER localization (Figure 19B) and did not influence substrate binding to CNX in control experiments (Figure 19C). Remarkably, similar to CNX WT the binding deficient Y/T/L→V mutant caused no detectable

ER stress as well and showed normal ER localization (Figure 19 A and B). The ALFA-tag is lysine-free and thus allows for the uncompromised use of lysine-crosslinkers like DSSO in co-immunoprecipitation experiments. CNX has a large number of well-dispersed lysine's which should allow for *in situ* DSSO-crosslinking (Figure 19D). Indeed, DSSO crosslinking revealed many CNX crosslinks that can be immunoprecipitated using ALFA-tag nanobodies. Interestingly, differences can be observed between the crosslinked interactome of WT CNX and the binding deficient variant (Figure 19E).

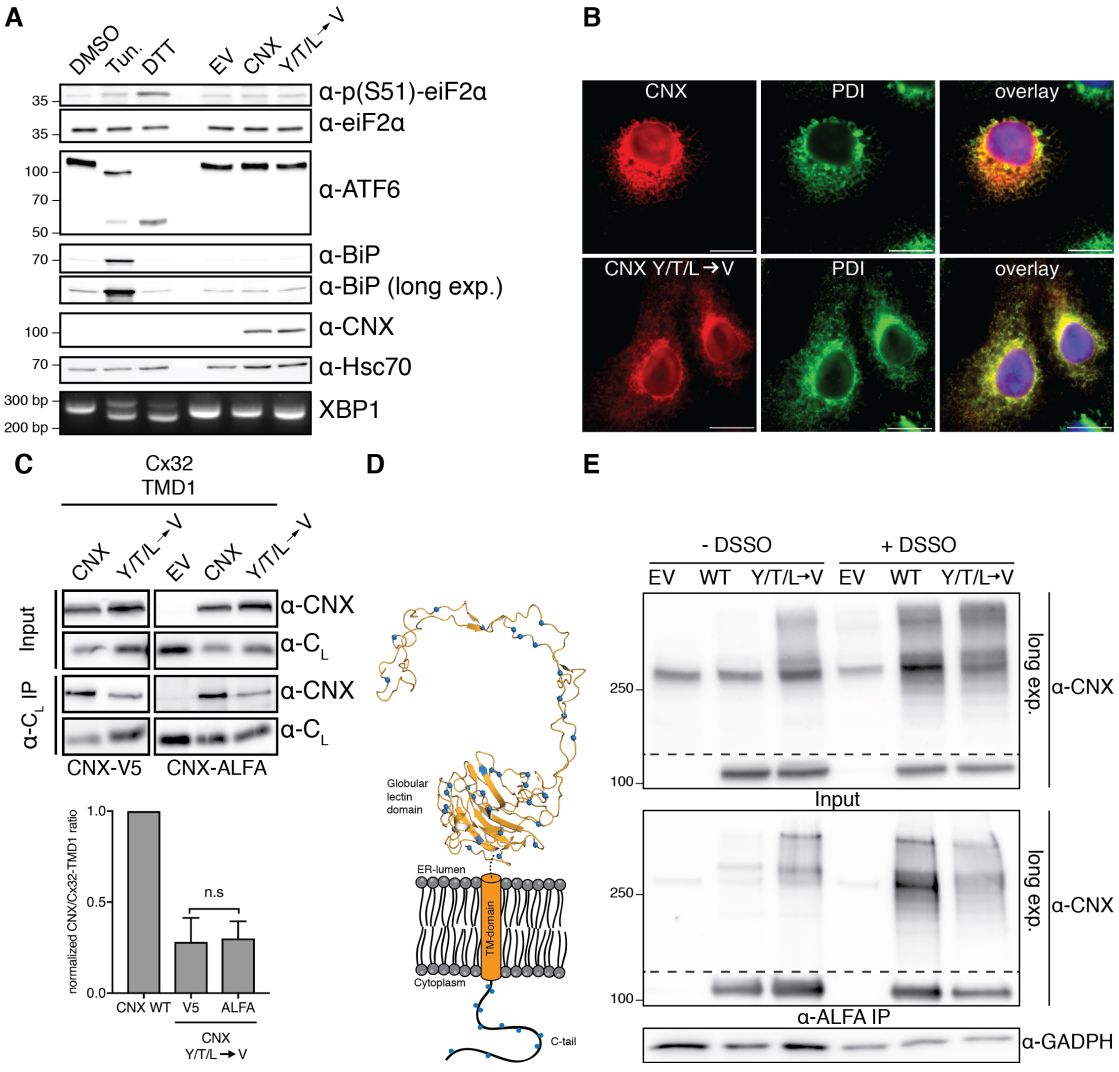


Figure 19 Functional analysis of CNX-V5 and CNX-ALFA expression and interaction behavior in the HEK293T CNX KO context. (A) UPR activation was assessed in CNX knockout cells transfected with mock plasmid, and incubated with DMSO (vehicle control), tunicamycin (Tun.), or DTT. CNX, or its binding-deficient mutant were transfected into the CNX knockout cells (right side) and activation of the three branches of the mammalian UPR was assessed. **(B)** COS-7 cells were transfected with the indicated CNX ALFA-tagged constructs and

fluorescence microscopy was performed using PDI (green) as an ER marker. CNX WT constructs and CNX mutant variants deficient in substrate binding were detected with anti-ALFA-FluoTag-X2 nanobodies (red) and show ER localization. Nuclei were stained with DAPI (blue). Images are representative of cells from at least three different biological replicates. Scale bars correspond to 20 μm. **(C)** Interaction of CNX with Cx32-TMD1 remains unaffected independent of different epitope tags fused to the C-terminus of CNX and lack of endogenous CNX in HEK293T CNX knockout cells. Cell lysates of HEK293T CNX KO cells co transfected with Cx32-TMD1 and either EV, CNX WT or the interaction deficient version CNX Y/T/L→V along with C_L immunoprecipitates were analyzed for C_L-tagged Cx32-TMD1 and co-immunoprecipitating CNX mutants containing either a V5 or an ALFA-tag. The graph shows results from four independent experiments (mean ± SEM, N ≥ 4, n.s.: not significant, two-tailed Student's t tests). **(D)** Schematic of CNX showing lysine residues, which can be crosslinked by DSSO. Individual lysine residues are indicated by blue dots. Exact location of lysine residues is shown in the crystal structure of the luminal domain of CNX whereas the position of lysines in the cytoplasmic tail region represents an approximation. **(E)** Immunoblot analysis following *in situ* DSSO crosslinking in CNX deficient cell lines transiently transfected with plasmids expressing CNX or the YTL→V TMD mutant compromised in intra-membrane client binding, both containing a C-terminal ALFA-tag. Cell lysates and ALFA-tag immunoprecipitates were immunoblotted against CNX to detect co-immunoprecipitating DSSO crosslinks.

After revealing DSSO-dependent CNX crosslinks in the immunoblot analysis, we performed a global analysis of the CNX interactome in CNX deficient HEK293T cells. To this end, we transfected our ALFA-tagged CNX WT construct into CNX deficient HEK293T cells, crosslinked protein complexes with DSSO, and lysed the cells with 1% NP-40 buffer. Subsequently, we immunoprecipitated the CNX proteins via the ALFA tag and performed affinity-enrichment mass spectrometry experiments. Our LC-MS/MS analysis identified several different membrane proteins interacting with CNX. Remarkably, many of the functional interaction partners of CNX we have identified are not glycoproteins, which renders the nature of their interaction interesting. Confirming the specificity of our experimental setup, we found the PDI family members ERp57 and ERp29 but also EDEM1 to interact with CNX (Oda, Hosokawa et al. 2003, Pollock, Kozlov et al. 2004, Nakao, Seko et al. 2017)

