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FUNCTION AND REGULATION OF THE ENDOPLASMIC RETICULUM CHAPERONE GRP170

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2 Summary

Secretory and transmembrane proteins originate in a dedicated cellular compartment, the Endoplasmic Reticulum (ER). In the environment of this organelle these proteins fold into their native structure, starting as an elongated amino acid chain, acquire posttranslational modifications and eventually assemble with additional subunits before leaving the ER. All of these processes are orchestrated and monitored by resident proteins of the ER, the chaperones. Chaperones also decide whether a protein will be secreted or is degraded, a process called ER quality control (ERQC). Two major chaperone systems exist in the ER: the lectins calnexin and calreticulin, and the Hsp70 superfamily members BiP and Grp170. This work focused on understanding the functions of Grp170, a protein involved in many biological processes and disease states *via* poorly characterized molecular mechanisms.

BiP and Grp170 were assigned to the same superfamily based on a similar domain composition. However, Grp170 possesses additional domains and thus belongs to the subgroup of large Hsp70s. Both, BiP and Grp170 are the only members of their respective subgroups in the ER, whereas several orthologs of both proteins are present in the cytosol. In the past decades it was demonstrated that BiP binds ATP, and that this nucleotide-state induces a low affinity for its protein substrates. Once a substrate is bound, ATP is hydrolyzed to ADP, resulting in an increase in binding affinity. Thus, in order to release substrates so that they can fold or be degraded, ADP has to be exchanged to ATP by nucleotide exchange factors (NEFs). Whether Grp170 and other large Hsp70s also served as chaperones that bound to substrates directly, as suggested by their Hsp70-like domain organization, became unclear after the surprising discovery that large Hsp70s can function as NEFs for Hsp70s, such as BiP. In addition, since all hitherto described potential Grp170 substrates were also BiP substrates, it was unclear which function (chaperone *vs.* NEF) Grp170 might be playing in the identified Grp170:BiP:substrate complexes.

In the first part of this work we demonstrated that Grp170 binds to incompletely folded protein substrates *in vivo* in the absence of BiP, arguing that it bound to these substrates directly as a *bona fide* chaperone. In contrast to BiP, which dissociates from its substrates in the presence of ATP, Grp170 remains bound. Instead of a nucleotide regulating the binding of substrates to Grp170, we found that this interaction is modulated by the unique domains of the large Hsp70s that set them apart from conventional Hsp70.

One central question of the ERQC process is how chaperones distinguish between incompletely folded and terminally misfolded proteins. For conventional Hsp70s it has been established using *in vitro* peptide screens and *in silico* studies that they

recognize approximately 7-9 amino acid stretches of alternating hydrophobic residues. However, this question has not been addressed *in vivo* and for large Hsp70s the only information in regard to binding specificity is based on two *in vitro* studies with a few peptides that suggested a preference of large Hsp70s for aromatic residues. In order to dissect the binding sites of BiP and Grp170 within their substrates *in vivo*, we designed and established a novel *in vivo* peptide expression library covering two substrates that interact with both of these chaperones. For the first time, we directly demonstrated that BiP binds to multiple sequences throughout its substrate proteins *in vivo* with different apparent affinities, consistent with predictions that BiP binding sites are likely to occur ca. every 16 amino acids. In contrast, Grp170 bound only to very few well-defined peptides, and the binding was restricted to unanticipated domains, suggesting that Grp170 binding sites may have been specifically selected against in domains that were critical to the evolution of antibody molecules for quality control purposes. In agreement with previous studies, most Grp170 binding sites were particularly enriched in aromatic residues. Together these insights represent a crucial step towards understanding which sites are recognized by these chaperones *in vivo* within a substrate and whether these specific sites encode an ERQC code that decides about the fate of substrates in the ER.

The demonstration that Grp170 functions as a chaperone in the ER with a different regulation and substrate binding specificity compared to BiP posed the question whether Grp170 is also involved in a similar co-chaperone network of ERdj proteins as BiP. It became clear that also in that regard there are differences, because Grp170 does not possess the critical residue that was shown to be the basis for the interaction of BiP with the ERdj co-chaperones. Instead, the fact that Grp170 is a heavily glycosylated protein in contrast to the unglycosylated BiP attracted our attention. Indeed, our data suggests that the Grp170 glycosylation sites are involved in its interaction with Sel1L, a transmembrane protein involved in the degradation of terminally misfolded proteins, which might help explaining the biological functions of Grp170.

In summary, within this work we gained important insights into the function of Grp170 as a chaperone and its regulation, emerging evidence about its interaction network in the ER and began to dissect for the first time the precise *in vivo* binding sites of Grp170 and BiP within their substrates.

3 Zusammenfassung

Sekretorische und Membranproteine werden in einem auf die Proteinfaltung spezialisierten zellulären Organell synthetisiert, dem Endoplasmatischen Retikulum (ER). Im Kontext des ER falten diese Proteine in ihren nativen Zustand, von einer unstrukturierten Aminosäurekette ausgehend. Sie erhalten posttranslationale Modifikationen und assemblieren ggf. mit weiteren Untereinheiten bevor sie das ER verlassen. Diese Prozesse werden von dezidierten Proteinfaltungshelfern, den Chaperonen, kontrolliert und unterstützt. Die Chaperone beeinflussen entscheidend ob ein Protein sekretiert oder abgebaut wird, ein Prozess der als ER Qualitätskontrolle (ERQC) bekannt ist. Zwei wesentliche Chaperon-Systeme existieren im ER: Die Lektinchaperone Calnexin und Calreticulin, die Oligosaccharide auf Proteinen erkennen, sowie BiP und Grp170, die beide zur Hsp70 Superfamilie gehören. Grp170 wurde mit zahlreichen biologischen Prozessen als auch Krankheiten in Verbindung gebracht, wobei die zu Grunde liegenden molekularen Mechanismen weitgehend unverstanden sind. Der Fokus dieser Arbeit bestand deshalb darin, Einsichten in die Funktion und Regulation von Grp170 auf molekularer Ebene zu erlangen.

Aufgrund grosser Ähnlichkeiten in der strukturellen Organisation ihrer Domänen wurden BiP und Grp170 in einer Superfamilie zusammengefasst. Da Grp170 zusätzliche Domänen im Vergleich zu BiP besitzt gehört es zu der Unterfamilie der grossen Hsp70 Chaperone. Sowohl BiP als auch Grp170 sind die einzigen Vertreter ihrer jeweiligen Unterfamilie im ER, jedoch sind mehrere Orthologe einer jeden Subgruppe im Zytoplasma vorzufinden. In den vergangenen Jahrzehnten wurde gezeigt, dass BiP ATP bindet und dadurch seine Affinität für Substratbindung verringert wird. Sobald ein Substrat bindet, wird ATP zu ADP hydrolysiert, und BiP besitzt nun eine hohe Substrataffinität. Damit ein Substrat wieder freigesetzt und somit falten oder abgebaut werden kann, wird ADP gegen ATP von den Nukleotid-Austauschfaktoren (NEF) ausgetauscht. Nach der überraschenden Entdeckung, dass Grp170 und andere grosse Hsp70 als NEF für Hsp70 Chaperone wie BiP dienen, wurde es unklar, ob grosse Hsp70 Proteine auch als Chaperone agieren, die direkt Substrate binden können, wie es ihre strukturelle Organisation nahelegt. Zudem gelten alle bisher identifizierten potentiellen Substrate von Grp170 auch als BiP Substrate. Aus diesem Grund kam die Frage auf, ob Grp170 in Grp170:BiP:Substrat Komplexen als NEF oder als Chaperon agiert.

Der erste Teil dieser Arbeit zeigt, dass Grp170 inkomplett gefaltete Proteinsubstrate *in vivo* in Abwesenheit von BiP binden kann. Diese Ergebnisse legen nahe, dass Grp170 diese Substrate als ein Chaperon bindet. Im Gegensatz zu BiP, das bei Zugabe von ATP von seinen Substraten abdissoziiert, blieb Grp170 gebunden. Die erhaltenen Daten zeigen, dass anstatt durch ATP die Bindung von Grp170 an Substrate

durch diejenigen Domänen von Grp170 beeinflusst wird, welche charakteristisch für grosse Hsp70 Proteine sind. Damit konnte gezeigt werden, dass die Substrat:Grp170 Wechselwirkung intramolekular reguliert wird.

Eine zentrale Frage der ERQC ist, wie Chaperone zwischen noch nicht gefalteten und terminal fehlgefalteten Proteinen unterscheiden. *In vitro* Peptidscreens und *in silico* Studien haben aus ca. 7-9 Aminosäuren bestehende Erkennungssequenzen für Hsp70 Chaperone etabliert, die aus alternierenden hydrophoben Resten aufgebaut sind. Analoge Erkenntnisse existieren *in vivo* bislang nicht, und für grosse Hsp70 Proteine sind lediglich zwei *in vitro* Studien mit wenigen untersuchten Peptiden verfügbar, die eine Präferenz für aromatische Reste nahelegen. Um die Bindungssequenzen von BiP und Grp170 innerhalb ihrer Substrate *in vivo* zu bestimmen, wurde eine neuartige *in vivo* Peptidbibliothek etabliert, die auf Sequenzen von zwei BiP und Grp170 Substraten basiert. Zum ersten Mal konnten wir demonstrieren, dass BiP an mehrere Sequenzen mit unterschiedlichen Affinitäten im Substrat im Kontext des ER bindet. Diese Erkenntnis ist im Einklang mit der Vorhersage, dass BiP-Bindungssequenzen etwa alle 16 Aminosäuren auftreten. Im Gegensatz zu BiP bindet Grp170 nur sehr wenige Peptide und die Bindung war auf bestimmte Domänen beschränkt. Da einige spezifische Domänen in der ERQC der Antikörperassemblierung eine Rolle spielen, könnte dies eine Selektion für oder gegen Grp170 Bindestellen im Laufe der Evolution implizieren. In Übereinstimmung mit vorhergehenden Studien enthielten die meisten Grp170 Bindesequenzen eine erhöhte Anzahl an aromatischen Aminosäuren. Diese gewonnenen Einsichten sind von kritischer Bedeutung, um die Bindungsspezifität von Chaperonen innerhalb ihrer Substrate *in vivo* zu verstehen und bieten die Möglichkeit ggf. einen ERQC Code, welcher über das Schicksal eines Substrats entscheidet, zu entziffern.

Die Einsichten in die Grp170 Funktion als Chaperon, dessen Substratbindung anders als bei BiP reguliert wird, warf die Frage auf, ob Grp170 durch andere co-Chaperone reguliert wird, als es für BiP und dessen Netzwerk aus ERdj Proteinen etabliert wurde. In der Tat wurden auch in dieser Hinsicht signifikante Unterschiede festgestellt, da die kritische Aminosäure, die die Basis für die BiP:ERdj Interaktion darstellt, in Grp170 nicht vorhanden ist. Stattdessen besitzt Grp170 zahlreiche Glykosylierungsstellen, im Gegensatz zu BiP, welches unglykosyliert ist. In vorläufigen Experimenten konnten wir zeigen, dass der Glykosylierungsstatus von Grp170 für dessen Bindung an Sel1L, ein am Proteinabbau beteiligtes Membranprotein, eine Rolle spielt.

Im Laufe dieser Arbeit konnten wichtige Einsichten in die Funktion von Grp170 als Chaperon und dessen Regulation gewonnen werden. Zudem wurden neue Erkenntnisse über dessen Rolle ER Chaperon-Netzwerk gewonnen. Darüber hinaus

konnten erstmals die Bindungssequenzen innerhalb verschiedener Substrate von Grp170 und BiP *in vivo* auf der Basis einer zellulären Peptidbibliothek analysiert werden.

4 Introduction

4.1 The Endoplasmic Reticulum: the Cell's Factory for Secretory and Transmembrane Proteins

Every cell needs to interact with its environment in order to survive. This is largely mediated by transmembrane (TM) cell surface and secretory proteins. All eukaryotic cells have developed a dedicated organelle for the synthesis of these proteins: the endoplasmic reticulum (ER). Additionally, the resident proteins of specific organelles, such as the ER, ER-Golgi intermediate compartment (ERGIC), Golgi, endosomes and lysosomes, have their origin in the ER (Zimmermann et al, 2011). Proteins enter the ER as an unfolded polypeptide chain acquiring all features of a fully folded, assembled and functional protein before traversing further along the secretory pathway. Therefore, the ER must provide an environment optimized for folding of proteins, which will in most cases ultimately fulfill their function outside the cell: it (i) provides a large pool of proteins that assist unfolded proteins in their folding, the chaperones; (ii) is more oxidizing than the cytosol and thus more similar to the extracellular space, which contributes to the formation of disulfide bonds that stabilize folded proteins and complexes, and also possesses an ion composition more similar to the extracellular space; (iii) possesses a machinery for co-translational N-linked glycosylation, which serves to stabilize proteins in the extracellular space and restricts the possible folding pathways available while in the ER due to their bulky and hydrophilic nature (Braakman & Bulleid, 2011; Gidalevitz et al, 2013). The translation of proteins destined for the ER takes place in the cytosol. Therefore, these proteins encode a signal sequence, which initiates their targeting and translocation into the ER through a proteinaceous transmembrane channel called the translocon. This signal sequence comprises 15-30 amino acids and is typically divided into a hydrophobic core with one or more amino-terminally located positively charged residues and a polar, but uncharged residue in the C-terminal region that serve to properly orient the signal sequence in the translocon (Devillers-Thiery et al, 1975; von Heijne, 1983; von Heijne, 1984; Zimmermann et al, 2011; Benham, 2012).

4.1.1 Targeting and Translocation of nascent proteins into the ER

Three pathways of protein translocation from the cytosol into the ER exist in mammalian cells (Figure 1): cotranslational and posttranslational translocation (Wickner & Schekman, 2005; Zimmermann et al, 2011; Benham, 2012; Park & Rapoport, 2012) and the recently identified tail-anchored (TA) proteins (GET) pathway in which a C-terminal transmembrane domain (tail) is integrated into the membrane and the N-terminal region is oriented to the cytosol (Denic, 2012; Johnson et al, 2013). All three pathways are largely conserved between mammals and yeast, but differences exist in proteins and steps involved as well as the preferences for the pathway used. Since this PhD thesis describes studies on the mammalian ER, the mammalian system will be the focus in chapters to follow. In mammals cotranslational translocation is the predominant protein translocation pathway, followed by posttranslational translocation (Zimmermann et al, 2011; Benham, 2012) and only approximately 5% of membrane proteins of the secretory pathway engage the GET pathway (Denic, 2012) (Figure 1).