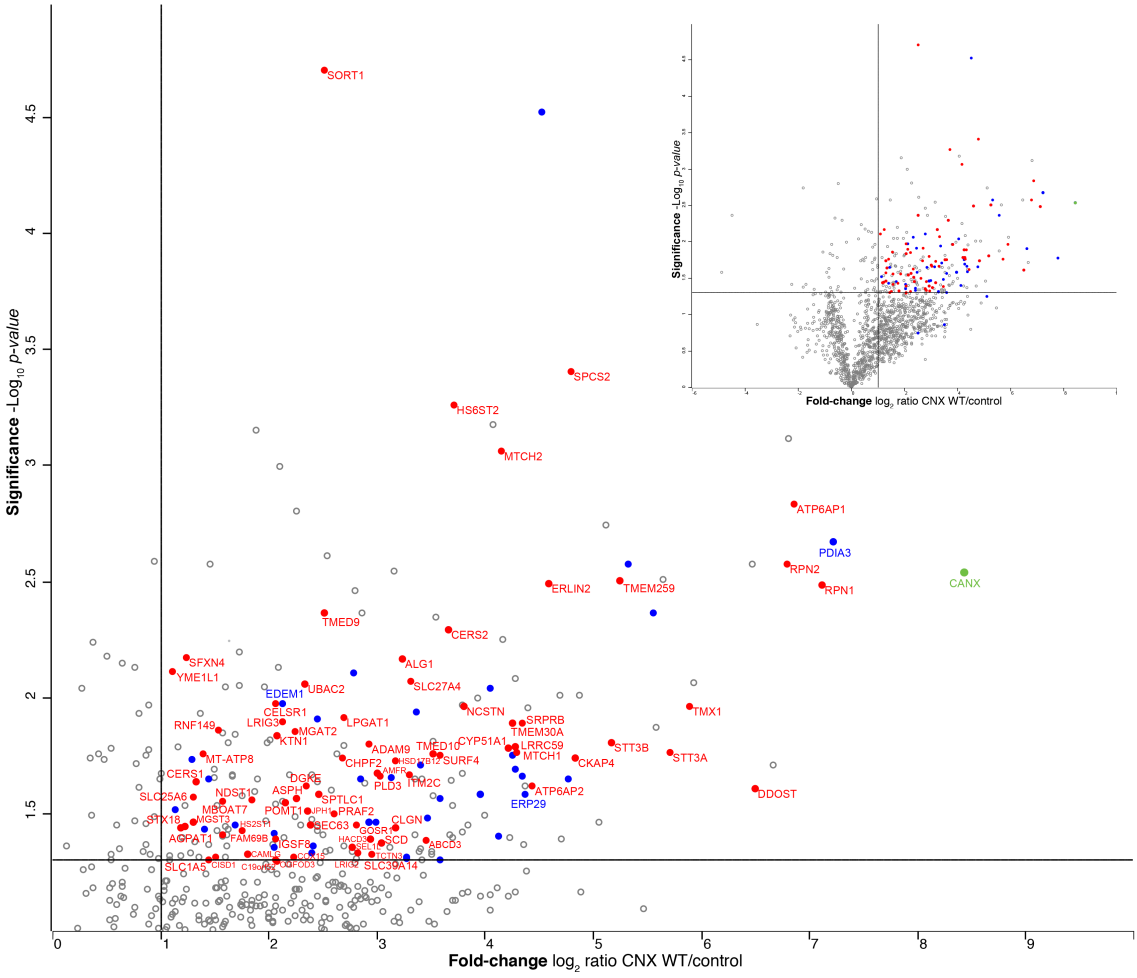


Figure 20 Global analysis of functional interaction partners of CNX. Volcano plots derived from LC-MS/MS analysis of ALFA-tagged CNX, immunoprecipitated in 1% NP-40 buffer from transfected CNX deficient HEK293T cells after DSSO crosslinking and compared to EV control co-IPs. Depicted is an enlarged image section as well as the associated complete volcano plot in the upper right part. Significantly enriched proteins and corresponding -log10 p-values were plotted. The respective proteins are denoted with their Uniprot gene names. CNX is shown in green and membrane proteins with the GO-term annotation "*integral to membrane"* are highlighted in red. ER proteins with the GO-term annotation "*endoplasmic reticulum"*, regardless of whether they are localized to the ER membrane or lumen are highlighted in blue. However, only known CNX interaction partners are indicated with their names in the enlarged image section. Cut-off values (solid lines) in the volcano plot were defined as $\log_2 = 1$ (2-fold enrichment) and $-\log_{10}$ = (p-value) of 1.3 (Data obtained from Nina Bach, Siebers Lab).

4 Discussion

This study describes a novel mechanism of intra-membrane substrate recognition by molecular chaperones as a basis for quality control of IMPs in the ER. By structural, mechanistic and systematic analyses we define features within the TMD of CNX and the TMD of its substrates that allow CNX to bind its clients in the lipid bilayer and thereby protect them from degradation. Importantly, efficient intra-membrane substrate recognition by CNX requires the presence of a certain recognition motif, which appears to be conserved in other intra-membrane chaperons involved in intra-membrane quality control.

Remarkably, several intra-membrane chaperones are known to interact with their substrate via direct helix-helix interaction, including the PAT complex or EMC. In the case of the EMC, it could be shown that this complex has specificity for multi-pass membrane proteins with polar and slightly hydrophobic TM domains or those with destabilizing features such as charged or aromatic residues. To date, however, the detailed mechanism of substrate recognition for both intra-membrane chaperones is poorly understood (Meacock, Lecomte et al. 2002, Shurtleff, Itzhak et al. 2018, Chitwood and Hegde 2020, Miller-Vedam, Brauning et al. 2020). Hence, the results of this study could help to elucidate how intra-membrane chaperones recognize transmembrane defects inside the lipid bilayer.

In addition, in this study, we show for the first time a global analysis of the substrate repertoire of CNX.

4.1 Principles of intra-membrane chaperoning processes by CNX

Historically, the function of CNX in recognizing and processing misfolded substrate proteins has mostly been limited to its lectin function, which resides in the globular N-domain (Hammond, Braakman et al. 1994, Hebert, Foellmer et al. 1995). In addition to the canonical function of CNX, however, there is increasing evidence that its transmembrane segment participates in intra-membrane quality control and chaperoning processes, as direct interaction with various membrane proteins such as MHC-I molecules, PLP, the tetraspanin CD82, or disease-related mutants of PMP22 has been shown, directly affecting their degradation, assembly or targeting to multilamellar bodies (Margolese, Waneck et al. 1993,

Cannon and Cresswell 2001, Swanton, High et al. 2003, Korkhov, Milan-Lobo et al. 2008, Yamasaki, Hara et al. 2014). It is important that these intra-membrane recognition processes are not limited to glycoproteins but rather take place in a lectin-independent manner. Nevertheless, mechanistic insights into the interactions of CNX's TMD with its clients were lacking. This seems particularly important considering that incorrect folding and assembly of IMPs and destabilizing mutations within TMDs are linked to various human diseases. In addition, most studies of canonical degradation and quality control pathways so far have focused on proteins where the misfolded lesion is either in the ER lumen or in the cytosol, but not on factors that interact with unstable TM helices.

Using Cx32 as a model protein, we were able to show in this study that CNX differentiates between the correctly folded and misfolded state of clients, indicating that the folding and assembly status of transmembrane segments in the membrane is the center of the recognition mechanism. Furthermore, specifically stronger binding to misfolded, CMT1X disease-causing mutants of Cx32 than WT reveals that CNX functions as a true molecular chaperone for non-glycosylated membrane proteins like Cx32. Interestingly, when looking at the individual transmembrane segments in isolation, CNX preferentially binds the first TMD of Cx32. In full-length Cx32, however, we observe the strongest binding of CNX to mutants that carry mutations in the third TM segment. This might indicate that these mutants, although properly integrated into the lipid bilayer, lead to structural changes or exposure of a buried recognition motif in TM1, which, consequently, facilitates binding of CNX. To test this hypothesis, it would be useful to study the direct interaction between CNX and individual TM mutants. Unfortunately, however, we were not able to study directly the TM mutants individually, as they were unstable in the membrane. Importantly, Cx32 is not a glycoprotein; thus, recognition of the chaperone must be lectin-independent. Indeed, utilizing a minimal CNX construct that only contains the CNX TMD we could show, that the transmembrane domain of CNX is necessary and sufficient for binding clients in the membrane independently of the ER-luminal lectin domain or C-terminal tail. Accordingly, this indicates that the CNX TMD contains the relevant features essential to detect defective transmembrane domains. As a

result, CNX could play an essential role in the ER-mediated quality control for many membrane proteins that are neither glycosylated nor have luminal degrons

4.2 Intra-membrane client recognition motifs for CNX

Although several intra-membrane chaperones have been identified over the last few decades, and CNX has also been assigned such a function, the detailed mechanism of substrate recognition by such factors inside the membrane plane is poorly understood. In contrast, for example, it has long been known that particularly exposed hydrophobic residues in the ER lumen or cytosol represent recognition motifs for luminal ER chaperones or ERAD factors. Furthermore, particularly relevant, most of the studies carried out analyzing intra-membrane chaperoning processes used naturally evolved membrane proteins as a model. However, this excludes an unbiased study on the definition of intra-membrane recognition motifs since TM proteins may engage multiple topologically and functionally distinct layers of quality control. Using a more simplified model, a single pass ER membrane protein reporter system termed CoMem, we were able to show for the first time in a systematic study under clear and defined conditions that the CNX TMD can differently recognize non-glycosylated clients with well-defined lesions in the membrane. Interestingly, our study reveals a highly distinct binding pattern for CNX to different TM substrates. Although we see significant changes in the binding pattern for most of the different amino acids that have been replaced against the central Val residue in the CoMem TM domain, there is no clear consistent pattern of stronger or weaker interaction within each amino acid group (non-polar, polar, positively and negatively charged). Nonetheless, except for Phe, Pro, and Trp, non-polar amino acids seem to have no pronounced effect on CNX binding. Interestingly enough, Phe and Trp have an aromatic system, which could explain the difference and Pro, which will be discussed below, has the property of destabilizing helical structures. At the same time, most of the polar and charged residues introduced into the central position of the CoMem TMD seem to reduce the binding of CNX. This is surprising since one might assume that exposed charged or unpaired polar residues, which on the one hand fulfill essential structural and functional roles but on the other can be potentially dangerous to the cell, are specific mediators of interaction with CNX or other intra-membrane chaperones. However, the most substantial interaction

is observed when the CoMem TMD is equipped with an Arg residue at the central position, which in comparison to most other amino acids is likely charged in membranes due to resonance stabilization. In contrast, we observed the weakest interaction if Pro is in this position.

Remarkably, in addition to our *in vivo* data, MD simulations revealed an altered binding behavior between the CoMem TMD mutant with a centrally located Arg residue and CNX. The simulation data shows that this mutant has a different orientation and, compared to CoMem WT, binds to CNX with the back of its helix. Although this finding does not directly explain the stronger interaction between CoMem-Arg and CNX observed in our experiments, it is still interesting. Additionally, MD simulation revealed that CoMem-Arg resides in a more tilted position within the membrane compared to CoMem WT and CNX. This is presumably due to the interaction between the positively charged side chain in the center of the CoMem-Arg TMD and the negatively charged head groups of the phospholipid bilayer, which exerts a pulling effect on the helix. In general, tilting of TMDs within the membrane might contribute to allow for a large interaction interface between two adjacent helices. In the light of these findings, it seems conceivable that the simulated tilting effects develop differently when the charge is shifted from a central position to the distal ends of the TM helix, whereby the distance of both charges to each other is varied. This is particularly interesting because only the center of the membrane is completely hydrophobic and the rest contains a mixture of polar lipid head groups and water molecules. Hence, this could explain the positional dependence of CNX binding to the different CoMem-Arg TMD variants observed in our experiments. Unfortunately, we did not include the CoMem-Lys variant in our MD simulations, but it would be interesting to see whether this mutant shows a different behavior despite a similar positive charge in the side chain in the central position, which would contribute to explaining the difference to the CoMem-Arg mutant. In this context, it appears additionally interesting that most of the mutations that cause disease result from the introduction of Arg, but not Lys, residues into TMDs (Fink, Sal-Man et al. 2012).

Similar to the CoMem-Arg variant, we recapitulated our *in vivo* data for the CoMem-Pro mutant by MD simulations. Interestingly, MD simulations shows that this mutant is less successful in developing stable interactions with CNX, which

might explain why we observe less interaction between CoMem-Pro and CNX in our experiments. It is noteworthy that Pro acts as a helix breaker and introduces kinks in TM helices, as is the case in CNX´s TM helix. Despite its propensity to break the helix, this Pro residue, however, is conserved in the CNX family, which is why it is believed to play an important functional role (Lakkaraju, Abrami et al. 2012). At first, we hypothesized that introducing a Pro residue into the helix of our reporter system could compensate for the Pro-induced kink in the CNX TM domain, thus leading to a stronger interaction but the opposite seems to be the case, as our *in vivo* and MD simulation data shows. Nonetheless, the position of the Pro residue and thus the kink introduced into the helix seems to have a decisive influence on the interaction with CNX because the more central the position within the membrane, the less interaction with CNX.

Although our simplified reporter system provides ideal conditions for the systematic definition of intra-membrane recognition motifs for CNX, it is not well suited to determine the biological consequences of binding. The CoMem reporter system is an artificially designed protein that lacks important secondary binding motifs which normally would enable interaction with chaperones or ERAD factors for example. Consequently, such factors would be excluded when determining downstream processes. To this end, more complex and naturally evolved model proteins such as Rho or Cx32 are required. However, although Cx32 has been shown to interact with CNX, Rho is more attractive as a substrate because of CNX's involvement in its quality control (Rosenbaum, Hardie et al. 2006). Thus, Rho was selected to address the biological consequences of CNX binding.

4.3 A conserved recognition motif in the CNX TMD is required for intra-membrane client recognition

After we have demonstrated direct helix-helix interactions between either the CNX TMD and the CoMem TMD segment or with the first TMD segment of Cx32 (Cx32-TMD1) *in vivo*, we used these interacting systems to define structural motifs for intra-membrane Calnexin:client recognition by MD simulations. Strikingly, computational modeling revealed that similar residues which involve a Tyr, a Thr, and a Leu in the CNX TMD region are required for interaction of CNX with both substrates. Furthermore, the identified residues reside on the same side of the CNX TM helix, suggesting that these residues represent a molecular

recognition motif that is xYxxTxxL and enables the CNX TMD to interact with the substrate helix inside the lipid bilayer. In fact, mutating these residues to presumably inert Val or Ala residues significantly reduced the interaction between CNX and different substrates tested in our study, including CoMem, Rho, Cx32, and Cx32-TMD1, which showed the most drastic effect. Interestingly, the recognition process seems to be position-dependent since shifting the motif to a more N-terminal position results in a reduced interaction of CNX with its substrate.

Based on the observation that the identified residues are sufficient to interact with many different substrates, we wondered whether the xYxxTxxL interaction motif is only found in CNX or exists in other membrane-localized factors which mediate intra-membrane substrate recognition. It is worth mentioning that although Leu seems to play a role in the CNX-mediated substrate recognition process, as the deletion of all three residues together causes a stronger phenotype than the deletion of Tyr and Thr alone, this residue does not seem to fit the picture. This is because, within TM domains, structural or functional tasks are mainly mediated by charged or polar amino acids, including Tyr or Thr, but usually not by non-polar amino acids such as Leu, rendering its effect interesting. In contrast, aromatic residues such as Tyr are crucial for many molecular recognition and assembly processes due to their unique structure and thus highly conserved in TM segments (Ramachandran, Tweten et al. 2004, Fink, Sal-Man et al. 2012). Furthermore, previous studies have shown that dimerization of TM segments is significantly increased by cation - π interactions, which could explain the comparatively strong interaction between CNX and CoMem-Arg (Johnson, Hecht et al. 2007). Importantly although such a scenario would at first glance contradict our simulation data, in which we observe a changed topology for the CoMem-Arg mutant, one has to consider that it is impossible to find every possible complex between CNX and CoMem-Arg by simulation. For this reason, we focused our comparison on a truncated xYxxTx recognition motif, in which x represents non-polar / hydrophobic residues that are important for the residues Tyr and Thr to be on the same helical face.

Surprisingly, a brief preliminary bioinformatic analysis revealed that different membrane proteins contain the recognition motif on different positions in their TM

domains, including Asi2, the P5A ATPase and partly EMC4. Of note, although the recognition motif in EMC4 contains a Asn residue instead of an Thr residue, this amino acid is also polar and, moreover, faces the same helical side as Tyr.

Protein	xYxxTx recognition motif
CNX	V**Y**IL**T**V
EMC4	V**Y**LIG**N**L
Asi2	V**Y**MV**T**A
P5A ATPase	V**Y**LG**T**L

Important for our study, all of the proteins mentioned above are involved in the quality control of membrane proteins and bind substrates via their TM domain. At least for EMC and the P5A ATPase, substrates and TM features such as hydrophobicity are known that enable recognition, but, similar to CNX, features in the TM segment of these proteins, which are required for client binding to occur, are unknown.

The membrane-spanning Asi complex, which consists of Asi1, Asi2, and Asi2, is the third most important ER-ubiquitin-ligase complex in yeast alongside Hrd1 and Doa10. Interestingly, while Asi1 and Asi3 contain a RING domain, Asi2 does not have a functional domain but has been shown to recognize misfolded and mistargeted membrane proteins at the inner nuclear membrane by direct binding the substrate TMD. Subsequent substrate ubiquitination and Cdc48-dependent membrane extraction facilitate proteasomal degradation (Natarajan, Foresti et al. 2020). Interestingly, EMC4 composed of three TMDs, is the subunit of the EMC complex that mediates the insertion of membrane proteins with moderately hydrophobic transmembrane domains into the ER membrane and is therefore crucial for the function of the entire complex (Shurtleff, Itzhak et al. 2018). Although the P5A ATPase preferentially interacts with moderately hydrophobic transmembrane domains, similar to the EMC, its dislocase activity mediates the opposite function. After binding directly to the TM segment of a misdirected or misoriented membrane protein, these are removed from the membrane in an ATP dependent manner (McKenna, Sim et al. 2020). However, to definitively confirm the existence of the xYxxTx motif among various intra-membrane chaperons and

quality control factors in the end, a broader bioinformatics approach and further experimental testing is required.

4.4 The protective function of CNX on membrane proteins

To address the biological functions of intra-membrane client recognition by CNX, we used Rhodopsin as a model substrate. Our data show that if intra-membrane client recognition by CNX is disturbed, as is the case in the YTL→V mutant, the binding of Rho to the chaperone is significantly reduced. Strikingly, caused by this reduced binding, Rho degradation is significantly accelerated, which argues for a protective role of CNX in intra-membrane chaperoning processes. The protective function of CNX on Rho becomes more evident if CNX is completely absent, causing an even stronger effect on Rho degradation and suggests that CNX mediated intra-membrane and lectin-based client binding works synergistically. Notably, our observation that CNX is essential for Rho biogenesis since Rho expression levels in CNX k/o cells are hardly detectable contradicts previous studies (Rosenbaum, Hardie et al. 2006, Kosmaoglou and Cheetham 2008). Thus, possible causes for these differences will be discussed in the following.