A. Cotranslational Translocation into the ER

Proteins utilizing the cotranslational translocation pathway generally encode their signal sequence at or near the N-terminus (Figure 1*A*). As soon as the hydrophobic sequence of the nascent protein emerges from the ribosome, it is recognized by the signal recognition particle (SRP). The SRP, a complex composed of a 7S RNA scaffold and six polypeptides, slows translation upon binding to the A site of the ribosome and targets the ribosome-mRNA-nascent protein complex to the ER membrane (Wickner & Schekman, 2005; Benham, 2012). Once at the ER membrane, the SRP binds to the α– subunit of the SRP receptor, which is anchored in the membrane *via* its β-subunit (Benham, 2012). This in turn positions the 60S subunit of the ribosome at the translocation channel Sec61, where it forms a tight complex (Walter et al, 1982; Alder & Johnson, 2004; Mandon et al, 2009; Benham, 2012; Park & Rapoport, 2012). Upon GTP hydrolysis, the SRP is released from its receptor and the ribosome, allowing translation to resume. The energy used for translation translocates the elongating polypeptide chain through the ribosome tunnel directly into the aqueous interior of the Sec61 channel. Three Sec61 subunits, α, β and γ, form this proteinaceous channel spanning the lipid bilayer, which forms a barrier between the cytosol and the ER lumen. The ten α-helical transmembrane

(TM) segments of the α-subunit form the central pore, while the γ-subunit links the two halves of the α-subunit, and the β-subunit makes only a few contacts with the α-subunit and is less well conserved.

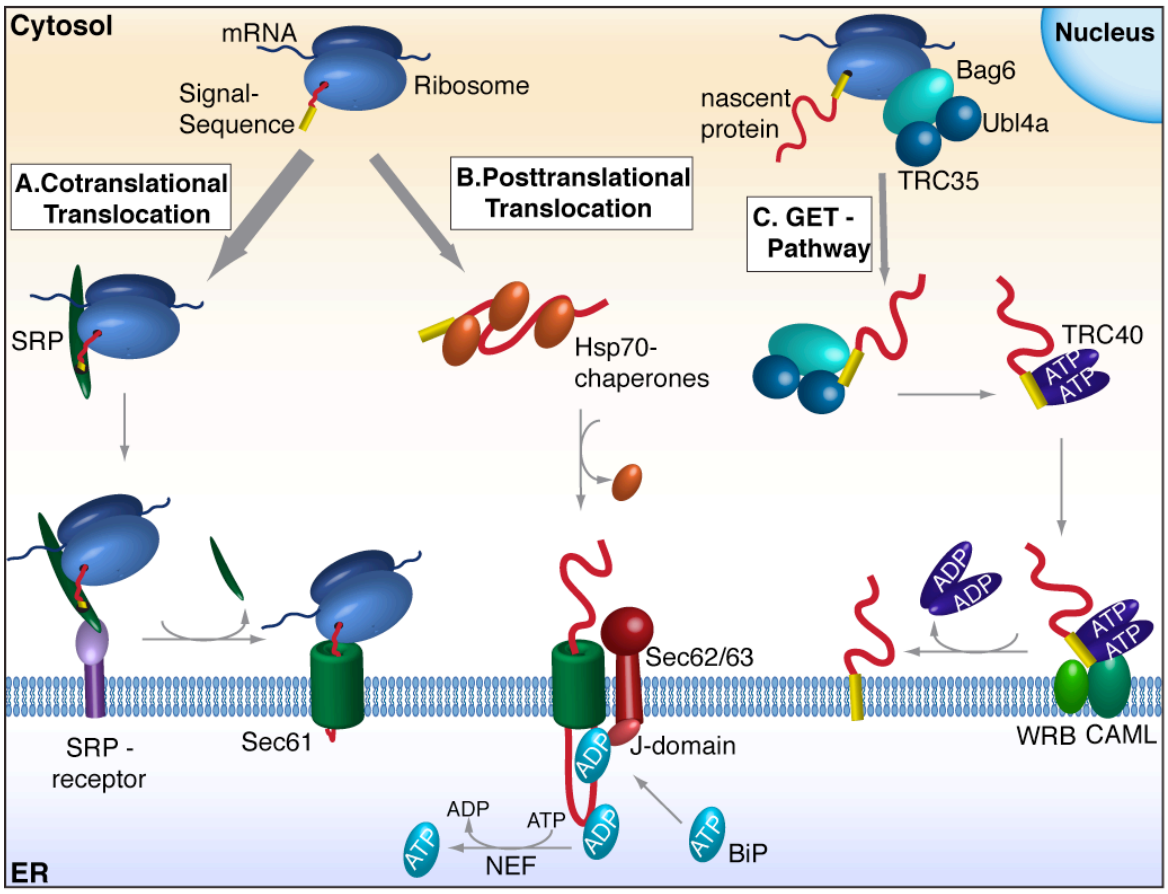

Figure 1. Translocation pathways of proteins from the cytosol into the ER in mammals.

Three major translocation pathways facilitate the entry of proteins into the ER lumen or integration into the ER membrane: cotranslational translocation (A), posttranslational translocation (B) and the GET pathway (C). Only distinctive key factors of each pathway are depicted. See text for further details.

During subsequent translocation of the amino acid chain through Sec61 into the ER lumen, the signal sequence remains in contact with the Sec61 channel, while the rest of the nascent protein continues to enter the ER lumen co-translationally. The signal sequence is in most cases cleaved at some point by the signal peptidase (Evans et al, 1986), and if the protein does not possess further hydrophobic TM regions, the soluble protein is then released into the ER lumen when translation is completed. If the signal

sequence remains uncleaved, it can serve to anchor the protein in the ER membrane with the N-terminus facing the cytosol and the rest of the protein in the ER lumen (type II membrane protein) (Andersson & von Heijne, 1994; Pool et al, 2002; Zimmermann et al, 2011). If the signal sequence is cleaved but a second sequence that is sufficiently hydrophobic to serve as a transmembrane domain occurs later in the sequence, the polypeptide will become an integral membrane protein that might ultimately be expressed on the cell surface (type I membrane protein) (Andersson & von Heijne, 1994). For the integration of transmembrane proteins, the α-subunit harbors a lateral gate to the hydrophobic environment of the lipid bilayer. Since the interior of the channel is hydrophilic, the hydrophobic TM segments of a translocating protein can exit the channel *via* the lateral gate and partition into the ER membrane (von Heijne, 2005; Mandon et al, 2009; Park & Rapoport, 2012).

B. Posttranslational Translocation into the ER

The posttranslational translocation pathway (Figure 1B) is used by proteins with a signal sequence of low hydrophobicity, which is not recognized by the SRP, or by particularly small proteins containing usually less than 75 amino acids (Zimmermann et al, 2011). This pathway has been primarily studied in yeast, whereas the details of this pathway in mammalian cells is not well understood (Walter & Johnson, 1994; High, 1995; Park & Rapoport, 2012). In this scenario the polypeptide chain is completely translated in the cytosol. Chaperones of the Hsp70 family and co-chaperones of the Hsp40 family recognize hydrophobic sequences on the polypeptide chain during translation and keep the nascent chain in an unfolded, translocation competent state and prevent it from aggregation. In addition to the Hsp70 system, in the posttranslational translocation pathway of small secretory proteins also calmodulin was shown to maintain these proteins in a translocation competent state (Shao & Hegde, 2011). Once the protein binds to the translocation complex composed of the Sec61 channel and the Sec62/63 transmembrane proteins, the cytosolic chaperones are released and protein movement into the ER lumen is initiated. However, the mechanism of pulling the protein into the ER is less clear as the energy for translocation is no longer provided by chain elongation. One mechanism involves the ER lumenal Hsp70 family chaperone, immunoglobulin heavy-chain binding protein (BiP), which has been shown to bind to the emerging polypeptide chain of at least some proteins and keep them from sliding back into the cytosol. BiP is bound to a lumenal site on Sec61 and serves to seal the translocon until

the ribosome forms a tight seal on the one hand and on the other hand it is readily available once a nascent chain emerges in the ER (Hamman et al, 1998). BiP is an ATP-driven chaperone and as soon as it binds to the nascent protein, its intrinsic ATPase activity is stimulated by the J-domain of Sec63. Once ADP-bound BiP remains associated with the nascent protein it either simply prevents it from slipping back to the cytosol or in some cases a second BiP molecule might bind more C-terminally to the emerging amino acid chain. In the latter case the protein would be pulled step by step by this ratcheting mechanism into the ER lumen. The release of ADP and subsequent rebinding of ATP are critical to this process, as BiP ATPase mutants are defective in translocation (McClellan et al, 1998). The presence of BiP nucleotide exchange factors (NEFs) accelerate this reaction, which in turn releases BiP so that the polypeptide chain can continue to enter into the ER lumen (Zimmermann et al, 2011). However not all proteins are bound by BiP. The binding of the integral membrane lectin chaperone calnexin (CNX) to nascent glycoproteins may serve a similar function. Regardless which mechanism is engaged by the translocating protein, the factors on the ER lumenal side are indispensable for its successful unidirectional import into the ER lumen (Nicchitta & Blobel, 1993).

C. The GET Pathway

In contrast to the co- and posttranslational translocation pathways, proteins taking their route to the ER *via* the GET pathway encode their signal sequence near or at the C-terminus (Figure 1*C*) (Kutay et al, 1993; Favaloro et al, 2008). As a consequence, the translation of these proteins is almost or entirely complete before it can be recognized as a substrate destined for the ER. The ~20 amino acid α-helical signal sequence of these proteins thus serves as a TM domain, which ultimately becomes an anchor in the ER membrane (Denic, 2012). Although TA proteins represent a small percentage of TM proteins, they are functionally diverse and are often involved in essential functions such as apoptosis, vesicular transport or protein translocation (Denic, 2012; Johnson et al, 2013). Instead of the SRP playing the guiding role, the TM domains of TA proteins are posttranslationally recognized by a complex composed of BCL2-associated anthanogene 6 (Bag6), TRC35 and Ubiquitin-like protein 4a (Ubl4a). This pre-recognition complex then associates with 40-kDa component of the TMD recognition complex (TRC40) and hands over the TA protein. Interestingly, the Bag6-complex can play a role

not only in the biogenesis of TA proteins, but also in the degradation of mislocalized membrane proteins (Hessa et al, 2011). In this scenario Bag6 recruits the cytosolic ubiquitination machinery instead of TRC40 *via* its UBL domain and initiates degradation of its bound protein (Hessa et al, 2011). Hence, Bag6-TRC35-Ubl4a fulfills a quality control function by sorting its substrate either towards degradation or membrane integration. Proteins bound to TRC40 and destined for ER integration are delivered to a receptor in the ER membrane composed of the tryptophan-rich basic protein (WRB) (Vilardi et al, 2011) and calcium-modulating cyclophilin ligand (CAML). TRC40 releases the TA protein upon ATP-hydrolysis and most likely the protein is directly inserted into the lipid bilayer *via* its TMD (Denic, 2012; Johnson et al, 2013).

4.1.2 Protein Folding and Modification in the ER

The environment of the ER is likely optimized to meet specific qualitative and quantitative demands required by various proteins in distinct cell types and must adjust to the diverse and changing conditions they may encounter. Nevertheless the process of folding for all proteins in a cell depends on the same biophysical laws elucidated by *in vitro* folding studies. An understanding of which biophysical forces drive a protein from an elongated amino acid chain into its three dimensional structure explains the evolution of the various chaperone classes residing in the ER (Figure 2).

A. Protein Folding in vitro

One of the hallmark studies to understand how proteins obtain their native conformation was performed by Anfinsen and colleagues (Anfinsen et al, 1961). After using mercaptoethanol and urea to reduce and denature the eight-cysteine containing protein ribonuclease, they observed an almost complete renaturation into the native enzyme under optimal conditions of polypeptide concentration, pH and oxygen when urea was diluted. Based on this study it was concluded that the amino acid sequence itself contains all the necessary information for generating the secondary and tertiary structure of a protein (Anfinsen et al, 1961), which, together with other studies, led the authors to posit the famous thermodynamic hypothesis (Anfinsen, 1973). In general, it states that protein folding can be depicted as a process where an unfolded polypeptide chain (U) undergoes a transition to partially folded intermediate states (I) to ultimately reach its

native state (N) (Figure 2A) (Wagner & Kiefhaber, 1999; Walter & Buchner, 2002; Compiani & Capriotti, 2013). Within this process, the state of an elongated amino acid chain with exposed hydrophobic, or hydrophilic residues for TM proteins, is energetically unfavorable. Whereas burying the hydrophobic residues within the structure of a protein as it folds leads to the much more energetically favorable native state. Intramolecular interactions that initiate the folding of a polypeptide are mostly non-covalent, like hydrophobic and charge-charge interactions as well as hydrogen bonds and salt bridges. Additionally, covalent interactions such as the formation of disulfide bridges by two cysteines (Compiani & Capriotti, 2013; Nick Pace et al, 2014) play an important role. Together these interactions lead to the formation of α-helices and β-sheets, the two major secondary structure elements of a protein. The packing of the secondary structure elements into a three dimensional entity is required to achieve the native state. If several subunits or polypeptide chains assemble to form the final protein or protein complex, this is called the quaternary structure.

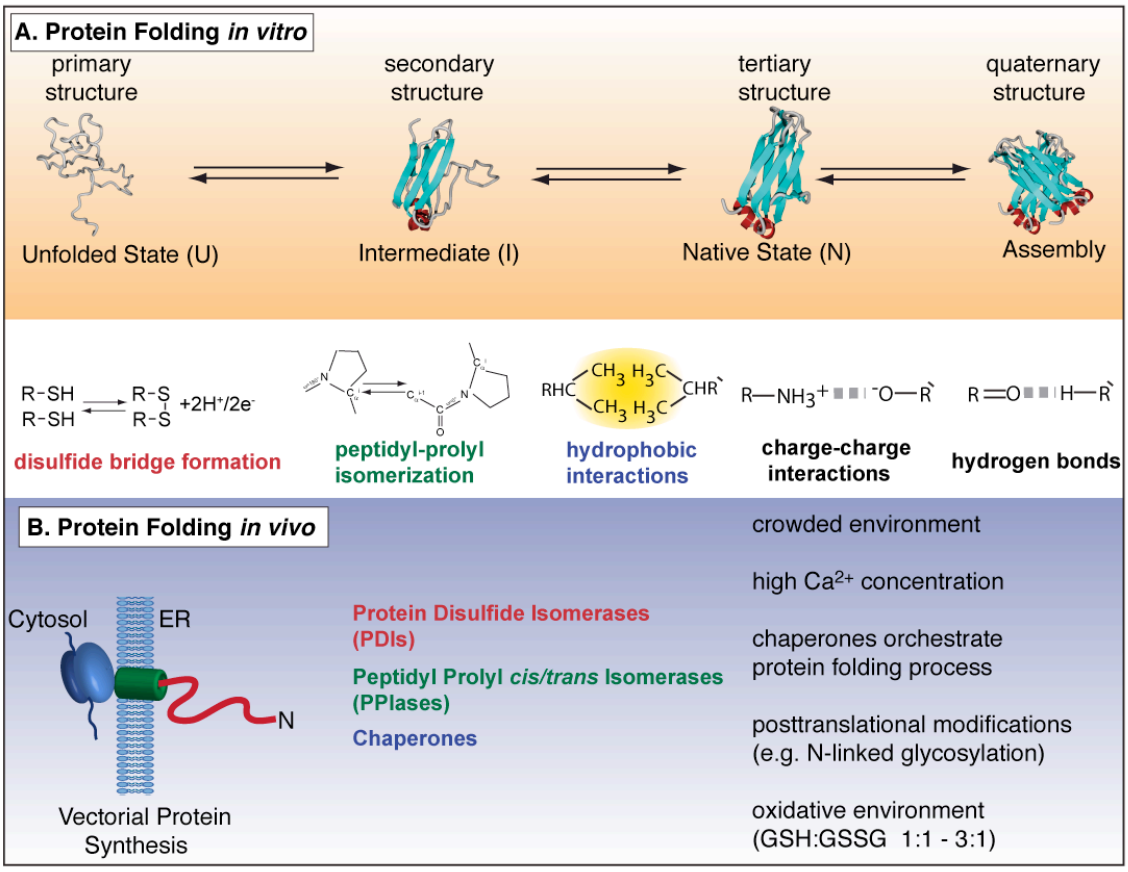

Figure 2. Comparison of Protein Folding in vitro *vs.* in vivo in the ER.