Rhodopsin is a member of the GPCR-family and is found in the rod photoreceptor cells of the retina. During photoactivation by the absorption of light, Rho converts the energy provided by the photons into a chemical reaction which ultimately leads to an electrical signal that stimulates signal transduction processes in the nervous system (Hubbard and Kropf 1958). In addition, during photoactivation, Rho is subject to structural changes that cause the rearrangement of specific helices and thus creates a small binding pocket for a heterotrimeric G-protein on the cytoplasmic side of the receptor, which initiates the phototransduction cascade (Zhou, Melcher et al. 2012). Importantly, Rho is a glycoprotein and undergoes N-linked glycosylation at two asparagine residues near the N-terminus, which are crucial for the correct folding of the receptor. Mutations in these residues and 120 other known point mutations are responsible, among others, for autosomal dominant retinitis pigmentosa (ADRP) and night blindness (Sung, Davenport et al. 1991, al-Maghtheh, Gregory et al. 1993, Kawamura, Colozo et al. 2012). While point mutations affecting the C-

terminus still fold normally, however, cause mistargeting, mutations in the transmembrane domains lead to protein misfolding and ER retention. Interestingly, although Rho has been identified as a direct CNX substrate in different studies, the role of CNX in the quality control of mutant, misfolded Rho seems to be very different among different organisms. While studies in *Drosophila* examining loss of function mutations in CNX suggest that Rho biogenesis essentially depends on CNX, the chaperone is dispensable for Rho to mature in mammalian cells (Rosenbaum, Hardie et al. 2006, Kosmaoglou and Cheetham 2008). It is important to note that *Drosophila* rhodopsin differs significantly from that of vertebrates. For example, maturation essentially requires N-glycosylation of one of the two glycosylation sites, but in its mature form, Rho is non-glycosylated (Murray, Fliesler et al. 2009). This contrasts with mammalian Rho, which does not require glycosylation for correct folding (Kaushal, Ridge et al. 1994, Saliba, Munro et al. 2002). Furthermore, *Drosophila* CNX only shares 49 % sequence identity with human CNX and differs significantly in its TMD regions, which all together can explain the observed differences. However, since we also used a mammalian system in our study, the reason for the different observations regarding the importance of CNX for the biogenesis of Rho must be found elsewhere. Indeed, the difference can be explained by the findings of our study. Results around the group of *Kosmaoglou et al.* are based on the use of a functionally deficient CNX cell line in which a conserved Asp required for calcium ion coordination, two Cys residues involved in a surface-exposed disulfide bond required for substrate binding and residues that form the glucose binding pocket are deleted (Denzel, Molinari et al. 2002, Kosmaoglou and Cheetham 2008). Notably, the truncated CNX version still contains the N-terminal ER-targeting sequence, the C-terminal ER retention motif, and, more importantly for our studies, the TMD region. Given that we observe synergistic client binding activities of CNX with both its lectin domain and TM region, it seems conceivable that CNX´s TM domain can compensate for a dysfunctional lectin domain. Hence no effect on Rho biogenesis is observed. Moreover, the exact mechanism of CNX´s chaperoning function is not known. It is possible that during the biosynthesis of client proteins, the CNX TMD functions as a placeholder, shielding problematic residues in order to support the development of the

correctly folded state of the substrate. Consequently, in a model like this, the biological function of CNX is determined even stronger by its TM domain. Future studies using our minCNX construct, which lacks CNX´s entire globular domain and only contains the TM domain, could address this question. In this context, it seems interesting that in *Drosophila,* the intra-membrane chaperone and insertase EMC not only interacts with CNX but is also essential for the biogenesis of Rho (Satoh, Ohba et al. 2015).

Another finding that supports the role of CNX as an intra-membrane chaperone is the observation that the association of CNX with Rho is highly dependent on its conformation since CNX preferentially associates with misfolded Rho compared to the correctly folded WT (Noorwez, Sama et al. 2009). Remarkably, the strongest association can be observed with mutants containing mutations in TM regions causing severe misfolding of the entire protein instead of mutations occurring in the N-terminal region. A similar effect of CNX binding was observed in studies on Cx32, however, the difference between the correctly folded WT and a Cx32 TM mutant causing an altered topology of the protein was not as severe (Coelho, Stahl et al. 2019). Importantly, it has to be considered that in these scenario´s stronger binding of CNX to mutant Cx32 or Rho is not necessarily caused via stronger binding to the mutated TMD helix but can also be the result of general exposure of TMD helix binding sites and the involvement of additional factors which target the misfolded mutants. Nevertheless, since we also observed a direct and stronger CNX binding to Cx32 mutants that are still integrated into the membrane correctly, one would expect faster degradation for such misfolded TM mutants than the WT in CNX k/o cells co-transfected with our binding deficient CNX mutant.

4.5 Global analysis of the CNX substrate repertoire

Using DSSO-crosslinking coupled to mass spectrometry, we show a global analysis of the CNX substrate repertoire for the first time. Among the interacting ER-localized substrates, we find the PDI family members ERp57 and ERp29 besides EDEM1, which functions in the degradation of misfolded ER proteins. Importantly, for all of these proteins, the interaction with CNX has been well documented, which validates the results of our analysis (Oda, Hosokawa et al.

2003, Pollock, Kozlov et al. 2004, Nakao, Seko et al. 2017). In addition, we were able to identify a large number of membrane proteins to interact with CNX. Remarkably, around half of the identified membrane proteins (51,31%) that interact with CNX in our mass spectrometry analysis are not glycoproteins, according to UniProt. This finding indicates that CNX recognizes a variety of membrane protein clients regardless of their glycosylation state, presumably through direct interaction with their transmembrane domains. Thus, our global analysis of CNX´s substrate repertoire supports the various other reports that describe a lectin-independent function of CNX (Cannon and Cresswell, 2001, Coelho et al., 2019, Swanton et al., 2003).

Importantly, however, although we can observe a direct interaction between the TM helix of CNX and a client (CNX:client) in our study, we cannot rule out whether other co-factors are involved in the client recognition and interaction process of CNX (CNX:X:client). Therefore, future studies should reveal if client binding is exclusively mediated by the TM domain of CNX. In addition, it would be interesting to reveal whether CNX´s protective function that we were able to observe for Rhodopsin also applies to other interaction partners we have identified in our MS analysis, as this would foster the role of CNX as an intra-membrane chaperone for non-glycosylated membrane proteins.

4.6 Final conclusion

In this study, we reveal principles of intra-membrane substrate recognition by the molecular chaperone CNX that contradict the previous assumption that client binding of CNX occurs exclusively outside the membrane. We show that CNX directly recognizes misfolded client proteins via its single TM domain and reveal that this intra-membrane client recognition process depends on a unique motif present in the CNX TM domain. Remarkably, the functionality of this motif is not only a prerequisite for efficient client binding, but also protects clients from being degraded.

Based on the structural and biochemical data presented in this study, we propose the following model for the various chaperone activities of CNX (Figure 21).

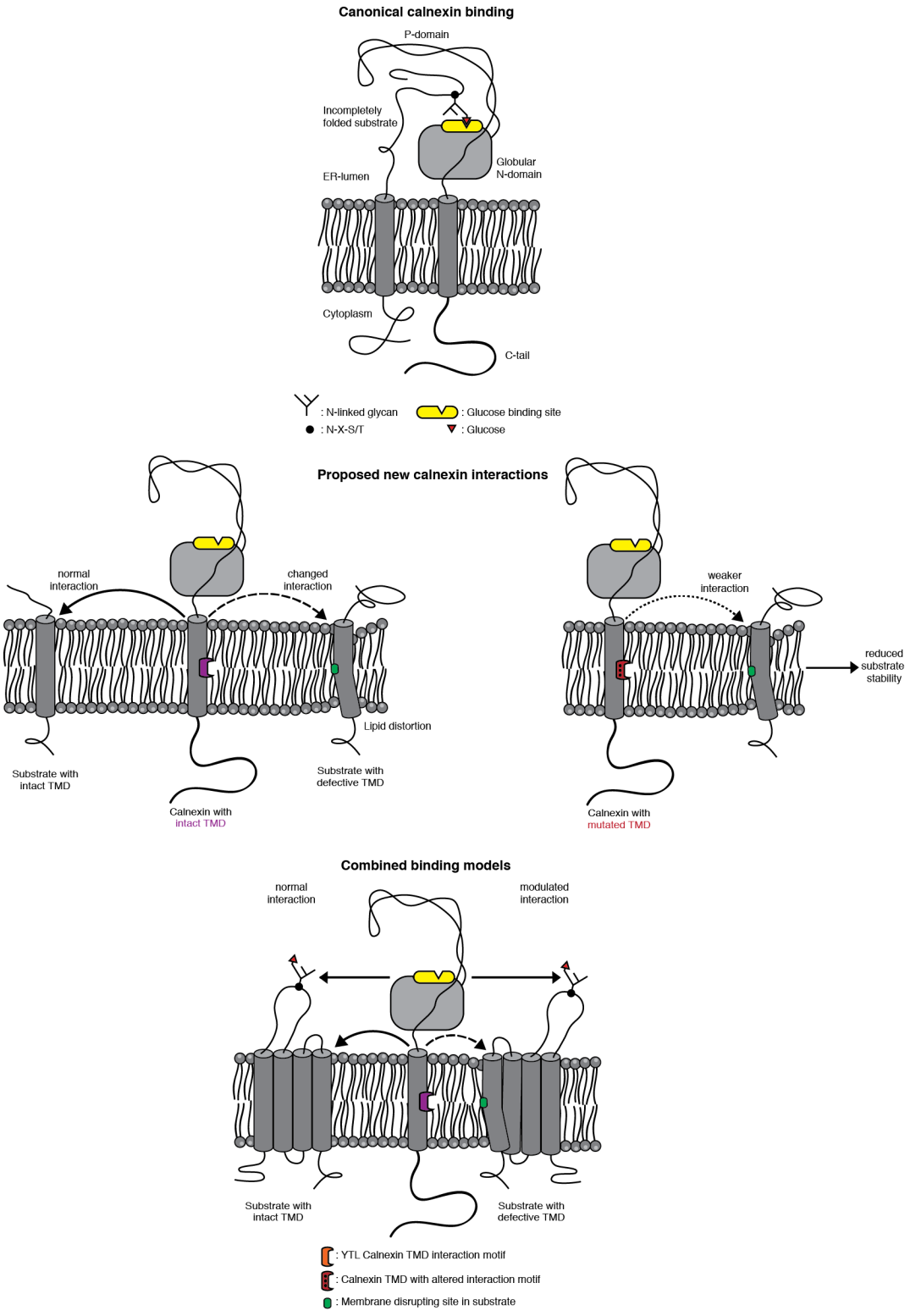


Figure 21: The combined function of CNX´s lectin and TM domain in the recognition and quality control of misfolded membrane proteins. According to its canonical function in the biogenesis of membrane proteins, CNX binds immature and incompletely folded glycoproteins via a glucose binding site located in its globular domain. In addition, however, CNX functions as an intra-membrane chaperone for non-glycosylated membrane proteins, especially those containing

a misfolded or defective TMD. The recognition of intra-membrane defects essentially depends on the functionality of an interaction motif located in CNX´s TMD and protects clients from degradation. In contrast, a dysfunctional TMD interaction motif with reduced ability for substrate binding within the membrane causes increased instability of the client.

5 Materials and methods

Unless specified otherwise, chemicals and reagents were obtained from Applied Biosystems, Bio-Rad, GE Healthcare, Life technologies, Merck, Millipore, New England Biolabs, Promega, Roche, Serva, Sigma and Thermo Scientific. Sterile flasks and sterile and de-ionized water as well as sterile solutions were used in all protocols and experiments described.

5.1 Molecular biology techniques

5.1.1 DNA design and modification

DNA constructs

CoMem WT constructs, minCNX and CNX constructs were obtained from GeneArt Gene Synthesis (ThermoFisher) in a pcDNA3.4 TOPO expression vector optimized for mammalian expression. An illustration of the amino acid sequence and construct details of WT CoMem and minCNX constructs and their respective controls that lack the TMD domain can be found below (Figure 22 A-D) The TM sequence of CoMem WT was designed by multiple sequence alignment of 200 human single pass plasma membrane proteins (152 type I and 48 type II orientation) using Unipro U Gene software whose TM sequences were obtained from Membranome 2.0 Database. Human CNX sequence was obtained from UniProt and complemented with a C-terminal epitope tag. For construction of the minCNX construct, human CNX TM sequence was obtained from UniProt, its TM helix reconciled according (Hessa, Meindl-Beinker et al. 2007) to avoid artificially shortening of the TM sequence and finally complemented by endogenous amino acids flanking the TM region N and C-terminally. Cx32 cDNA was obtained from Origene and cloned into a pSVL vector (Amersham). C_L-Cx32-TM1 reporter constructs were synthesized by GeneArt Gene Synthesis (Thermo Fisher) and cloned into a pSVL vector. Monomeric Sec61 mCherry was provided from Prof. Dr. Linda Hendershot (St. Jude Children Research Hospital, TN, USA).

Individual construct components of the designed proteins and N as well as C-terminal epitope tags are separated by $(GGGS)_2$, $(GSGS)_2$ or $(GGGA)_2$ linker. Cloning into mammalian pSVL expression vector was performed using suitable

restriction enzymes and T4 DNA ligase (Promega). Introduction of epitope tags and point mutations was carried out via site-directed mutagenesis PCR using overlapping, complementary mutagenesis primers. Pfu polymerase (Promega) reaction and subsequent DpnI (NEB) digest of the template plasmids were utilized to generate mutated vectors, which were transformed into competent E. coli cells (DH5α, Thermo Fisher) cells for amplification. All constructs were verified by sequencing prior to use (Eurofins Genomics).

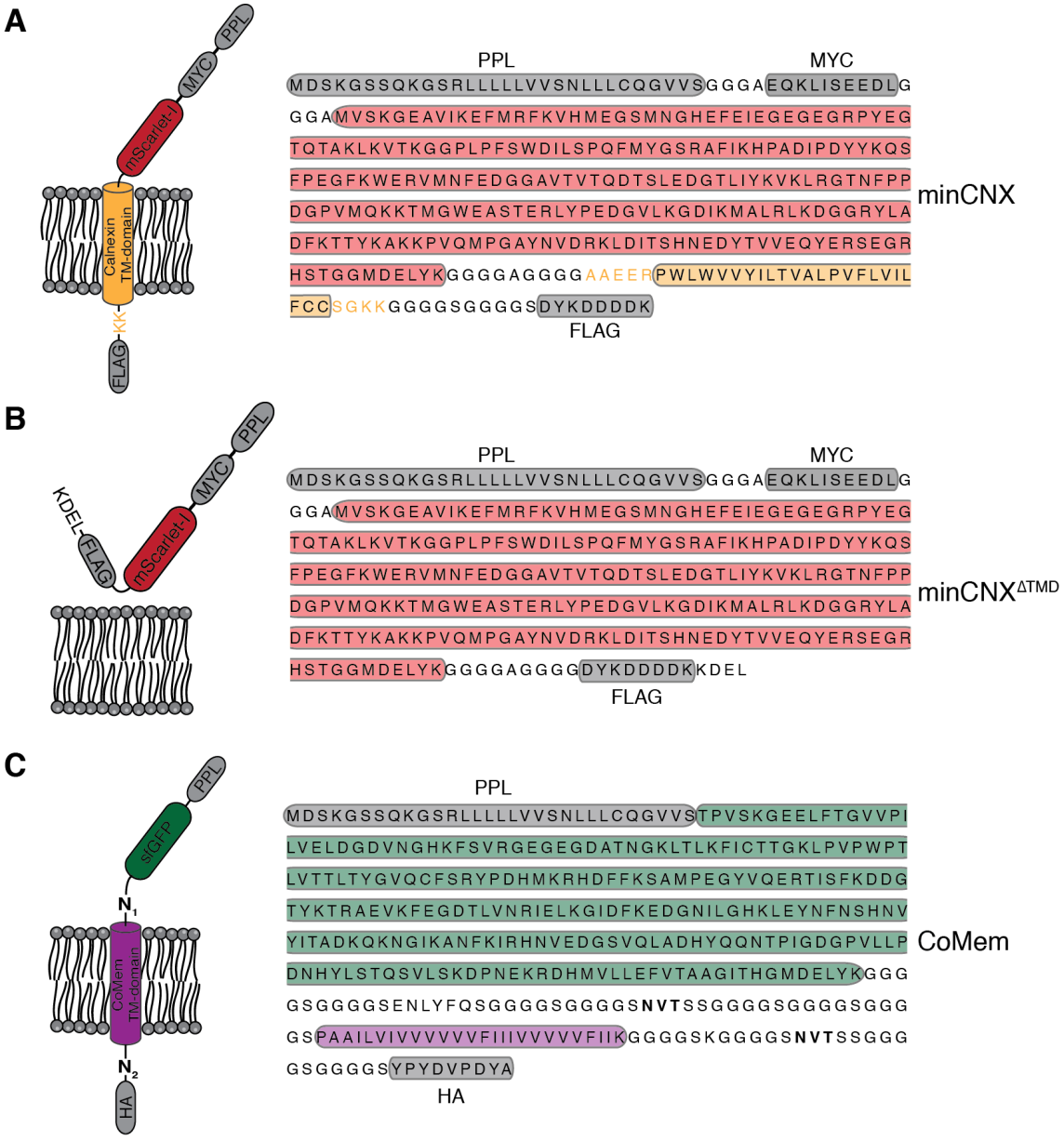


Figure 22: Schematic of the minCNX, minCNX$^{\Delta TMD}$, CoMem and CoMem$^{\Delta TMD}$ construct: Illustration of the amino acid sequence and construct details of **(A)** minCNX, **(B)** of minCNX$^{\Delta TMD}$, **(C)** of CoMem and **(B)** of CoMem$^{\Delta TMD}$. To further promote a type I orientation of CoMem, an additional Lys residue (in addition to the C-terminal one in the consensus sequence) was placed between the TMD and the NVT reporter site according to the positive inside rule (von Heijne and Gavel 1988).