Three major models have been defined to describe the transition of a protein from an unfolded to a folded state: the hydrophobic collapse model, the nucleation-condensation model and the framework model (Dill, 1985; Itzhaki et al, 1995; Compiani & Capriotti, 2013). In the first scenario, hydrophobic forces lead to a chain collapse prior to formation of secondary structure elements. In the second model, the formation of a few defined key interactions establishes a folding nucleus around which the rest of the protein folds, while in the last model secondary structure elements form in several regions of a protein in parallel. However, it is important to keep in mind that the native state of a protein is not a rigid structure. Many proteins undergo conformational changes in processes such as allosteric communication within one protein (Motlagh et al, 2014) or fold upon interaction with other subunits (Lee et al, 1999; Feige et al, 2009), and the class of intrinsically disordered proteins (IDPs), which lack a persistent structure, has greatly expanded over the last decade (Forman-Kay & Mittag, 2013).

B. Protein Folding in vivo

While all of aforementioned aspects of protein folding identified from *in vitro* studies also apply in the cell, the conditions under which they occur *in vivo*, e.g. high protein concentration and synthesis taking place, are significantly different (Figure 2*B*). First, the environment of the ER is extremely crowded possessing protein concentrations ranging from 100-400 mg/ml and contains a large variety of different proteins at various stages of their folding process (Araki & Nagata, 2011; Braakman & Hebert, 2013). As a consequence, even a short exposure of hydrophobic regions of a nascent protein would likely lead to irreversible aggregation with other hydrophobic regions within the protein or with other proteins. Second, the compartmentalization of a cell dictates a vectorial entry of a nascent protein into the ER from N- to C-terminus (Figure 2*B*). This in turn means that, in contrast to a full-length polypeptide chain used in *in vitro* folding studies, not all parts of a protein that might need to interact for productive folding are present in the ER lumen or even synthesized when folding can already begin at the N-terminus. Additionally, folding is also kinetically restricted by a translation elongation rate of 4-5 residues per second in eukaryotes (Braakman & Bulleid, 2011), which is slower than e.g. the kinetics of secondary structure formation (Bieri et al, 1999; Ferguson & Fersht, 2003; Fierz et al, 2007). Conversely, tertiary structure is often achieved *in vivo* at much faster rates than observed *in vitro* (Anfinsen, 1973). Indeed, leaving a protein to fold on its own under *in vivo* conditions would be unlikely to succeed and the consequences

would be detrimental to the cell. Eukaryotic cells have thus evolved an optimized environment for protein folding in the cell and in particular in the ER, enabling the secretion of up to several thousands proteins per second and still meeting stringent qualitative standards (Masciarelli & Sitia, 2008). How well the ER has evolved to meet these requirements has already been mentioned by Anfinsen who observed that renaturation of ribonuclease *in vitro* takes hours, whereas addition of ER components reduces this process to two minutes (Anfinsen, 1973). In the years following this observation the explanation was found in the discovery of different classes of chaperones, which are dedicated protein folding helpers. BiP was the first ER chaperone to be discovered in any organism, followed by glucose-regulated proteins, Grp94 and Grp170, and the lectins, calnexin (CNX) and calreticulin (CRT) (Haas & Wabl, 1983; Lee et al, 1984; Sorger & Pelham, 1987; Hendershot et al, 1988; Lin et al, 1993; Ou et al, 1993; Nauseef et al, 1995). These chaperones coordinate and orchestrate specific steps during the folding process as well as assembly of several subunits if necessary (Araki & Nagata, 2011; Braakman & Bulleid, 2011; Brodsky & Skach, 2011; Feige & Hendershot, 2011; Gidalevitz et al, 2013).

Exposed hydrophobic stretches of nascent or incompletely folded proteins are shielded against aggregation by chaperones on the one hand and on the other hand these regions are kept in a folding competent state if posttranslational folding is required. The most prominent member fulfilling this function in the ER is BiP, which belongs to the Hsp70 chaperone family and will be discussed together with other chaperones in more detail in the following sections. In addition to chaperones, folding enzymes, which often also possess chaperone activity, further aid the protein folding process. One example is the family of protein disulfide isomerases (PDIs), which can oxidize, isomerize and reduce disulfide bridges (Appenzeller-Herzog & Ellgaard, 2008; Bulleid & Ellgaard, 2011). In addition, peptide bonds preceding the amino acid proline exist in a *cis-trans*-equilibrium in the absence of structural constraints, with ~80% of this bond populating a *trans* conformation. In the context of a given native protein structure, however, one particular isomer is often required. The necessary isomerization process is slow and *in vivo* catalyzed by peptidyl-prolyl isomerases (PPIases), another class of folding enzymes (Lu et al, 2007). An additional modification unique to the ER is the covalent attachment of glycan complexes to asparagine residues (N-linked) of proteins. The oligosaccharyl-transferase complex (OST) responsible for glycosylating nascent polypeptide chains resides in close proximity to the Sec61 channel (Kelleher & Gilmore, 2006; Roboti &

High, 2012; Pfeffer et al, 2014) and usually co-translationally transfers an oligosaccharide complex from a dolichol donor to Asn-X-Ser/Thr (X = any amino acid except Pro) sequons on nascent proteins (Kornfeld & Kornfeld, 1985). However, more recently post-translational glycosylation has been described for some proteins (Shrimal et al, 2013), and additional rare glycosylation sequons that are targeted for glycosylation, e.g. N-X-C, have been identified (Zielinska et al, 2010). This modification fulfills several purposes: it (i) contributes to the stability of secretory proteins outside the cell; (ii) is important for the interaction with glycan-binding proteins (GBPs) in the extracellular space; (iii) is used as a quality control tool within the CNX/CRT cycle in the ER (Dalziel et al, 2014); (iv) facilitates interaction of some proteins with PDIs (Brodsky & Skach, 2011); and (v) plays a critical role in promoting correct folding by restricting the possible folding pathways.

C. The Protein Folding Environment of the ER

The ER environment developed to be more similar to the extracellular environment than the rest of the cell, as many of the proteins synthesized here will ultimately function outside the cell. Adaptations include a much higher oxidizing potential, which can lead to the oxidation of cysteine pairs on proteins. The resulting disulfide bridges are an important feature of secretory proteins, since they significantly contribute to the overall stability of proteins (Appenzeller-Herzog & Ellgaard, 2008; Bulleid & Ellgaard, 2011; Nick Pace et al, 2014). The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) is lower (GSH:GSSG between 1:1 and 3:1) than in the cytosolic compartment and provides the redox buffer system utilized by the PDI family members to form, isomerize and reduce disulfide bonds (Appenzeller-Herzog, 2011). One of the major regulators of disulfide bond formation is considered to be the flavoprotein Ero1, which includes the broadly expressed Ero1α and the tissue specific Ero1β in higher eukaryotes (Tu & Weissman, 2004; Sevier & Kaiser, 2008). However, since mice lacking both Ero1 isoforms are viable, other alternative systems such as peroxiredoxin IV (PRDX4) exist and are also important in the redox cascade (Zito et al, 2010; Wang et al, 2012; Cao et al, 2014). The electrons for the oxidation of cysteines are accepted by O_2, which results in the formation of one molecule of H_2O_2 per disulfide bond formed. The generation of these reactive oxygen species (ROS) would be harmful to the cell and the discovery that H_2O_2 can be re-used to oxidize PRDX4 or PDIs directly converting H_2O_2 to H_2O provided an explanation of how the cell neutralizes its load of ROS (Tavender & Bulleid, 2010).

In addition to the mitochondria, the ER is one of the major Ca^{2+} storage sites in the cell and as such plays an important role in Ca^{2+}-homeostasis and signaling (Meldolesi & Pozzan, 1998). Many ER resident proteins possess high- and low-affinity Ca^{2+}-binding sites and at the same time secretory proteins acquire their conformation in the presence of higher Ca^{2+} concentrations. Tight control of the Ca^{2+} concentration in the ER is provided by two main Ca^{2+} efflux channels, inositol1,4,5-triphosphate receptors (IP3Rs) and ryanodine receptors (RyRs) as well as influx channels called sarcoplasmic reticulum Ca^{2+}-ATPases (SERCAs) (Meldolesi & Pozzan, 1998; Brini et al, 2012; Bublitz et al, 2013). Several interactions between factors involved in the redox system and Ca^{2+}-homeostasis indicate that the Ca^{2+} flux into the ER is linked to its lumenal redox condition (Araki & Nagata, 2011). These include the inhibition of the IP3R type 1 (IP3R1) receptor by the PDI family member ERp44, or the regulation of SERCA2b-dependent Ca^{2+}-oscillations by another PDI member, ERp57, thereby providing an antiapoptotic function during cellular stress responses. On the other hand, a proapoptotic response is promoted by Ero1α *via* an indirect activation of IP3R1 and a concomitant Ca^{2+}-release into the cytosol.

To maintain its fine-tuned environment, the ER can also control its size and protein content by initiating a response known as the Unfolded Protein Response (UPR). The UPR is controlled by three transmembrane sensors and transducers residing in the ER membrane, inositol-requiring enzyme 1 (Ire1), RNA-dependent protein kinase-like ER kinase (PERK) and activating transcription factor 6 (ATF6). In case of the accumulation of unfolded proteins or a high demand in protein folding capacity, the UPR sensors initiate the UPR upon which the ER is expanded, chaperone levels are increased and protein translation is transiently inhibited to restore homeostasis (Mori, 2009; Walter & Ron, 2011; Gardner et al, 2013). Taken together, the ER harbors a powerful protein folding machinery that orchestrates protein folding while at the same time is able to respond to changes in demand to maintain an optimal ER folding environment.

4.1.3 ER Quality control (ERQC), ER associated degradation (ERAD) and COPII-mediated export from the ER

Although many proteins originating in the ER are ultimately released into the extracellular space, most of them acquire their native structure in the ER and thus tight

quality control measures have evolved to monitor the success of their maturation, a process known as ER quality control (ERQC). A failure in ERQC in the best case would result in a protein that is not functional outside the cell, however in the worst case this protein could either possess inappropriate and sometimes dangerous functions outside the cell, e.g. incompletely assembled antibodies that possess effector functions but that do not bind the appropriate antigen (Feige et al, 2010), or cause toxic aggregates, which would not be readily resolved once outside the cell, and e.g. form amyloids (Feige et al, 2008; Placido et al, 2014). The nature of the ERQC lies in the distinction between unproductive and productive protein folding. In the first scenario proteins are disposed of in a process known as ER-associated degradation (ERAD) and in the latter proteins are released to traverse further along the secretory pathway in COPII-coated vesicles (Figure 3).

ERQC: The lectin and Hsp70 Protein Quality Control systems in the ER

Two major chaperone systems serve to triage proteins between secretion and degradation fates during ERQC: the lectin chaperones CNX and CRT (Ellgaard et al, 1999; Helenius & Aebi, 2004; Olzmann et al, 2013) and the Hsp70 chaperone system centered around BiP (Hendershot, 1990; Ni & Lee, 2007; Dudek et al, 2009; Otero et al, 2010) (Figure 3).

A. Calnexin/Calreticulin ER Quality Control

Proteins enter the CNX/CRT cycle as the co-translationally attached N-linked oligosaccharide composed of $Glc_3Man_9GlcNAc_2$ (Glc: glucose, Man: mannose, GlcNAc: N-acetylglucosamine) on nascent proteins is acted on by glycosidases I and II (GlcI and II). GlcI and II remove the two outermost glucose residues and thereby create the motif $Glc_1Man_9GlcNAc_2$ that is in turn recognized by CNX/CRT (Hebert et al, 1995a; Trombetta & Helenius, 1998; Hebert et al, 2005) (Figure 3*A*). The oxidoreductase ERp57, which is associated with CNX/CRT, can facilitate the formation and isomerization of disulfide bonds to aid in the maturation of the substrate protein (Oliver et al, 1997). Due to its proximity to the Sec61 translocon, the transmembrane protein CNX interacts with substrates cotranslationally, in contrast to its soluble counterpart CRT, which binds to its clients posttranslationally (Hebert et al, 1995b; Daniels et al, 2003).

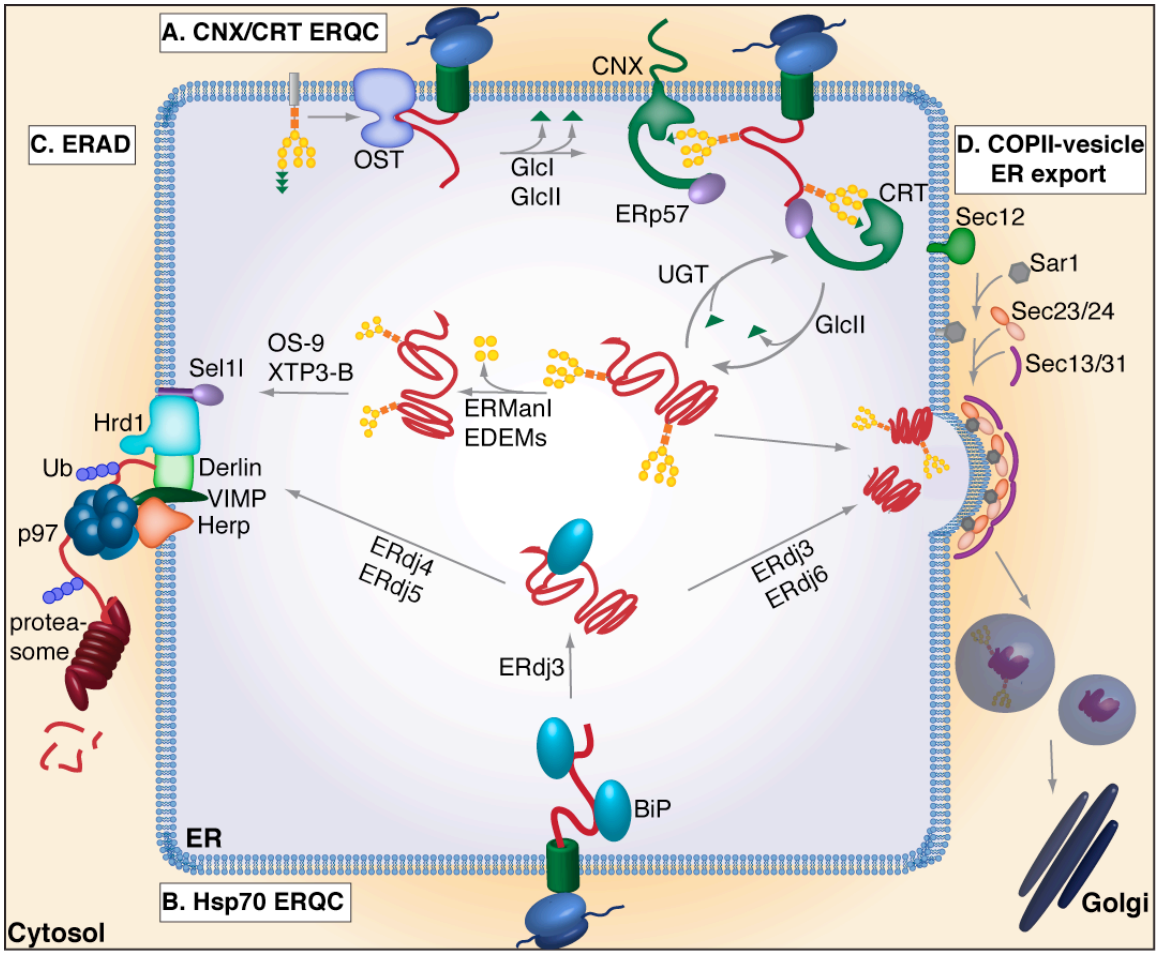

Figure 3. Protein folding environment of the mammalian ER.
Two major quality control systems reside in the ER: the lectins CNX/CRT (A) and the Hsp70 system centered around BiP (B). Their functions are aided by further co-factors whose major members are depicted here. The ERQC system decides whether a protein meets the qualitative standards and can be secreted *via* COPII-coated vesicles (D). In case a protein fails to fold it is degraded in a process called ERAD (C). The $Glc_3Man_9GlcNAc_2$ oligosaccharide is depicted *via* following color code: green triangle (Glc), yellow circle (Man), orange square (GlcNAc).

Once GlcII removes the third and last glucose, the protein exits the CNX/CRT cycle and can move on along the secretory pathway. However, if not yet completely folded, the enzyme UDP-Glc glycoprotein glycosyltransferase (UGT) recognizes both unfolded regions in proximity to N-linked glycan as well as the $Man_9GlcNAc_2$ glycan structure and transfers a single glucose back onto the oligosaccharide. This sends the protein back into the CNX/CRT cycle and provides it with another opportunity to fold. Mannosidases of the glycosylhydrolase 47 (GH47) family such as the ER Mannosidase I (ERManI), ER degradation-enhancing alpha-mannosidase like protein 1 (EDEM1), EDEM2 and EDEM3

compete with the de- and re-glycosylating GlcII and UGT and remove four mannose residues from the oligosaccharide generating a $Man_5GlcNAc_2$ oligosaccharide (Oda et al, 2003; Merulla et al, 2013). Thereby the ability of UGT to reglucosylate the unfolded protein is prevented, and instead the unfolded glycoprotein is now recognized by one of the two ERAD-associated lectins, osteosarcoma amplified 9 (OS-9) and XTP3 transactivated protein B (XTP3-B). The competition between mannosidases and UGT defines a time window for a protein to fold and prevents futile CNX/CRT cycling. OS9 and XTP3-B possess mannose 6-phosphate receptor homology (MRH) domains, two of which are present in XTP3-B and one in OS9, which play a role in the recognition of the modified, mannose-exposed glycan structure. The soluble mannosidases in the ER lumen connect the substrate to the ER resident, membrane integral ERAD machinery, which retrotranslocates proteins from the ER into the cytosol for proteasomal degradation. The transmembrane protein suppressor/enhancer of Lin-12-like (Sel1L) acts as a scaffold for the mannosidases and the E3 ligase hydroxymethylglutaryl reductase degradation protein 1 (Hrd1), which possesses a cytoplasmic E3 RING finger ubiquitin ligase domain (Christianson et al, 2008; Hampton & Sommer, 2012; Merulla et al, 2013). Mutations in the MRH domains of OS9 and XTP3-B interfere with their binding to Sel1L (Christianson et al, 2008), while both can still recognize aberrant non-glycosylated and glycosylated proteins despite mutated MRH-domains (Araki & Nagata, 2011). This suggests that the MRH-domains of OS9 and XTP3-B can also play a role in their interaction with Sel1L (Christianson et al, 2008) besides the ones with ERManI/EDEM1-3 trimmed oligosaccharides on ERAD substrates (Olzmann et al, 2013).

B. Hsp70 ER Quality Control

Since not all proteins synthesized in the ER are glycosylated, a second major ERQC system exists in the ER with BiP as its central player (Figure 3*B*). Unlike the lectin chaperone system, BiP recognizes only exposed hydrophobic stretches, a feature common to all incompletely folded soluble proteins, as these are ultimately buried within the native protein structure. For this reason BiP fulfills global functions in the ER ranging from translocation of nascent proteins from the cytosol into the ER (Figure 1) *via* nascent protein folding to ERAD and others (Figure 3).

The best-characterized example for BiP mediated ERQC is its role during the assembly of antibodies, which in their simplest form are composed of two heavy (HCs)

and two light chains (LCs). For a long time it was appreciated that plasma cells could secrete free LCs and fully assembled IgG molecules, while incompletely assembled HCs were retained in the cell (Morrison & Scharff, 1975; Mains & Sibley, 1982; Bole et al, 1986). Based on earlier studies from individuals with Heavy Chain Deposition Disease, the C_H1 domain in the HC, its first constant domain, was identified to be responsible for the HC retention in the cell (Seligmann et al, 1979). In fact, BiP was discovered because it was bound to unassembled and incompletely assembled Ig intermediates (Haas & Wabl, 1983; Bole et al, 1986). *In vivo* studies demonstrated the reason why HCs were not secreted on their own: BiP was bound to the unfolded C_H1 domain (Hendershot et al, 1987), which could only fold when LC assembled and BiP was released (Lee et al, 1999). BiP is retained in the ER due to the presence of a KDEL ER retention sequence at its C-terminus, which is captured by the KDEL receptor, in case BiP leaves the ER, and transports it back (Munro & Pelham, 1987; Dean & Pelham, 1990; Lewis & Pelham, 1990). Thus, as long as the C_H1 domain remains unfolded and associated with BiP, it is retained in the ER.

In vitro studies provided the molecular mechanism for this process: LC assembly induces a folding nucleus in the C_H1 domain and a concomitant BiP release, and the folding process of C_H1 is further accelerated by cyclophilin B (CypB), a PPIase, which is important for the isomerization of at least one critical proline residue within C_H1 (Feige et al, 2009). Together these studies unveiled the elegant ERQC mechanism of antibody assembly that allows the secretion of only fully assembled antibody molecules. However, not only antibodies utilize this ERQC mechanism, as early on folding intermediates of other proteins were found to be associated with BiP as well (Ng et al, 1989; Machamer et al, 1990; Ng et al, 1992). This in turn also means that the ERQC machinery has not only evolved diverse chaperone members for efficient protein folding, but proteins have also developed protein folding mechanisms that utilize these chaperones to ensure only properly matured proteins are transported in e.g. the secretion of fully assembled molecules. Despite the elucidation of the different ERQC networks, one central and intriguing question still remains: how does the ERQC system distinguish between terminally misfolded proteins and those that have not yet had the opportunity to fold? Whether the answer lies in the motifs encoded in the amino acid sequence within proteins themselves, protein folding pathways, members of the ERQC system or a combination of all, remains to be determined.

A network of ER-localized, DnaJ-related co-chaperones, the ERdjs, of which seven members have been identified so far, plays a decisive role in the fate of BiP client proteins (Otero et al, 2010). Two ERdj members, ERdj4 and ERdj5, were reported to direct BiP substrates towards the ERAD pathway (Shen et al, 2002; Dong et al, 2008; Ushioda et al, 2008; Hagiwara et al, 2011; Lai et al, 2012; Oka et al, 2013). The connection of ERdj4 to ERAD is based on the observation that its over-expression decreases the half-life of mutant surfactant protein C, whereas, when ERdj4 levels are reduced, the mutant protein is stabilized (Dong et al, 2008). Further support came from the recently discovered association of ERdj4 with Derlin-1, a transmembrane component of the ERAD machinery (Lai et al, 2012). ERdj5 belongs to the ERdj family as well as to the PDI family due to its N-terminal J-domain and its four thioredoxin-like domains at the C-terminus (Ushioda et al, 2008; Araki & Nagata, 2011). As the most reducing ER PDI family member ERdj5 reduces disulfide bonds of ERAD substrates, so that these can be transported back into the cytosol through a retrotranslocation channel, but in other cases it can isomerize non-native disulfide bonds required for final folding of proteins like LDLR (Oka et al, 2013). ERdj5 interacts not only with BiP, but also with components of the lectin ERQC system such as EDEM1 (Araki & Nagata, 2011). In contrast to ERdj4 and 5, other ERdjs, such as the ER lumenal ERdj3 and 6 assist BiP in its pro-folding function (Shen & Hendershot, 2005; Rutkowski et al, 2007; Jin et al, 2008; Petrova et al, 2008; Jin et al, 2009).

Exit from the ER: into the cytosol via the ERAD machinery or to the Golgi via COPII-coated vesicles

The function of the ERQC system is to decide whether a protein has folded correctly and can be allowed to exit the ER in membrane vesicles for further transport along the secretory pathway (Figure 3*D*) or has failed this process and should be retrotranslocated back into the cytosol to be degraded by the 26S proteasome (Figure 3*C*).

C. ER-associated Degradation (ERAD)

The retrotranslocation of an ERAD substrate protein involves the ER lumenal ERQC system, a channel for its retrotranslocation from the ER lumen or extraction from the ER

membrane and a protein degradation machinery in the cytosol (Figure 3*C*). Hence, a large multicomponent protein complex must be assembled on and within the ER membrane to provide all of these functions. On the ER lumenal site binding of the lumenal ERQC members to the lumenal domain of Sel1L initiates the retrotranslocation of an ERAD substrate, whereas the transmembrane domain of Sel1L ties it to other membrane integrated components of the ERAD translocation machinery. The central role of Sel1L in this process was further demonstrated by a recent study showing that Sel1L is indispensable for mammalian ERAD both in animals and in cultured mouse embryonic fibroblast cells (Sun et al, 2014). The transmembrane region of Sel1L is connected to the E3 ligase Hrd1 and it also nucleates the formation of a complex composed of further membrane ERAD components, such as Der1-like proteins, three of which are present in mammalian cells (Derlin 1-3), valosin-containing protein (VCP)/p97-interacting membrane protein (VIMP) and Herp (Hampton & Sommer, 2012; Bagola et al, 2013; Metzger et al, 2013; Olzmann et al, 2013). The protein channel, which ERAD substrates use to cross the ER membrane, has not been clearly identified yet and several proteins have been proposed to fulfill this function. The list of candidates include the Sec61 channel, Hrd1, which might be involved in retrotranslocation of soluble as well as membrane proteins, (Brodsky & Skach, 2011) and Derlin 1-3, after they had been identified as members of the rhomboid pseudoprotease family possessing a compact helical bundle of six TM domains (Greenblatt et al, 2011; Olzmann et al, 2013; Mehnert et al, 2014). It also remains unclear whether a single channel plays a central role in retrotranslocation of all proteins, similar to Sec61 in ER import, or whether the channels are rather substrate specific and are made up of different subsets of components as has been observed for the three Derlin members (Lilley & Ploegh, 2004; Ye et al, 2004).

Once the substrate has been threaded into the retrotranslocon, energy is required to fully extract it from the ER and target it for recognition by the proteasomal degradation system. This entails at least a two-step process. The first involves the attachment of ubiquitin (Ub) to amino acids exposed to the cytosol *via* either one of two broad-spectrum E3 ligases (Hrd1 and gp78) (Liang et al, 2003; Nadav et al, 2003) or several more substrate-specific E3-ubiquitin ligases (Hassink et al, 2005; Younger et al, 2006; Stagg et al, 2009). In fact it appears that multiple types of amino acids including lysines, serines and threonines can be ubiquitinated on ERAD substrates (Ishikura et al, 2010; Shimizu et al, 2010), increasing the chance that an exposed amino acid can be readily modified. The adaptor protein VIMP connects the homohexameric VCP/p97

complex, an AAA-ATPase to the ER membrane on the cytosolic site (Lilley & Ploegh, 2005). P97 recognizes ubiquitin chains on emerging ERAD substrates and actively pulls them out of the ER into the cytosol in an ATP-dependent manner. The membrane protein Herp in turn is in complex with p97 as well as ubiquitinated substrates and 26S proteasome (Okuda-Shimizu & Hendershot, 2007). Once the polyubiquitin-destruction tag is recognized by the 26S proteasome, the polypeptide chain of the ERAD substrate protein is cleaved in the cavity of the proteasome (Gallastegui & Groll, 2010).

D. COPII-mediated vesicle export from the ER

Once a protein has passed the quality threshold set by the ERQC it is released from the chaperone machinery to travel further along the secretory pathway be it the Golgi complex, endosome, lysosome, peroxisome, the cell surface or to remain in the ER and function as a resident ER protein (Figure 3*D*). The export from the ER is mainly mediated *via* Coat Protein complex II (COPII)-coated vesicles (Barlowe et al, 1994; Sudhof, 1995; Younger et al, 2006; Zanetti et al, 2012). Five proteins constitute the COPII machinery, driving vesicle formation from the ER membrane: Sar1, Sec23, Sec24, Sec13 and Sec31 (Barlowe et al, 1994; D'Arcangelo et al, 2013). All of these proteins assemble on the cytosolic face of the ER membrane and drive the budding of a vesicle from the ER membrane, the concentration of cargo in nascent buds and the vesicle cleavage from the ER (Gillon et al, 2012; Zanetti et al, 2012; D'Arcangelo et al, 2013). The vesicle formation is a step-by-step process and is initiated by the recruitment of the GTPase Sar1 *via* its ER membrane tethered guanine nucleotide exchange factor (GEF) Sec12. ER membrane resident Sar1 in turn interacts with the heterodimeric Sec23/24 complex, in which Sec23 binds to Sar1 and Sec24 is a cargo-binding adaptor (D'Arcangelo et al, 2013). Association of Sec23 with Sec31 of the Sec13/31 heterodimer starts the formation of a cage-like outer coat layer by the polymerization of Sec13/31. As more COPII components bind to this complex an inner and an outer coat layer composed of Sec23/24 and Sec13/31, respectively, is formed. The vesicle release from the ER membrane depends on GTP hydrolysis by Sar1, whose GTPase activity is activated by Sec23 and further promoted by Sec31 (Zanetti et al, 2012; D'Arcangelo et al, 2013). Once a complete vesicle is formed, the COPII-coat components are released and recycled before the vesicle can fuse with the next compartment of the secretory pathway, the ER-Golgi intermediate compartment (ERGIC) (Brandizzi & Barlowe, 2013).

4.2 Chaperones: the Protein Folding Conductors of the Cell

A subset of ER chaperones are organized in large chaperone complexes which enables them to fulfill their functions synergistically (Meunier et al, 2002). Considering that a given protein may simultaneously require chaperones to shield hydrophobic unfolded regions, as well as enzymes to catalyze the formation of disulfide bonds or to isomerize prolines (Figure 2), this adds another layer to protein folding efficiency of the ER. Resident ER proteins that facilitate the maturation of nascent substrate proteins in the ER can be divided into the following families based on their main function: (i) the PDI family of oxidoreductases; (ii) the PPI family of peptidyl-prolyl *cis/trans* isomerases; (iii) the lectin family of glycan binding proteins; (iv) glucose-regulated proteins (Grps), a chaperone family specific for hydrophobic patches on unfolded regions on protein substrates; and (v) the various co-chaperones that regulate the activities of the chaperones (Braakman & Bulleid, 2011). In addition there are a number of ER folding factors, which are either client specific (Braakman & Bulleid, 2011) or are up-regulated under certain differentiation related conditions (Shimizu et al, 2009; van Anken et al, 2009).