CRISPR

CNX-deficient cells were generated using the CRISPR/Cas9 system. The vector pSpCas9(BB)-2A-Puro (PX459) V2.0 was provided from Feng Zhang (plasmid 62988; Addgene, Cambridge MA) (Ran, Hsu et al. 2013). The design of guide RNA was performed using the German Cancer Research Center (DKFZ) E-CRISP design tool. In order to prevent the occurrence of off-target effects two different guide RNA sequences for the CNX target gene were designed

gRNA1: 5´-CACCGCTTGGAACTGCTATTGTTG-3´

gRNA2: 5´-CACCGTGGTTGCTGTGTATGTTAC-3´

and subsequently cloned into PX459 using a combined restriction/ligation approach according to published protocols (Ran, Hsu et al. 2013). In the following, HEK293T cells were transfected using GeneCellin (Eurobio) according to the manufacturer's protocol and cultured for two days. Selection of cells was carried out through to the addition of 1,5 μg/mL puromycin for 72 hours. Subsequently, single colonies were isolated and CNX protein levels were determined by immunoblotting. To ensure a complete knockout of the target gene and loss of the respective protein this process was repeated during several passages. Finally, genomic DNA, from clones were CNX protein levels were completely absent, was isolated according to the manufacturer´s protocol

(PureLink Genomic DNA Kits, Invitrogen). Subsequently, the genomic CRISPR/Cas9 target area was amplified by the usage of specific primers and the obtained amplicons were sequenced and the successful CNX knockout verified by NGS CRISPR amplicon sequencing (CCIB DNA Core, Massachusetts General Hospital, Boston, MA).

5.1.2 Cell culture techniques

Cell culture and transient transfections

HEK293T (ECACC) and COS-7 (ECACC) cells were cultivated in Dulbecco's Modified Eagle's Medium ((DMEM), high glucose (Sigma-Aldrich), supplemented with 10 % (v/v) fetal bovine serum (Biochrom) and 1% (v/v) antibiotic-antimycotic solution (25 µg/ml amphotericin B, 10 mg/ml streptomycin, 10,000 units of penicillin (Sigma-Aldrich)) at 37 °C in a 95% humidified atmosphere containing 5% CO_2. Transient DNA transfections were performed by chemical transfection (HEK293T) or via electroporation (COS-7). 24 h prior to transfection, 250.000 HEK293T cells were seeded per p35 plate (pre-coated Corning BioCoat Poly-D-Lysine 35 mm #354467 or uncoated Nunclon multidish 6 well plates, Thermo Scientific #140685 coated with 50 µg/ml Poly-D-Lysine solution (Gibco A38904-01) per well according to manufacturer's instructions or 275.000 per p60 plate (Tissue Culture Dish 60, TPP). HEK293T cells were transfected using GeneCellin (Eurobio) according to the manufacturer's protocol. For transient transfections using pcDNA (highly expressing vectors) the amount of DNA was reduced to half the amount suggest by the manufacturer. When performing co-transfections, the amount of transfected DNA was divided equally or splitted in a 3:1 ratio (vector plasmid with weaker promotor:vector plasmid with stronger promotor) in case of different promotor strength.

For passaging, cells were washed with PBS (Sigma-Aldrich) and subsequently incubated with trypsin solution (Sigma-Aldrich) for 5 min at 37°C before transfer in fresh, pre-warmed medium.

Cell lysis

Cells were harvested 24 h after transfection. All cell lysis steps were performed on ice or at 4 °C using ice-cold solutions unless otherwise indicated. Cells were washed twice with PBS and then lysed for 20 min by adding 1 ml (p60) or 500 µl (p35) lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 % (w/v) NaDOC complemented with either 1% (w/v) Digitonin (Sigma) for samples analyzing membrane-embedded interactions with CoMem and Cx32 constructs or 1% (v/v) NP-40 (Sigma) for samples analyzing membrane-embedded interactions with CL-Cx32-TM1 and mScarlet-I-CNX as well as CNX constructs) when conducting co-immunoprecipitation experiments or NP-40 buffer for other experiments. All buffers were supplemented with cOmplete protease inhibitor cocktail (Roche) prior to lysis. The resulting lysate was centrifuged for 15 min at 15,000 x g and the supernatant was complemented with 5x Laemmli buffer containing 10 % (v/v) β-mercaptoethanol (reducing). Finally, samples dedicated to the analysis for membrane proteins were incubated for 30 min at 50°C, except samples involving Cx32 proteins were incubation was performed for 30 at 37°C. In contrast, sample lysates for the analysis of soluble proteins were boiled for 10 min at 95°C.

5.1.3 Cell culture based *In vivo* assays

Cycloheximide chase analysis

Cells were prompted to express the indicated constructs overnight. Inhibition of protein biosynthesis and determination of protein half-live was performed by the replacement of complete medium with medium containing 50 µg/ml Cycloheximide (Sigma-Aldrich) 24 h after transfection. Subsequently lysates were collected at different time points as described below. Negative controls were prepared in the same manner without the addition of CHX (t = 0 sample).

Induction of ER stress

The induction of UPR was carried out by the addition of 5 µg/ml tunicamycin (Sigma-Aldrich) in medium and incubation for 6h prior cell lysis or the supplementation of pre-warmed medium with 10 mM DTT (Sigma-Aldrich) for one hour. Cleavage analysis of the activating transcription factor 6 (ATF6) based on

immunoblotting was performed following immunoprecipitation to increase the amount of detectable protein. Determination of endogenous phosphorylation events by immunoblotting (total amount and phosphorylation at Ser51 only) of the eukaryotic initiation factor 2 (eIF2) was performed following cell lysis using lysis buffer additionally supplemented with 1 x phosphatase inhibitor mix (Serva). To assess XBP1 splicing, RNA was extracted using RNeazy Mini Kit (Qiagen) in RNase free environment. Subsequently RT-PCR of the purified RNA was performed using OligodT20 Primer (18418020,Thermo Fischer) and SuperScript III Reverse Transcriptase (18080044, Thermo Fischer). Following amplification of the XBP1 transcript of the resulting cDNA, XBP1 splicing events were analyzed on 2% agarose gels. Positive controls were included.

5.1.4 Fluorescence Microscopy

Immunofluorescence

Determination of the intracellular localization of target proteins was carried out by immunofluorescence microscopy using either electroporated or transiently transfected COS-7 cells.

Seeding and transfection

Electroporation of COS-7 cells was carried out using a X2 Gemini electroporator (BTX) according to manufacturer´s instructions and protocol. For transient transfection of COS-7 cells, 36 µl DMEM containing 3.6 µg DNA were mixed with 1.2 µl TorpedoDNA transfection reagent (ibidi) and incubated for 15 min at RT. Subsequently, 200 µl of COS-7 cell suspension were added and mixed gently. In both cases, following electroporation or transient transfection, 30 µl of the resulting suspension of 4 x 10^5 cells/ml were applied per inlet of a pre-warmed µ-Slide IV 0.4 (ibidi) and the µ-Slide was incubated for 3 h at 37 °C, 5% CO_2. According to manufacturer´s instructions, medium was replaced 3 h after seeding. Therefore, 60 µl complete DMEM were added per reservoir, and the µ-Slides were incubated for additional 48 h at 37 °C, 5% CO_2.

Staining

For fixation, all liquid was removed from all reservoirs and channels and 60 µl glyoxal fixation solution (20% EtOH, 7.825% glyoxal, 0.75% acetic acid) were added to each channel (Richter, Revelo et al. 2018). Samples were then incubated for 30 min on ice and thereafter further 30 min at RT. The reaction was quenched by aspirating the fixation solution and adding 60 µl of 100 mM NH_4Cl (Sigma-Aldrich) and subsequent incubation at RT for 20 min. Samples were then washed twice for 5 min with 100 µl 4 °C PBS. Permeabilization of cells and simultaneous blocking of epitopes was conducted by the addition of 60 µl of blocking solution (2.5% BSA (Sigma-Aldrich), 0.1% Triton X-100 in PBS) to the samples which were then incubated for 5 min at RT following three further washing steps at RT. As an ER-marker, 30 µl of anti-PDI antibody conjugated to Alexa Fluor 488 at 1:50 dilution in blocking solution were added and incubated for 1 h at RT in the dark. The antibody solution was washed out using 100 µl PBS and the samples were thereafter washed three times. All liquid was removed and 25 µl of DAPI solution (Sigma-Aldrich) (0.01% in PBS) were added to stain Nuclei and incubated for 2 min. The samples were then washed three times with PBS, the liquid was aspirated, and mounting medium (ibidi) was added to cover the inlets of the slides.

Microscopy

Imaging was performed on a DMi8 CS Bino inverted widefield fluorescence microscope (Leica) using a 100 x (NA = 1.4) or 63 x (NA = 1.4) oil immersion objective. The employed dichroic filters were chosen to image Alexa 488 and sfGFP (GFP channel; excitation/bandpass: 470/40 nm; emission/bandpass: 525/50 nm), mScarlet-I and Sec61mCherry (TXR channel; excitation/bandpass: 560/40 nm; emission/bandpass: 630/75 nm), or DAPI (excitation/bandpass: 350/50 nm; emission/bandpass: 460/50 nm). For image analysis and processing, the LAS X (Leica) analysis software and ImageJ (NIH) where used. Adjustments of acquired images were restricted to homogenous changes in brightness and contrast over the whole image.