4.2.1 The Protein Disulfide Isomerase (PDI) and Peptidyl-Prolyl Isomerase (PPI) Families

The name of the PDI family is based on its founding and best-characterized member, protein disulfide isomerase (PDI). To date over 20 family members have been identified in the mammalian ER (Appenzeller-Herzog & Ellgaard, 2008; Bulleid & Ellgaard, 2011; Braakman & Hebert, 2013). The unifying feature of the PDIs is the presence of a thioredoxin-like (Trx) domain possessing the characteristic catalytic amino acid motif CXXC. PDI possesses two enzymatically active (a and a´) and two inactive (b and b´) Trx domains. While the first two domains perform the actual redox function, the more C-terminal b´ domain has the ability to bind hydrophobic peptides (Appenzeller-Herzog & Ellgaard, 2008; Braakman & Hebert, 2013). Accordingly, PDI fulfills a dual function as an oxidoreductase and a chaperone. The composition of the amino acids located between the active site cysteines influences the redox potential of the PDI protein, allowing some members to preferentially catalyze the formation of disulfide bonds. In contrast, the redox potential of others allows them to reduce non-native bonds in proteins that are in

the process of folding or to aid in unfolding regions of proteins that fail quality control so that they can be retrotranslocated for degradation (Feige and Hendershot, 2011). Other members, such as ERp57, also have a peptide binding b´ domain, but in this case instead of the b´ domain serving to bind unfolded proteins, ERp57 uses this domain to bind to CNX or CRT. Studies with different members of the PDI family suggest some redundancy in their oxidoreductase function and a rather broad substrate range (Rutkevich et al, 2010). Some specificity comes with additional domains, as is the case for the J-domain in ERdj5, which is important for its interaction with BiP and thus BiP substrates, or the b´ domain in the ERp57-CNX/CRT association, making ERp57 more specialized for glycoproteins (Feige & Hendershot, 2011). Nevertheless, many members of the PDI family await detailed characterization of their function in the ER chaperone network and one of the central questions is why so many PDI family members are present.

The PPIase family is a poorly characterized protein family *in vivo*, which includes the cyclophilins, such as the cyclosporine A-binding cyclophilin B (CypB), and FK506-binding proteins (FKBPs) (Braakman & Bulleid, 2011; Gidalevitz et al, 2013; Boudko et al, 2014). Most insights gained so far demonstrate either association with an unfolded substrate or genetic necessity in yeast (Gidalevitz et al, 2013). The requirement for PPIases during protein folding is underscored by the fact that *cis-trans* proline isomerization can take seconds to minutes at room temperature *in vitro* and is accelerated significantly by the addition of PPIases (Feige et al, 2009; Feige & Buchner, 2014). For example, the murine C_H1 antibody domain has three prolines that are in the *cis*-state in the native protein. Their isomerization represents a rate-limiting step in the *in vitro* folding of this domain, which is accelerated by the addition of CypB. Isomerization of one of these proline residues was found to be critical for the folding, assembly and secretion of antibodies from mammalian cells, arguing that CypB may also play an important role *in vivo* (Feige et al, 2009). In keeping with this possibility, CypB was found to be part of a large chaperone complex associated with unfolded Ig heavy chains (Meunier et al, 2002). PPIases also interact with six members of the PDI family as well as BiP, CNX, CRT and Grp94, the ER ortholog of the cytosolic chaperone Hsp90 (Sorger & Pelham, 1987; Gidalevitz et al, 2013), and play a role in the general protein quality control (Feige & Buchner, 2014). Nevertheless, the cellular functions of most PPIase family members remain to be elucidated.

4.2.2 Lectins, the Glycan Binding Protein Family in the ER

A number of carbohydrate-binding proteins, the lectins, are present in the ER. These play a role in folding, ERQC and ERAD of glycoproteins (Figure 3*A* and *C*). ER resident lectins include: CNX and CRT, GH47 family members ERManI and EDEM 1-3, Malectin, a membrane protein that associates with misfolded glycoproteins (Qin et al, 2012), GlsI, UGT and MRH domain containing lectins, GlcII, OS-9 and XTP3-B (Pearse & Hebert, 2010; Castonguay et al, 2011). Recently a new mechanism of quality control in yeast was described, the O-mannosylation pathway (Xu et al, 2013). In this pathway a mannose residue is added to a serine or threonine residue and terminates the folding of nonglycosylated proteins by a similar triage mechanism as has been established for N-linked glycans. However, it remains to be shown whether mammalian orthologs of the yeast O-mannosyltransferases POMT1/2 fulfill a similar function in the ER and whether other mannosidase-recognizing lectins, such as the MRH-domain containing OS-9 and XTP3-B, might be involved in that process (Kleizen & Braakman, 2013).

4.2.3 Glucose-regulated Proteins in the ER: Grp78, Grp94 and Grp170

The name of the glucose-regulated proteins (Grps) is based on their induction by glucose starvation in cells, which led to their discovery in the mid-1970s, in combination with their molecular weight (Olden et al, 1978; Lee, 2014). All of the Grp proteins belong to the heat shock protein (Hsp) family and are closely related to several orthologs in the cytosol. Grp78, which was soon identified to be identical to BiP (Hendershot et al, 1988), and Grp170 belong to the Hsp70 superfamily and both will be described in dedicated sections to follow.

In contrast to its cytosolic Hsp90 orthologs, which have been characterized in detail in the past decades (Rohl et al, 2013), the precise function of the ER resident Grp94 is still poorly understood. Interestingly, in yeast and mammalian cells two Hsp90 isoforms exist in the cytosol (Rohl et al, 2013), while only a single family member, Grp94, is present in the mammalian ER (Marzec et al, 2012), and no Hsp90 protein is found in the yeast ER. Like other Hsp90s, Grp94 possesses three domains: an ATP-binding N-terminal domain (NTD), a middle domain (MD), and a C-terminal domain (CTD), which supports homodimerization of Grp94. ATP binding in the NTD influences the opening

and closing of Grp94 (Dollins et al, 2007). Grp94 was found to form complexes containing BiP and Grp170 (Meunier et al, 2002), as well as to bind OS-9 independent of this complex (Christianson et al, 2008). Gene knock-out studies revealed that Grp94 is an essential protein, with deficient mice exhibiting lethality at days E6.5-7.5 during embryonic development (Wanderling et al, 2007). However, in contrast to cytosolic Hsp90 for which several hundred substrates, including primarily kinases and transcription factors, have been identified (Rohl et al, 2013), only a few *bona fide* substrates for Grp94 have been described so far, despite the fact that Grp94 is one of the most abundant proteins of the ER (Marzec et al, 2012). The list of Grp94 substrates described so far includes, Toll-like receptors, integrins, thyroglobulin and insulin-like growth factors-I and II. However, it remains unclear which sequences or structural motives are recognized by Grp94 and whether it also binds to metastable, near-native states as was shown for Hsp90. The interactions of Grp94 with the ER Hsp70, BiP, as well as with lectin components of the ERAD machinery, support an important, but still poorly understood role for Grp94 in the ERQC process.

4.3 BiP, the conventional Hsp70 Member of the ER Hsp70 Superfamily

The Hsp70 chaperone BiP, the first ER chaperone to be identified (Haas & Wabl, 1983; Bole et al, 1986; Hendershot et al, 1987), is the major molecule of the other main chaperone system present in the ER besides the lectin chaperones CNX and CRT (Figure 3*B*). BiP belongs to the class of conventional Hsp70s, of which several members are also present in the cytosol. In the past decades the importance of BiP became more and more evident as its functions were dissected. These range from a role in protein translocation, protein folding and assembly, ERAD, regulation of the ER localized UPR transducers, and contribution to the ER calcium stores that are essential for many signal transduction pathways. Like all Hsp70 family members, the ability of BiP to bind and release unfolded protein substrates is tightly regulated by a cycle of ATP binding, hydrolysis, and nucleotide exchange (Figure 4). And as with all other family members, a number of co-factors exist to regulate this cycle. However, only in the past decade have nucleotide exchange factors (NEFs) for BiP been identified, Grp170 and Sil1. Data obtained for these NEFs have shed light not only on the mechanism of BiP assisted

protein folding in the ER, but also on Hsp70 family members that reside throughout the cell.

4.3.1 A resting state of BiP

Changes in the extracellular environment or different developmental processes can result in large variations in the load of unfolded or misfolded proteins in the ER. BiP as one of the major ER chaperones must therefore be readily available in times of need. Differentially modified and assembled forms of BiP have been identified in the ER and are suggested to help cope with altering cellular conditions. In the absence of stress, BiP has been shown to be a major ADP-ribosylated protein in mammalian cells (Carlsson & Lazarides, 1983; Hendershot et al, 1988) and is thought to also be phosphorylated (Welch et al, 1983; Hendershot et al, 1988) (Figure 4*A*).

However, when the load of unfolded proteins increases in the ER, the amount of modified BiP decreases (Carlsson & Lazarides, 1983; Hendershot et al, 1988; Leustek et al, 1991; Chambers et al, 2012). Accordingly, it has been suggested that the ADP-ribosylated or phosphorylated forms of BiP represent a pool of inactive molecules which can quickly be reactivated when needed (Carlsson & Lazarides, 1983; Leno & Ledford, 1989; Freiden et al, 1992). In agreement with this idea, only monomeric unmodified BiP is bound to substrates (Hendershot et al, 1988; Freiden et al, 1992). Despite this and other (Carlino et al, 1992; Satoh et al, 1993; Gaut, 1997) circumstantial evidence suggesting that BiP's functions *in vivo* might be regulated by post-translational modifications, it remains unclear how modifications of BiP are physiologically regulated. Although neither the kinase nor ADP-ribosyltransferase or the enzymes that would remove these modifications have been identified, the ADP-ribosylation site of BiP could be mapped to two arginine residues within its SBD (Chambers et al, 2012). As mutation of these two residues abolished all post-translational modifications of BiP, the phosphorylation signal reported in earlier studies has been suggested to be due to the phosphates present in the ADP-ribose moiety (Chambers et al., 2012). Substitution of these two arginine residues with bulky amino acids that mimicked ADP-ribosylation interfered with substrate binding and thus could explain earlier observations showing that only unmodified BiP is bound to substrates (Freiden et al, 1992). In other studies, recombinant BiP was found to form oligomers that could be dissociated with substrates and ATP (Carlino et al, 1992; Blond-Elguindi et al, 1993b), suggesting that BiP oligomers

might also represent an inactive pool that could readily be reshuffled into its functional ATPase cycle in the ER when unfolded proteins began to accumulate (Figure 4).

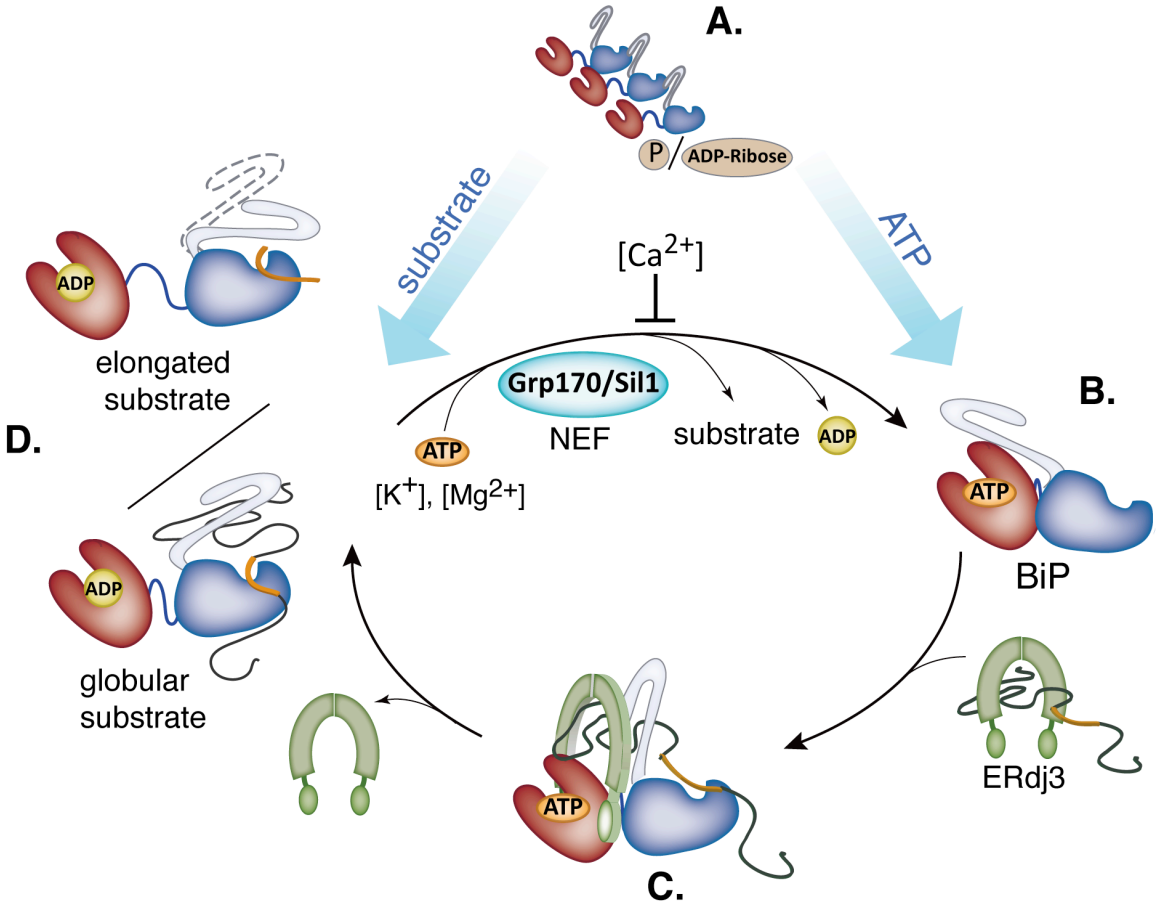

Figure 4. The ATPase cycle of the Hsp70 chaperone BiP and its regulation (see text for details).

4.3.2 The ATP-bound state of BiP

Like all Hsp70's BiP is an ATP-dependent molecular chaperone (Munro & Pelham, 1986; Kassenbrock & Kelly, 1989; Dorner et al, 1990; Clairmont et al, 1992; Hendershot et al, 1995). A large variety of BiP mutants, recent single molecule studies and a plethora of data on other Hsp70 family members now allow us to describe the ATPase cycle of BiP in quite some detail (Figure 4). Once ATP binds, BiP enters a state of low substrate affinity with high on and off rates for substrate binding (Flynn et al, 1989; Kassenbrock &

Kelly, 1989). Structural insights into the ATP-bound state of Hsp70s were recently provided by two crystal structures of *E. coli* Hsp70 DnaK (Kityk et al, 2012; Qi et al, 2013). To stabilize the transient ATP-bound state of Hsp70 in both studies the mutation T199A was introduced into the nucleotide binding pocket thereby significantly reducing the intrinsic ATPase activity, but maintaining the allosteric coupling between the nucleotide binding domain (NBD) and substrate binding domain (SBD). The ATP-bound state of Hsp70 was further stabilized by two disulfide bonds between the NBD and the lid domain (Kityk et al, 2012) or replacement of the peptide-binding loop in the SBD by a shorter loop (Qi et al, 2013). Both structures demonstrate that the α-helical lid domain docks on the NBD, thereby completely exposing the SBD for potential substrates to bind (Figure 4*B*). These conformational changes are induced by a complex allosteric mechanism that transmits information on the nucleotide bound state from the NBD to the SBD and on the substrate occupancy state from the SBD back to the NBD (Zuiderweg et al, 2012). This reciprocal information transfer is best understood for the *E. coli* Hsp70 DnaK (Mayer & Bukau, 2005; Bukau et al, 2006; Swain & Gierasch, 2006; Mayer, 2010). ATP binding leads to a closure of the otherwise quite dynamic NBD around the nucleotide (Flaherty et al, 1990; Zhang & Zuiderweg, 2004; Rist et al, 2006; Woo et al, 2009; Wisniewska et al, 2010; Macias et al, 2011). The presence of ATP in the NBD is then signaled to the SBD where it seems to open the substrate binding cavity by increasing SBD flexibility and lid opening (Rist et al, 2006; Swain et al, 2007).

It is still not clear, exactly how the nucleotide binding state is transmitted from the NBD to the SBD. Different signal transmission pathways seem to exist between these two domains in the Hsp70 molecule and details of these pathways seem to vary for different Hsp70 family members (Mayer & Bukau, 2005; Bukau et al, 2006; Sousa & Lafer, 2006; Swain & Gierasch, 2006; Mayer, 2010; Young, 2010). However, three different themes have emerged for how interdomain communication occurs. In DnaK, the bound nucleotide is sensed by residues in the NBD that lie in close proximity to the bound ATP and this information is thought to be transmitted *via* a Pro residue, which likely undergoes *cis*/*trans* isomerization, to an interface at the surface of the NBD that interacts with the SBD (Vogel et al, 2006a). Interestingly, all residues of the proposed sensor-relay system are conserved in BiP (Wisniewska et al, 2010; Macias et al, 2011). However, the impact of mutations of the corresponding residues has not been investigated in BiP. In addition to this putative Pro-focused sensor-relay system, Thr37 in the NBD plays a particularly important role as a nucleotide sensor in BiP (Wei et al,

1995), likely due to a direct interaction of its hydroxyl group with the γ-phosphate oxygen of the bound ATP (Sousa & McKay, 1998). How Thr37 signals to the surface of the NBD is not currently known, but it has been shown that once ATP binding is transmitted to the NBD surface, it influences the interaction of positively charged residues on the NBD with negatively charged residues on the opposing surface of the SBD (Vogel et al, 2006a; Awad et al, 2008). The third known relay that signals nucleotide binding from the NBD to the SBD is mediated by the conserved hydrophobic linker, which connects the two domains. Upon ATP binding, the linker binds to a cleft in the NBD, which is important in transmitting the nucleotide state of the NBD to the SBD and increases ATP hydrolysis of the NBD once bound to the cleft (Rist et al, 2006; Vogel et al, 2006b; Swain et al, 2007; Zhuravleva & Gierasch, 2011). Although some details of the NBD-SBD interface and regulation by further factors vary for different Hsp70s (Jiang et al, 2005; Swain et al, 2007; Ahmad et al, 2011), the combination of these conserved allosteric signaling pathways, which are most likely interconnected, results in a compaction of the NBD and SBD domains onto each other and an opening of the SBD lid upon ATP binding (Mapa et al, 2010; Mayer, 2010; Young, 2010; Marcinowski et al, 2011; Kityk et al, 2012; Qi et al, 2013). Of note, ATP hydrolysis itself is not necessary for the conformational changes leading to the opening of the SBD but binding of ATP in combination with potassium ions is sufficient for these changes (Palleros et al, 1993; Wei et al, 1995; Wilbanks & McKay, 1995) and further insights obtained from the structures of an ATP-bound Hsp70 (Kityk et al, 2012; Qi et al, 2013) now provide an explanation for this observation.

4.3.3 ATP hydrolysis and the ADP state

BiP has a very low intrinsic Mg^{2+}-dependent ATPase activity (Kassenbrock & Kelly, 1989; Wei & Hendershot, 1995; Mayer et al, 2003). Once ATP is hydrolyzed, the SBD-NBD interface is broken and both domains behave more independently of each other. The degree of domain separation in the ADP state seems to vary for different Hsp70's. In DnaK the SBD and NBD act independently when in the ADP state with only transient contacts between these two domains (Swain et al, 2007; Chang et al, 2008; Bertelsen et al, 2009; Mapa et al, 2010). Conversely, in the case of a bovine Hsc70, extensive contacts between the SBD and the NBD are detected even in the ADP state (Jiang et al, 2005), whereas single molecule measurements on mitochondrial Hsp70 (Mapa et al,

2010) and BiP (Marcinowski et al, 2011) argue for a heterogeneous ensemble of conformations. Taken together, in the ADP state, the NBD and SBD seem to be in a dynamic distance distribution with a general trend towards domain separation. Finally, not only the distance between the SBD and NBD seem to vary in the ADP bound state of Hsp70s, but in the case of DnaK, mitochondrial Hsp70, and BiP the lid also appears to populate open and closed conformations with a general trend towards more closed conformations (Mapa et al, 2010; Marcinowski et al, 2011; Schlecht et al, 2011). Thus, in contrast to the ATP state characterized by close NBD-SBD contact and an open lid, the ADP state seems to be more heterogeneous within a single type of Hsp70 as well as more diverse between individual Hsp70s.

4.3.4 Substrates in the ATPase Cycle of BiP

The binding of substrates has a very interesting impact on the ATPase cycle of Hsp70 proteins. It has long been appreciated that binding of peptide substrates accelerates the ATP hydrolysis rate of BiP (Flynn et al, 1991; Wei et al, 1995) and other Hsp70s (Mayer & Bukau, 2005; Bukau et al, 2006; Sousa & Lafer, 2006; Swain & Gierasch, 2006; Mayer, 2010; Young, 2010). Most studies have been performed with small hydrophobic peptides that were thought to be good substrate mimics. In the case of BiP, doubt has been cast on the significance of these results, since although short peptides do stimulate BiP's ATPase activity, a completely unfolded BiP-binding protein did not (Mayer et al, 2003). Indeed, in the cell, the only known sources of small peptides are those that enter through TAP transporters to be delivered to MHC I molecules (Chapman & Williams, 2010) and signal peptides that may never fully enter the ER lumen, neither of which have been shown to interact with BiP. Thus, it is more likely that the substrates BiP encounters are elongated polypeptide chains that are in the process of translocation *via* the Sec61 channel, or proteins that can be unfolded, partially folded, misfolded or even aggregated. Recent work has shown that in the case of more globular substrates, in contrast to what has been observed for peptides, complete lid closing over the bound substrate does not necessarily occur and that this can result in direct interactions between the lid and the substrate, leading to even further extension of the conformational space accessible to the Hsp70's SBD (Marcinowski et al, 2011; Schlecht et al, 2011). Of note, when a peptide is used to bind to the ATP state of DnaK it shifts the protein structurally more towards the ADP state (Swain et al, 2007), whereas binding of

a larger polypeptide substrate to the ADP state of BiP induces a conformation that more closely resembles the ATP state in terms of NBD-SBD domain separation and lid opening (Marcinowski et al, 2011). Thus, substrate itself seems to shift the conformational state of the Hsp70's, playing an active role in the allosteric signaling mechanisms. As substrates apparently shift Hsp70s structurally away from the pure nucleotide-regulated states, they might facilitate transitions between them and thereby accelerate ATP hydrolysis.

In the cell, substrate transfer to Hsp70s is mediated by DnaJ-like co-chaperones that can further increase the ATPase rate of the Hsp70 molecule and thereby lock a substrate on the Hsp70 in its ADP state (Laufen et al, 1999; Matlack et al, 1999; Mayer & Bukau, 2005) (Figure 4*C* and *D*). Thus, J proteins can help define where in the ER substrates are bound by Hsp70s, how fast substrates are bound, and even in the selection of which substrates are bound (Kampinga & Craig, 2010). Indeed, some J proteins directly bind to substrates and deliver them to the Hsp70 molecule, whereas others simply stimulate ATP hydrolysis by the Hsp70 (Kampinga & Craig, 2010; Otero et al, 2010). Mechanistically J's might shift the Hsp70 molecule towards a pre-hydrolysis conformation, perhaps by destabilizing the aforementioned charged SBD-NBD-interface, thus facilitating ATP-hydrolysis or by either increasing interaction of the hydrophobic linker with the NBD or acting as a linker mimetic themselves thereby accelerating ATP hydrolysis (Jiang et al, 2005; Jiang et al, 2007; Awad et al, 2008). Conflicting models exist for the binding of J proteins and the detailed molecular mechanism of J action is still unclear (Jiang et al, 2005; Jiang et al, 2007; Swain et al, 2007; Ahmad et al, 2011) and may vary between different Hsp70s and their J co-factors. For BiP, it has also been shown that ERdj3, a major co-chaperone in *de novo* protein folding in the ER (Yu et al, 2000; Shen & Hendershot, 2005), opens up the lid of the SBD directly thereby facilitating binding of a substrate protein (Marcinowski et al, 2011). Conversely, ERdj3-induced lid opening induced release of small bound peptides, underscoring the difference between peptides and proteins as Hsp70 substrates in terms of Hsp70 behavior (Marcinowski et al, 2011). This shows that a J protein can directly alter the lid conformation of BiP to poise it for binding to more globular folding intermediates states, which are likely to be encountered in the lumen of the ER. It is possible that other ERdj proteins might behave differently to facilitate binding to extended polypeptides or to transfer proteins to the ER degradation machinery (Lai et al, 2012).

4.3.5 Re-entering the ATPase cycle

To enter a new round in the ATPase cycle and release bound substrate, BiP has to be refueled with ATP. The proper functioning of the BiP ATPase cycle is crucial for substrate maturation in the ER (Gaut & Hendershot, 1993; Hendershot et al, 1996) and the integrity of the entire organelle (Hendershot et al, 1995; Paton et al, 2006). It might thus come as no small surprise that the conditions of the ER are far from optimal for an efficient ATPase cycle of BiP. The optimal pH for BiP's ATPase activity is quite acidic, and at a more physiological ER pH of around seven, the intrinsic ATPase of BiP is particularly slow (Kassenbrock & Kelly, 1989). Even more striking, calcium, which is present at high concentrations in the ER, almost completely inhibits the ATPase activity of BiP *in vitro* (Kassenbrock & Kelly, 1989; Wei & Hendershot, 1995). Calcium has been shown to increase the affinity of BiP for ADP almost 1000-fold, thereby inhibiting nucleotide exchange and accordingly BiP´s ATPase activity (Lamb et al, 2006). In contrast to an *in vitro* setting, where hydrolysis is the rate-limiting step in the ATPase cycle of BiP (Mayer et al, 2003), nucleotide exchange is thus most likely the rate-limiting in the ER. Calcium might even poise BiP to enter an inactive phosphorylated state (Leustek et al, 1991). Indeed, substrates are not readily released from BiP *in vivo*, suggesting that BiP does not seem to continuously traverse through its ATPase cycle (Vanhove et al, 2001). Taken together, the setup of the ER, the need for substrate release and maybe even the reactivation of BiP from its resting state renders another class of accessory proteins for BiP completely indispensable: the nucleotide exchange factors (NEFs). NEFs exchange ADP against ATP and thereby decide how fast substrates are released from BiP, where release in the ER occurs and maybe even in which folding state substrates are released from BiP.

4.4 BiP´s NEF Sil1

4.4.1 The Identification of Nucleotide Exchange Factors for BiP

Although BiP's ATPase cycle would appear to be particularly dependent on co-factors to regulate its binding and release from substrates, until the late 1990s there were no resident ER proteins identified in any organism that had nucleotide exchange activity. Then three labs independently identified the first BiP exchange factor in three different organisms. First, in a synthetic lethal screen to identify genes that interacted with the SCR2-encoded 7S RNA component of SRP in the yeast *Yarrowia lipolytica*, Sls1p was identified (Boisrame et al, 1996; Boisrame et al, 1998). It was shown to be a lumenal ER protein that interacted with both Kar2p (yeast BiP) and Sec61, a component of the translocon. Sls1p is induced by both heat shock and ER stress, and deletion of *SLS1* resulted in dramatically decreased synthesis of secreted proteins, leading to the hypothesis that Sls1p played a role in the translocation of proteins into the ER lumen. It was not until 2000, that the same group isolated the homologue in *Saccharomyces cerevisiae*, which was previously categorized as a gene of unknown function, and demonstrated using recombinant proteins that Sls1p bound preferentially to the ADP bound form of yeast BiP. Sls1p stimulated BiP's ATPase activity in the presence of a DnaJ-like co-factor, Sec63p, by accelerating ADP release, thus qualifying it as the first identified NEF for yeast BiP (Kabani et al, 2000; Kabani et al, 2002a; Kabani et al, 2002b).

Meanwhile the ATPase domain of human BiP was used as bait in a 2-hybrid screen of a human liver cDNA library to identify proteins that might regulate its activity (Chung et al, 2002). This approach probably would not have succeeded except for a bit of serendipity. Although the bait vector was constructed with both a wild-type and mutant ATPase domains, the screen was conducted first with the mutant ATPase domain. The BiP associated protein, BAP, preferred to bind to a variety of BiP ATPase mutants in the mammalian ER, and the wild-type BiP ATPase domain failed to produce yeast colonies in the 2-hybrid assay when expressed with BAP, suggesting that BAP preferred a particular conformation or nucleotide bound state of BiP. Similar to studies with Sls1p (Kabani et al, 2000), BAP was found to interact preferentially with the ADP bound form of BiP and stimulate nucleotide release, thus driving the ATPase cycle forward (Chung et

al, 2002). From this point forward we will refer to the proteins of all three species as Sil1/Sil1p.

4.4.2 The Mechanism of Sil1 Exchange Activity

Sequence analyses revealed that yeast and mammalian Sil1 shared significant but low identity with cytosolic Fes1p and HspBP1, respectively (see Figure 5 for a Hsp70 NEF overview).