5.2 Biochemical techniques

5.2.1 Protein modification analysis

Deglycosylation experiments

Determination of N-linked glycan modifications was performed by the use of deglycosylation enzymes. Samples were digested for 1 h at 37 °C with either EndoH or $EndoH_f$ and PNGase F (NEB) according to the manufacturer's instructions. Negative controls were prepared in the same manner without the addition of enzymes. The digested proteins were thereafter supplemented with 5x Laemmli buffer and 10% (v/v) β-mercaptoethanol and boiled for 10 min at 95

5.2.2 Protein interaction analysis

DSSO Crosslinking

Transiently transfected cells were washed twice with cold PBS prior the addition of 2mM DSSO (Thermo Fischer) in PBS per sample, diluted from 100 mM stock DSSO in anhydrous DMSO (Thermo Fischer). Crosslinking was performed on ice for 1 hour including periodic steps of agitation. Inhibition of the crosslinking reaction was achieved by washing of the samples with quenching solution (50 mM Tris-HCL pH 8.0) for 15 min. In the following, cells were lysed as described in the cell lysis part using (50 mM Tris-HCL pH 7.4, 150 mM NaCl, 5% Glycerol, 1 % NP-40 and 1 mM $MgCl_2$) lysis buffer. Subsequently, immunoprecipitation and immunoblotting was performed.

Co-immunoprecipitation

The qualitative and quantitative detection of protein-protein interaction was carried out with co-immunoprecipitations (co-IPs). Prior to co-IP of target proteins, 2% of cell lysate were supplemented with 5x Laemmli buffer (Laemmli 1970) containing 2% (v/v) β-mercaptoethanol. The remaining lysate was then incubated for 2 h under constant rotation with the appropriate amount of antibody as presented in the table above and subsequently for 1 h with 30 µl protein A/G agarose beads (Thermo Fisher). Alternatively, for ALFA or FLAG immunoprecipitations, 30 µl of ALFA Selector ST Agarose (NanoTag

Biotechnologies) or 30 µl of M2 anti-FLAG affinity gel (Sigma-Aldrich), was applied to the lysate and incubated for 3 h under constant rotation at 4 °C. Beads were thereafter washed three times with 1 ml NP-40 wash buffer (50 mM Tris/HCl, pH 7.5, 400 mM NaCl, 0.5 % (w/v) NaDOC and 0.5 % NP-40) or Digitionin wash buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.5 % Digitonin) with centrifugation steps for 5 min at 4°C and 2.500 rpm in between. Proteins determined for MS were washed two times more in detergent-free wash buffer. Finally, proteins were then eluted by addition of 2x Laemmli buffer supplemented with 10% (v/v) β-mercaptoethanol and subsequent incubation at 95 °C for 10 min, or 30 min at 50 °C and 37 °C respectively as described above.

5.2.3 Gel electrophoresis and immuno-blot techniques

SDS-PAGE and immunoblotting

Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 7 to 14% polyacrylamide gels. The polyacrylamide gels were then either imaged directly using a Typhoon 9200 Variable Mode gel scanner (GE Healthcare) (Cy5 filter setting, excitation 633 nm, emission 670 nm, bandwidth 30 nm or GFP excitation 526 nm, emission 532 nm, short pass) or subsequently western blotted. Proteins were blotted overnight at 4 °C onto a methanol (Sigma-Aldrich) activated polyvinylidene difluoride (PVDF) membrane (Biorad) and subsequently, the membranes were blocked for a minimum of 3h at RT (or overnight at 4 °C) with Tris-buffered saline supplemented with skim milk powder and Tween-20 (M-TBST; 25mM Tris/HCl, pH 7.5. 150 mM NaCl, 5% (w/v) skim milk powder, 0.05% (v/v) Tween-20). Primary antibodies in M-TBST were applied at 4 °C overnight. After washing (1 x 5 min TBS, 2 x 5 min TBST, 3 x 5 min TBS), the blots were decorated for 1 h at RT with the respective HRP-conjugated secondary antibody (Santa Cruz) diluted in M-TBST (at 10-fold lower dilution than the respective primary antibody). Following incubation with secondary antibody, blots were washed an extra time according to the scheme mentioned above. Proteins were then visualized by the detection of chemiluminescence using Amersham ECL prime solution (GE Life Sciences) and a Fusion Pulse 6 imaging system (Vilber

Lourmat). Quantifications were conducted using Bio-1D software (Vilber Lourmat).

Antibodies

For development of western blots or immunofluorescence microscopy, the following primary antibodies and HRP-conjugated secondary antibodies or proteins were used at the dilutions listed in the table below.

Target	Company, catalog no.	Dilution
ATF-6	Abcam, 122897	1:500
BiP	Cell Signalin, C50B12	1:500
CNX	Biolegend, 699402	1:1000
CNX	Chemicon, MAB3126	1:1000
C_L	Southern Biotech, 1060-01	1:250
Cx32	Sigma-Aldrich, C3595	1:500
(Ser51)eIF2alpha	Cell Signaling, 9721	1:500
eIF2alpha	Cell Signaling, 9721	1:1000
FLAG	Sigma-Aldrich, F1804	1:1000
FLAG	Sigma-Aldrich, F7425	1:1000
GADPH	Santa Cruz, 47724	1:1000
Goat IgG	Santa Cruz, 2354	1:5000
HA	Biolegend, 902302	1:1000
Mouse IgG	Santa Cruz, 516102	1:10000
MYC	Merck Millipore, 4A6	1:1000
PDI, Alexa Fluor 488-conjugated	Cell Signaling, 5051	1:50
Rabbit IgG	Santa Cruz, 2357	1:10000
Rat IgG	Biolegend, 4054	1:10000
Rhodopsin	Invitrogen, MA1-722	1:250
RFP	Chromotek, 6G6	1:1000
V5	Biolegend, 680602	1:1000

For immunoprecipitation, the following antibodies were employed as listed in the table below.

Target	Company, catalog no.	Amount
C_L	Southern Biotech, 1060-01	3 µg
CNX	Biolegend, 699402	1.5 µg
HA	Biolegend, 901513	1.5 µg
MYC	Merck Millipore, 4A6	1.5 µg

5.2.4 Analytical techniques

Mass spectrometry

HEK293T CNX KO cells were seeded in P100 plates and cultivated for 24 h prior transfection with 10 µg of EV or ALFA-tagged CNX variants with a total of three replicates for each construct. Following DSSO crosslinking, cell lysis was performed in NP-40 lysis buffer as described above, however, additionally supplemented with 1 mM $MgCl_2$ and 5% Glycerol. Immunoprecipitations were performed in the same buffer using anti-ALFA Selector ST agarose beads as described above followed by two additional washing steps in detergent free buffer. During further proceedings, immunoprecipitated proteins destined for MS analysis were digested and eluted from beads prior to desalting and purification of the samples as otherwise described (Keilhauer, Hein et al. 2015). Nanoflow liquid chromatography-mass spectrometry (MS)/MS analyses were performed using a combination of an UltiMate 3000 Nano HPLC system (Thermo Fischer) together with an Orbitrap Fusion mass spectrometer (Thermo Fischer). Peptides were first loaded on an Acclaim C18 PepMap100 75 µm ID 2 cm trap column together with 0.1% FA before transfer to an Aurora reversed phase UHPLC analytical column, 75 µm ID 25 cm, 120 Å pore size, (Ionopticks). Columns were constantly heated at 40 °C. Subsequent separation was performed using a first gradient ranging from 5 to 22% acetonitrile in 0.1 % FA for 105 min followed by a second gradient ranging from 22 to 32% acetonitrile in 0.1% FA for 10 min at an overall flow rate of 400 nl/min. Peptides were ionized via electrospray ionization. Orbitrap Fusion was carried out in a top speed data dependent mode using a

cycle time of 3 s. Full scan (MS^1) acquisition (scan range of 300 – 1500 m/z) was performed in the orbitrap at a defined resolution of 120,000 as well as with an automatic gain control (AGC) ion target value of 2e5 whereby dynamic exclusion was set to 60 s. For fragmentation, precursors with a charge state of 2-7 and a minimum intensity of 5e3 were selected and isolated in the quadrupole using a window of 1.6 m/z. Subsequent fragment generation was achieved using higher-energy collisional dissociation (HCD, collision energy: 30%). The MS^2 AGC was adjusted to 1e4 and 50 ms were selected as the maximum injection time for the ion trap (with inject ions for all available parallelized time enabled). Scanning of fragments was performed by applying the rapid scan rate.