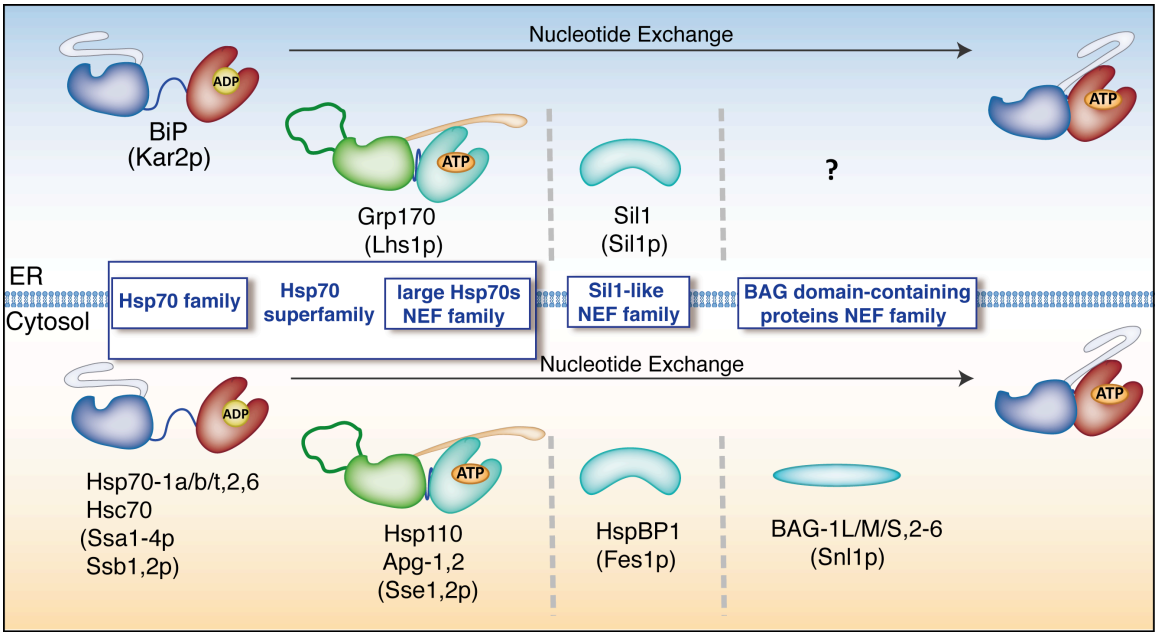

Figure 5. Overview over mammalian (*S.cerevisiae* in brackets) Hsp70 NEFs in the ER and the cytosol.

Data were beginning to accumulate at this time demonstrating that the cytosolic NEF proteins regulated the ATPase activity of the Hsp70s (Raynes & Guerriero, 1998; Kabani et al, 2002b), even though they bore no apparent structural relationship to the only previously identified Hsp70 nucleotide exchange factors, E. coli GrpE (Harrison et al, 1997) or eukaryotic cytosolic Bag domain proteins (Sondermann et al, 2001). Both GrpE and Bag-1, although structurally distinct themselves, bind to the cleft at the top surface of the Ib and IIb subdomains of the Hsp70 NBD and in a similar fashion “push” the IIb domain away from the Ib domain, thereby allowing nucleotide to be released (Bukau et al, 2006) (Figure 6). The structure of human HspBP1 could be solved only with domain II of Hsp70. It revealed that HspBP1 is composed of four armadillo-like repeats that wrap around subdomain IIb from the side and bind to it with higher apparent affinity than Bag-

1 (Shomura et al, 2005). When this structure was superimposed on the structure of the Hsc70 nucleotide-binding domain crystallized with ADP (Flaherty et al, 1990), it became clear that this orientation of HspBP1 would have severe steric conflicts with domain Ib in the intact nucleotide-binding domain. Thus, the data suggested that HspBP1 served not only to push the IIb subdomain away from Ib like GrpE and Bag-1 do, but was likely to additionally displace Ib, thus further destabilizing this domain and decreasing its affinity for nucleotide (Shomura et al, 2005). This implied mechanism of nucleotide release argued that unlike Bag-1, HspBP1 induced a major distortion of the Hsp70 ATPase domain.

More recently a structure of yeast Sil1p was solved with the complete nucleotide binding domain of Kar2p (Yan et al, 2011) (Figure 6*A*). Similar to HspBP1, Sil1p is composed of four armadillo motifs that wrap around the IIb lobe of the BiP ATPase domain. Importantly, this structure further revealed that Sil1 also makes contact with subdomain Ib causing lobe IIb, and to a lesser extent Ib, to rotate away from the bound nucleotide (Figure 6*A* and *B*), which confirmed the hypothesis put forward for the HspBP1 structure. In spite of their overall structural similarity, these two orthologs vary significantly in their regions of contact with their respective Hsp70 ATPase domains and their orientation of binding. In the case of Sil1, this binding to BiP leads to the disruption of a hydrogen bond between the two lobes that is likely to help hold them together (Yan et al, 2011). This causes lobe IIb to rotate away from the nucleotide binding pocket, which in turn disrupts the hydrogen bonds between BiP and ADP leading to its release. Thus, overall it appears that these two proteins use a fairly similar mechanism to release nucleotide.

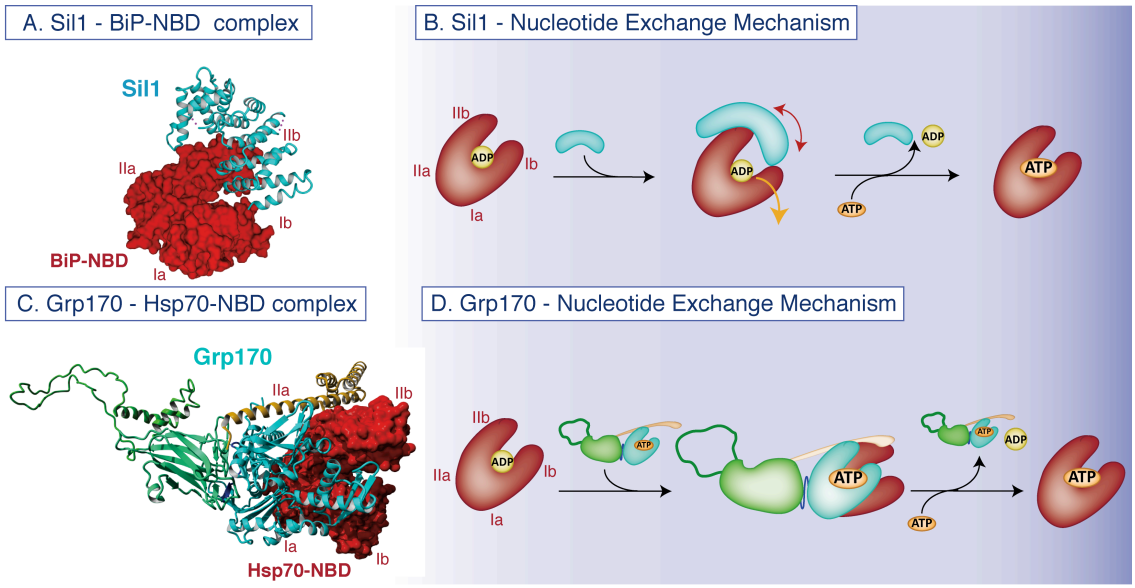

Figure 6. NEF mechanism of the BiP NEFs Sil1 and Grp170.

(A) The crystal structure of Sil1 bound to the NBD of BiP (Yan et al, 2011) depicts how the single domain NEF Sil1, consisting of four Armadillo-like repeats (cyan, shown in ribbon), wraps around lobe II of BiP's NBD (red, shown in surface representation) during the nucleotide exchange reaction. (B) Sil1 mediated nucleotide exchange mechanism in BiP. (C) The crystal structure of yeast Sse1p bound to the NBD of human Hsp70 (Polier et al, 2008) was used to model Grp170 (NBD in cyan, β-sheet and unstructured loop in green and α-helical domain in yellow, shown in ribbon) using Yasara Structure (www.yasara.org) and is shown bound to the human Hsp70 NBD from the Sse1p structure (red, shown in surface drawing). (D) Possible nucleotide exchange mechanism mediated by Grp170.

4.4.3 Cellular functions dependent on Sil1

A number of cellular functions have been identified that require Sil1 activity, which appears to vary by organism. In *S. cerevisiae*, where Sil1 is a UPR target, deletion of this gene results in a very modest induction of the UPR (Tyson & Stirling, 2000), whereas in *Y. lipolytica*, where it is a very minor UPR target, its deletion leads to a dramatic induction of the UPR (Babour et al, 2008). Activation of the UPR is consistent with Sil1 having a role in either protein folding or ERAD, and a few studies have been designed to examine this, which are described below. A mouse strain was identified in which a retrotransposon inserted into the Sil1 locus just downstream of exon 7 (Zhao et al, 2005). This resulted in a chimeric protein that no longer included the last 200 amino acids of the Sil1 protein, which encode BiP interacting domains. Examination of the cerebellum in this mouse revealed evidence of protein aggregates and inclusions containing ubiquitinated proteins in the Purkinje neurons. This correlated with up-regulation of BiP and CHOP transcripts, indicative of UPR activation, and resulted in Purkinje cell loss. Just previous to this, the gene defect responsible for Marinesco-

Sjögren syndrome (MSS), an autosomal recessive multi-system disease characterized by neurodegeneration, ataxia, and myopathy, was mapped to chromosome 5q31 (Lagier-Tourenne et al, 2003), which among several other genes contained the Sil1 locus. Further examination of the locus revealed that ~50-60% of individuals with classical MSS have mutations in both copies of their Sil1 gene (Anttonen et al, 2005; Senderek et al, 2005). While a number of different mutations have been identified throughout the gene, in most cases either a large portion of the Sil1 protein is missing and/or the mutant protein is destabilized (Anttonen et al, 2008; Howes et al, 2012). Although no studies are available to determine if there is evidence of UPR activation in tissues from MSS patients, electron micrographs of a skeletal muscle biopsy from an affected individual showed evidence of accumulated pleomorphic material associated with membranes (Senderek et al, 2005) that could represent misfolded, aggregated ER proteins. An alternative explanation for loss of Sil1 resulting in UPR activation in non-human species comes from a study performed in *Y. lipolytica* where BiP no longer interacted with Ire1 and a constitutive UPR activation was observed (Babour et al, 2008). However it was unclear if Sil1 was required for BiP binding to the transducers or if the loss of interaction was due to the accumulation of unfolded proteins or a reduction in the pool of free BiP. A more recent study revealed that Sil1 loss led to reduced insulin secretion in the mouse model (Ittner et al, 2014), but there are no reports to suggest that MSS patients have problems with glucose regulation.Taken together, many observations underscore an important function of Sil1, but it remains poorly understood on the molecular level.

4.5 Grp170, a two-faced NEF

Another protein similar to BiP, but larger in size due to additional structural features and thus belonging to the family of large Hsp70s, resides in the ER: Grp170. Based on the structural similarities, which include an N-terminal NBD followed by a β-sheet domain, that acts as a substrate binding domain (SBD) in conventional Hsp70s, and an α-helical domain at the C-terminus, large and conventional Hsp70s were combined into the Hsp70 superfamily (Figure 5). While several members of conventional as well as large Hsp70s are present in the cytosol, BiP and Grp170 are the only ER resident representatives of each class. In contrast to BiP, the functions of Grp170 and its role within the ER chaperone network are less well defined. It is known to serve as a NEF to regulate BiP´s function. Its structural organization, on the other hand, suggests it functions as a chaperone with the importance of either role not well understood *in vivo.*

4.5.1 Regulation and structural organization of Grp170

Perturbations of ER homeostasis, by deprivation of either glucose in chick fibroblasts (Olden et al, 1978) or reducing oxygen levels in human and rodent cell lines (Heacock & Sutherland, 1986), resulted in the induction of a high molecular weight protein which was thus named glucose-regulated protein of 170,000 kDa (Grp170) or oxygen-regulated protein of 150,000 kDa (ORP150), respectively. These were ultimately found to be the same protein, which will subsequently be referred to as Grp170. Cloning of the cDNA for hamster Grp170 revealed a related gene in *C. elegans* (Chen et al, 1996), and at about the same time a homolog was identified *in S. cerevisiae*, Lumenal Hsp Seventy, Lhs1p (Baxter et al, 1996; Craven et al, 1996; Hamilton & Flynn, 1996), demonstrating that Grp170 was conserved in eukaryotes. Like BiP, the canonical ER-localized Hsp70 chaperone, Grp170 contains an ER targeting signal sequence that is not present in the mature protein, an N-terminal NBD, which binds to ATP even more efficiently than BiP (Dierks et al, 1996), followed by a β–sheet domain, an α-helical domain, and an ER retention sequence at its C-terminus (Chen et al, 1996; Ikeda et al, 1997). Even though the NBDs between BiP and Grp170 are well conserved with ~29% amino acid identity, their C-terminal regions are quite different. The β–sheet and the α-helical domains of Grp170 share only 14% identity with the corresponding regions in BiP. Unlike BiP,

Grp170 possesses an acidic unstructured loop insertion in its β-sheet domain and a significantly extended and unstructured region at its C-terminus that in part account for its much larger size than BiP (Figure 5). Furthermore, inspection of the putative C-terminal SBD of Grp170 reveals significant differences compared to the corresponding region in BiP. This region in BiP forms an α-helical lid that stabilizes substrate binding to the β-sheet portion of the C-terminal SBD. However, structural studies on cytoplasmic large Hsp70s (Liu & Hendrickson, 2007; Polier et al, 2008; Schuermann et al, 2008) revealed that the α-helices comprising this region are too extended to allow the necessary kink to be formed so that this domain could serve as a lid. Instead, the extended α-helical domain in the large Hsp70s reaches out to embrace lobe II of the NBD of Hsp70 to presumably facilitate the nucleotide exchange reaction (Polier et al, 2008; Schuermann et al, 2008) (Figure 6*C* and *D*). Based on striking parallels between the ATP-bound structures of Hsp70s (Kityk et al, 2012; Qi et al, 2013) and large Hsp70s (Liu & Hendrickson, 2007; Polier et al, 2008; Schuermann et al, 2008), which have the α–helical domain docked on the NBD in common, it would be interesting to know which conformation large Hsp70s would adopt in an ADP-bound or apo-state.

In addition to these structural differences between canonical and large Hsp70s, there are a number of other distinctions. Unlike BiP, which is an unglycosylated chaperone, Grp170 has nine predicted glycosylation sites throughout the protein with the majority of them mapping to the C-terminal region (Park et al, 2003) that is presumed to support substrate binding. Furthermore, a highly conserved Arg present in the NBD of all known canonical Hsp70s, which is critical for interaction with DnaJ family members (Awad et al, 2008), is missing in all ER-localized large Hsp70 family members that have been identified. Interestingly, this Arg is present in all the cytosolic orthologs, suggesting that the functional regulation of the ER proteins might be distinct from other canonical and large Hsp70-type proteins.