5.3 Data evaluation

Mass spectrometry evaluation

MS raw files were analyzed with MaxQuant software (version 1.6.17.0) with most default settings and a protein database containing human sequences (downloaded July 2019 from Uniprot, taxonomy ID: 9606, 74349 entries). The FLAG tagged version of Calnexin was not implemented. The following parameter settings were used: PSM and protein FDR 1%; enzyme specificity trypsin/P; minimal peptide length: 7; variable modifications: methionine oxidation, N-terminal acetylation; fixed modification: carbamidomethylatio. The minimal number of unique peptides for protein identification was set to 2. For label-free protein quantification, the MaxLFQ algorithm was used as part of the MaxQuant environment: (LFQ) minimum ratio count: 2; peptides for quantification: unique. Statistical analysis was performed in Perseus (version 1.6.14.0). Proteins identified only by site, reverse hits or potential contaminants were removed. LFQ intensities were log2 transformed and data were then filtered for at least two valid values in the wt group. Then, missing values were imputed from normal distribution (width: 0.3, down shift: 1.8 standard deviations, mode: over whole matrix). The replicate groups were compared via a two- sided, two-sample Student's t test (S0 = 0, permutation-based FDR method with FDR = 0.05 and 250 randomizations). Enrichment values and corresponding –log10 P values

were plotted. Cut offs in the volcano plot were set to 2-fold enrichment and a p-value of 0,05.

Quantification and statistics

Immunoblots were quantified using the Bio-1D software (Vilber Lourmat). Binding of endogenous CNX to CoMem TMD helices was calculated as the ratio of intensities of the co-immunoprecipitated protein (CNX) to the overall intensity of immunoprecipitated CoMem (sum of both glycosylated and nonglycosylated species – beginning at the molecular weight of HA tagged nonglycosylated CoMem until the upper protein smear represented by the complex glycosylated species. In case of CoMem N_1Q, the area around the nonglycosylated species was included into quantification). Subsequently, to facilitate comparability of individual experiments, formed ratios were each normalized to the CoMem WT N_1Q (CoMem lacking the luminal glycosylation site) dataset of each experiment. The same approach was used analyzing binding of overexpressed CNX variants to Cx32 TM1 or Cx32 full length. To determine effects of CHX treatment on the decay of target proteins, the intensity at each time point was normalized to the intensity of the untreated sample with no CHX incubation (t= 0h). Half-life calculations from decay data were carried out by logarithmically linearizing the CHX decays and determination of the ln(0.5) value. Statistical analyses were performed using Prism (GraphPad Software). Where indicated, data were analyzed with two-tailed, unpaired Student's t-tests and differences were considered to be statistically significant when $p < 0.05$.

Sequence analysis

TM regions of Cx32 and CoMem WT as well as mutant variants were annotated by using ΔG prediction server v1.0 (Stockholm University). The same tool was used to predict the ΔG_{app} for helix insertions using the full protein scan mode.

5.4 Computational modelling

Molecular dynamic simulations / computational modeling

All MD simulations have been performed with the GPU accelerated version of PMEMD (Salomon-Ferrer, Gotz et al. 2013), part of the AMBER18 package (Case, Ben-Shalom et al. 2018). Amino acids have been described with ff14SB (Maier, Martinez et al. 2015) lipids with LIPID17 (unfortunately only LIPID14 has been published (Dickson, Madej et al. 2014)) and water molecules with TIP3P (Jorgensen, Chandrasekhar et al. 1983). The sequences of the helices are given in (Figure 22) All simulations have been prepared with the membrane builder (Wu, Cheng et al. 2014) of CHARMM-GUI (Jo, Kim et al. 2008) utilizing the AMBER-FF compatibility (Lee, Hitzenberger et al. 2020). The simulation boxes consisted of approx. 200 POPC molecules, 13500 water molecules with box dimensions (after equilibration and pressurization) of 84Åx84Åx95Å. Simulations were performed in a 0.15M KCl solution. Prior to sampling, the systems treated with the equilibration protocol, suggested by CHARMM-GUI (Table 1).

	Steps	Time Step	K Protein	K Lipid	K Dihed.	Temperature	Pressure
min	2000	minimization	10.0	2.5	250	---	---
Eq1	125000	1fs	10.0	2.5	100	303.15K	NVT
Eq2	125000	1fs	5.0	2.5	50	303.15K	NVT
Eq3	125000	1fs	2.5	1.0	50	303.15K	1 bar
Eq4	250000	2fs	1.0	0.5	50	303.15K	1bar
Eq5	250000	2fs	0.5	0.1	25	303.15K	1bar
Eq6	250000	2fs	0.1	0.0	0	303.15K	1bar

Table 1: Overview over the 7 equilibration steps performed for all simulations. The initial minimization is followed by six equilibration simulations with decreasing force constants (K) on positional restraints of amino acids (Protein, all atoms), positional restraints on lipid headgroups (Lipid, phosphorus atom) as well as restraints on the lipid dihedrals (Dihed).

In all cases, periodic boundary conditions were used and the temperature was set to 303.15K using the Langevin thermostat (Goga, Rzepiela et al. 2012) with a friction coefficient of 1.0 ps^{-1}. To sample NpT ensembles, the Berendsen manostat (Berendsen, Postma et al. 1984) was utillized and set to 1.0 bar with a

relaxation time of 1ps. All non-bonded interactions have been calculated directly up to a distance of 9Å, after which electrostatic interactions were treated with the particle mesh Ewald method (Darden, York et al. 1993), while long range van-der-Waals interactions were estimated by a dispersion correction model. To achieve time-steps of 4fs, the Shake algorithm (Andersen 1983) as well as hydrogen mass repartitioning (Hopkins, Grand et al. 2015) were used.

Prior to building the dimeric simulation systems, the Cnx, Cx32 and CoMem helices have been simulated in isolation for 1μs in a POPC bilayer in order to equilibrate the structure and to predict their orientation in the membrane. All simulation snapshots have been rendered using VMD (Humphrey, Dalke et al. 1996).

Cnx-CoMem dimerisation simulations

Initially, five simulations of one Cnx helix and one CoMem (WT) helix in the same simulation box, have been performed. Each of the five simulations was started with different relative orientations of the two helices which have been generated by hand using VMD. After preparing the simulation systems using CHARMM-GUI, the systems were equilibrated using the protocol in Table 1. Subsequently, unrestricted sampling simulations of at least 2.5μs have been performed. Successful complex formation was only sampled in one of the five simulations. To improve statistics, two additional independent simulations have been performed.

These simulations have been started from a snapshot of the successful simulation in which the two helices were not yet interacting with each other. In one of these two simulations, a similar complex was formed as in the previous, successful simulation. At this stage, all simulations have been sampled for at least 4μs.

To obtain binding statistics on the amino-acid level that are more generally valid, we also performed simulations with Cnx and two CoMem mutants that exhibit amino acid alterations in the TMD segment: V13P and V13R.

Several at least 4μs long simulations have been performed for both mutants, however, only two resulted in the formation of Cnx-CoMem interactions along the entire TMD: One simulation featuring V13R and one with the V13P mutant. Of

interest: While the V13P mutant binds to Cnx in a fashion very similar to WT CoMem, the V13R mutant uses an alternative interface to bind to Cnx. Cnx, on the other hand, binds all CoMem variants utilizing basically the same binding interface. Altogether, 22 μs stim-scale simulations of this system have been performed. However, for analysis we only selected the 4 simulations that exhibited broad TMD-TMD interactions between Cnx and CoMem. Data analysis was performed on 4μs long trajectories in each case.

Cnx-Cx32 dimerization simulations

For this system five, at least 4μs long simulations have been performed. The simulation set-up was identical to the one used for the Cnx-CoMem system. In four out of the five cases, we were able to sample at least transient interactions between the helices, involving the entire TMD of Cnx. These four simulations were then selected for data analysis on 4μs of the sampled trajectories.

Abbreviations

ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
BiP	Immunoglobulin heavy-chain binding protein
cDNA	Complementary DNA
CHX	Cycloheximide
C_L	Constant domain of the light-chain
CNX	Calnexin
COS	CV-1 in origin, and carrying the SV40 genetic material
CRT	Calreticulin
DMEM	Dulbecco´s modified Eagle medium
DNA	Deoxyribonucleic acid
DTT	Dithioreitol
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
Glc	Glucose
GlcNac	N-Acetylglucosamine
gp78	Cell surface glycoprotein of 78 kDa
HA	Hemagglutinin
Hrd1	HMG-CoA reductase degradation
IB	Immunoblot
IF	Immunofluorescence
IRE1	Inositol-requiring protein
IP	Immunoprecipitation
min	Minute
mL	Milliliter
MW	Molecular weight
NaDOC	Sodium deoxycholate
Nm	Nanometer
OST	Oligosaccharyltransferase
PAGE	Polyacrilamide gel electrophoresis

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDI	Protein disulfide-isomerase
PERK	Protein kinase RNA-like ER kinase
PNGase	Peptide:N-glycosidase
PTM	Post-translational modification
PVDF	Polyvinylidene fluoride
QC	Quality control
RIPA	Radioimmunoprecipitation assay buffer
RNC	Ribosome-nascent chain complex
RT	Room temperature
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SRP	Signal recognition particle
TBS	Tris-buffered saline
TM	Transmembrane
TRAM	Translocating chain-associated membrane protein
TRAP	Translocon-associated protein
Tm	Tunicamycin
UPR	Unfolded protein response
WT	Wild type
ß-Me	ß-mercaptoethanol

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