4.5.2 A possible chaperone function for Grp170

Although BiP and Grp170 share structural similarities, in contrast to BiP, where much is known about its various functions and how they are regulated (Chapter 4.3), we still lack a clear understanding of Grp170´s functions in the ER. In spite of the fact that the more C-terminal regions of Grp170 are quite different from the SBD of BiP, Grp170 was first found in a complex with Ig HCs (Lin et al, 1993), and subsequently shown to bind

thyroglobulin upon ER stress (Kuznetsov et al, 1997), the non-secreted α1-antitrypsin Z mutant (Schmidt & Perlmutter, 2005), and clusterin, a natural secretory protein in canine kidney derived MDCK cells (Bando et al, 2000). As all of these proteins also interact with BiP, and Grp170 is in a complex with BiP *in vivo* (Lin et al, 1993; Meunier et al, 2002), it is not possible to determine from these studies if the binding of Grp170 to substrates is direct or occurs indirectly *via* its association with BiP. In addition to full-length proteins, radiolabeled peptides that were translocated into microsomes *via* the TAP–transporter were shown to bind to Grp170 directly (Spee & Neefjes, 1997; Spee et al, 1999). Interestingly, in an *in vitro* system using purified proteins, murine Grp170 was significantly more efficient in preventing the aggregation of heat denatured luciferase than Hsc70 (Park et al, 2003), which again argues for direct binding to substrates. Similar data were obtained with yeast Lhs1p (de Keyzer et al, 2009). However, refolding studies conducted with murine Grp170 revealed that it failed to restore enzymatic activity to denatured luciferase in the presence of ATP and the cytosolic DnaJ family protein, Hdj-1. As noted above, mammalian Grp170 does not possess the conserved arginine in its NBD, which is required for interaction with DnaJ family proteins, and it is unclear if nucleotides influence the binding of large Hsp70s to substrates. Only when rabbit reticulocyte lysate was added to the reaction could the activity of luciferase be recovered in an ATP-dependent manner (Park et al, 2003), suggesting that either some of Grp170´s functions depend on further co-factors or interaction partners or that Grp170 maintains the denatured luciferase in a folding-competent state allowing Hsc70 in the reticulocyte lysate to then fold it. Studies to identify the domain(s) responsible for prevention of luciferase aggregation suggested that the binding of large Hsp70s to substrates might be more complex than that of canonical Hsp70s (Oh et al, 1999; Park et al, 2003). The deletion of the α-helical domain at the C-terminus of murine Grp170 did not alter its ability to prevent luciferase aggregation, however this domain alone was able to prevent aggregation (Park et al, 2003). This suggests that Grp170 may possess more than one domain that can inhibit protein aggregation. In contrast, the corresponding α-helical domains of either Hsp110 or Hsp70 failed to prevent protein aggregation (Park et al, 2003). It is unclear if this reflects intrinsic differences between Grp170 and Hsp110, or if the recombinant isolated domains behave differently within the context of the full-length proteins.

A number of recent studies have capitalized on Grp170's ability to bind peptides. It is being used to present antigens in various vaccine protocols (Wang et al, 2001; Park

et al, 2006; Wang et al, 2006; Huo et al, 2010; Wang et al, 2010; Yuan et al, 2012; Zuo et al, 2012), and the ability of a secretable form of Grp170 with bound peptides to stimulate anti-tumor immunity in cancer treatment has been tested (Manjili et al, 2006; Gao et al, 2008; Gao et al, 2009; Arnouk et al, 2010). Studies analyzing peptide binding specificity of various cytosolic large Hsp70s, including Hsp110, Sse1p and Sse2p (Goeckeler et al, 2008; Xu et al, 2012), revealed major differences compared to Hsp70s like BiP (Flynn et al, 1991; Blond-Elguindi et al, 1993b). Based on very limited data for yeast Sse1p, and human Hsp110, it appears that large Hsp70s may have a preference for peptides containing aromatic residues (Goeckeler et al, 2008; Xu et al, 2012), whereas *E.coli* DnaK, a classic Hsp70, prefers to bind peptides with alternating hydrophobic residues, suggesting that conventional Hsp70s and large Hsp70s might bind to dissimilar substrates or to different regions within a substrate. However, more direct comparisons are needed between compartmental pairs to establish the universality of this finding. The regulation of substrate affinity of Hsp70s by ATP binding and hydrolysis is a universal property of these proteins (Figure 4). However, conflicting data have been reported as to whether ATP influences peptide binding to the large Hsp70s. While ATP was reported to lower the affinity of Sse1p and Sse2p for a peptide (Goeckeler et al, 2008; Xu et al, 2012), the binding of another peptide by Sse1p was not influenced by the presence of ATP or ADP (Goeckeler et al, 2008). Thus far no data are available for the role of nucleotides in regulating substrate binding to ER orthologs.

4.5.3 Grp170 possesses nucleotide exchange activity

The first indications that Grp170 might possess nucleotide exchange activity for BiP were provided by a yeast genetic screen for suppressors of the severe growth defect observed in the *Δire1 Δlhs1* double mutant, which identified Sil1p (Tyson & Stirling, 2000). Interestingly, BiP was unable to suppress this phenotype, suggesting that Lhs1p might be functioning in this screen as something other than a molecular chaperone. The fact that yeast deficient either in Lhs1p or Sil1p were viable, while the *Δlhs1Δsil1* double mutation was lethal (Tyson & Stirling, 2000), led these investigators to suggest a common function for these two proteins. A few years later, Lhs1p was shown to serve as a NEF for Kar2p (Steel et al, 2004), revealing that the common function shared by these proteins was exchange activity. In subsequent years, NEF activity was detected for

purified canine Grp170 (Weitzmann et al, 2006), as well as other cytosolic large Hsp70s from a variety of organisms (Shaner et al, 2005; Dragovic et al, 2006; Raviol et al, 2006a; Raviol et al, 2006b; Shaner et al, 2006; Andreasson et al, 2008), thus adding a completely new and unexpected function to these proteins.

Interestingly, despite the similarity in domain organization and NBD between Grp170 and BiP, Sil1p does not physically interact with Lhs1p (Steel et al, 2004). While both NEFs promote nucleotide release from BiP, the binding of Lhs1p and Sil1p to Kar2p occurs through distinct but mutually exclusive interactions (Hale et al, 2010), suggesting they might compete for Kar2p binding. Studies to quantify levels of the two NEF proteins in the ER of canine pancreas revealed that Grp170 is present at much higher levels (~0.60 μM) than Sil1 (~0.005 μM) (Weitzmann et al, 2007). It is unclear if this is because Grp170 has additional functions or if the two proteins might differentially regulate nucleotide exchange to assist in distinct BiP functions, as was recently shown for the NEFs of cytosolic Hsp70s (Abrams et al, 2014), or under unique cellular conditions. Deletion of cytosolic NEFs in yeast revealed that the large Hsp70, Sse1p, is involved in most Hsp70 functions, including protein biogenesis and degradation, while Fes1p was more specialized and no significant phenotype was observed upon deletion of the other cytosolic large Hsp70, Sse2p, and the BAG domain-containing NEF, Snl1p (Abrams et al, 2014). Future studies aimed at dissecting how the two ER resident NEFs influence the functions of BiP *in vivo* will be helpful to understand the significance of the differences in the levels of Grp170 and Sil1 and how they assist BiP in its various functions.

Insights into the mechanism by which the large Hsp70s stimulate nucleotide exchange were first obtained in studies with yeast Lhs1p, which found that exchange activity requires addition of ER-localized DnaJ family proteins to stimulate ATP hydrolysis by Kar2p (de Keyzer et al, 2009). Crystallographic studies on Sse1p alone (Liu & Hendrickson, 2007) or bound to an Hsp70 NBD (Polier et al, 2008; Schuermann et al, 2008) revealed that the ATP bound form of these large Hsp70s interacts directly with the NBD of Hsp70 in a head to head manner with multiple contacts occurring between domains IIa/b and Ia/b of Sse1p with domains Ia/b and IIb of the Hsp70 protein. And as noted above, additional interactions with Hsp70 occur through the α-helical bundle present at the C-terminus of the large Hsp70, which reaches over to embrace the Hsp70 NBD, instead of serving as a lid for the substrate binding domain as occurs in canonical Hsp70's (Figure 6*C*). The result of these interactions is to "pin" subdomain IIb of Hsp70

between Sse1's α-helical domain and its own subdomain Ib and rotate it sideways to release nucleotide (Figure 6*D*). Release of ADP from Hsp70 is not sufficient to trigger dissociation of the Hsp70-NBD:Sse1p complex, arguing that rebinding of ATP to the Hsp70 NBD may be required (Polier et al, 2008; Schuermann et al, 2008). This is in keeping with studies showing that Lhs1p can bind to Kar2p in either the apo or ADP bound form but not the ATP bound state (de Keyzer et al, 2009).

Although a crystal structure for an ER large Hsp70 bound to BiP has not been reported, based on the conformational dynamics of the Ssa1p NBD induced by Sse1p, the mechanism of the nucleotide exchange employed by Lhs1p/Grp170 is likely to be quite similar to that of Sse1p (Andreasson et al, 2010). Hydrogen-Deuterium exchange assays revealed that Lhs1p formed interactions with the NBD of Ssa1 that were very similar to those occurring when Sse1p bound to the NBD of Ssa1, including the interaction of the α-helical bundle of the two NEFs with subdomain IIb of the Hsp70 protein. In addition, the residues that are critical for Sse1p's NEF function (Polier et al, 2008; Schuermann et al, 2008) are conserved in Lhs1p and Grp170, and mutation of these residues similarly affected exchange activity in Lhs1p (Andreasson et al, 2010). This study also revealed that ATP hydrolysis was not required by either of the large Hsp70s examined in this reaction. Together these data make a strong case for the ER-localized large Hsp70s using the same mechanism of nucleotide exchange as has been established for their cytosolic orthologs.

4.5.4 Biological functions requiring Grp170

A large variety of biological functions and diseases have been associated with Grp170 (Kusaczuk & Cechowska-Pasko, 2013), although molecular insights into the role of Grp170 in these processes are generally lacking. Loss or decreased expression of Grp170 inhibits the translocation of nascent polypeptide chains into the ER in a number of organisms (Baxter et al, 1996; Craven et al, 1996; Dierks et al, 1996; Hamilton & Flynn, 1996). In yeast, *lhs1* null cells exhibit a selective translocation defect for a subset of proteins, including preKar2p, prePDI, prepro-α-factor and preCPY, but did not affect the translocation of the SRP-dependent protein, dipeptidylaminopeptidase B (Baxter et al, 1996; Craven et al, 1996; Hamilton & Flynn, 1996). Preprolactin translocation assays conducted with mammalian proteoliposomes that were depleted of ATP-binding proteins

revealed that preprolactin import was inhibited. BiP and Grp170 were identified as the major ATP-agarose binding proteins. Translocation was restored when the microsomes were repleted with the ATP-binding proteins but not with purified BiP alone, leading the investigators to conclude that either Grp170 alone or in combination with BiP was required for the efficient import of this protein into the microsomes (Dierks et al, 1996). However, since BiP is also required for protein translocation into the yeast ER (Normington et al, 1989; Rose et al, 1989), it remains unclear whether Grp170 itself plays a direct role in protein translocation through a possible chaperone function or whether nucleotide exchange by Grp170 is a rate-limiting factor for BiP's role in protein translocation.

Grp170 has also been implicated in a variety of physiologically relevant protein secretion processes linked to disease states. The high expression of Grp170 in pancreatic islets is significantly reduced by fasting, suggesting that it may have a role in insulin biosynthesis (Kobayashi et al, 2000). In keeping with this possibility, it was found that diminishing levels of Grp170 in a pancreatic cell line resulted in an intracellular accumulation of insulin (Kobayashi & Ohta, 2005), although the data did not allow a distinction between this occurring as a consequence of impaired protein translocation into the ER, an inability of insulin to fold properly, or an inability of it to be released from BiP. The effects of both increased and decreased Grp170 expression on insulin production and blood glucose levels has also been examined using a number of normal mice as well as mouse models for type II diabetes. Increasing Grp170 levels with adenovirus delivered constructs increased insulin secretion and decreased blood glucose after feeding, whereas decreasing Grp170 expression with anti-sense constructs inhibited insulin secretion and resulted in significantly higher blood glucose levels (Nakatani et al, 2005). In another study Grp170 levels were modulated by crossing the diabetes-prone Akita mice to either $Grp170^{(+/-)}$ heterozygous mice or Grp170 transgenic mice (Ozawa et al, 2005). Decreased expression of Grp170 accelerated the onset of diabetes in this model, whereas transgene-enhanced expression of Grp170 resulted in lower body weight and improved glucose tolerance at young ages. However this effect was lost in older mice. Interestingly, there were no changes in the level of insulin in the pancreatic cells of either genetic model, which could suggest that the retained insulin was continuously being degraded in the animal model. More detailed insights into the molecular mechanisms by which Grp170 contributes to insulin secretion are needed and may provide important insights into the role this protein plays in type 2 diabetes.

Similar to its effects on insulin secretion, a number of studies have indicated a role for Grp170 in VEGF secretion, one of the most important proangiogenic factors. Decreased Grp170 levels in C6 glioma cells (Ozawa et al, 2001b) and macrophages (Ozawa et al, 2001a), achieved by expressing an antisense-Grp170 construct, resulted in an inability of both cell lines to secrete VEGF into culture media under hypoxic conditions. In the case of C6 cells, the intracellular levels of VEGF were dramatically increased, arguing for an inhibition of protein transport possibly due to improper maturation when Grp170 levels were reduced. Conversely, when Grp170 was over-expressed, cells secreted significantly higher levels of VEGF compared to non-transfected cells (Ozawa et al, 2001a; Ozawa et al, 2001b). Grp170 could be co-immunoprecipitated with VEGF and *vice versa* under hypoxic conditions, demonstrating an interaction between these proteins (Ozawa et al, 2001a; Ozawa et al, 2001b).

Grp170 levels have also been associated with various cytoprotective functions. Ischemia and atherosclerotic plaque formation lead to lower oxygen levels in the surrounding tissue, which induces ER stress and hypoxic pathways, resulting in cell death if not resolved. Transgenic mice over-expressing Grp170 lost significantly less brain tissue in response to ischemia (Tamatani et al, 2001), and upon kainate administration fewer animals suffered from seizure (Kitao et al, 2001) compared to wild type mice. The survival rate of neurons after hypoxic stress (Tamatani et al, 2001; Miyazaki et al, 2002) or after excitotoxicity upon glutamate administration (Kitao et al, 2001; Miyazaki et al, 2002) also increased when Grp170 was over-expressed, although the mechanism for this protection or function of Grp170 required is not known. Regulated cell death is an important event in many developmental phases, including in the brain (Hyman & Yuan, 2012). Somewhat counter-intuitively, Purkinje cells were shown to significantly up-regulate Grp170 during the peak phase of programmed cell death in brain development in mice. However, over-expression of Grp170 in the transgenic mice model led to increased survival of these cells resulting in a higher number of Purkinje cells compared to wild-type. Of note, this abnormal brain development resulted in an impaired motor coordination of these mice (Kitao et al, 2004).

In another pathological condition, atherosclerotic plaques, which deprive surrounding tissue of their oxygen supply, lead to up-regulation of hypoxic and ER stress pathways in segments of aortae with severe atherosclerosis (Tsukamoto et al, 1996; Sanson et al,

2008; Sanson et al, 2009). In particular macrophages, one of the key players in the pathogenesis of atherosclerosis within the atherosclerotic plaque (Moore & Tabas, 2011), showed significant induction of stress pathways including up-regulation of Grp170 (Tsukamoto et al, 1996). When Grp170 levels were reduced in cultured mononuclear phagocytes, the cells became more susceptible to hypoxic stress and even more so when this was combined with oxidized LDL (Tsukamoto et al, 1996), and in a microvascular endothelial cell line, over-expression of Grp170 prevented ER stress activation (Sanson et al, 2009) and inhibited apoptotic cell death (Sanson et al, 2008) triggered by oxLDLs. Hence, Grp170 promotes cell survival under a number of physiological stress conditions.

Taken together, many physiological processes and disease states have been linked to Grp170, while its role within the ERQC network remains poorly understood and mechanistic insights are very limited. The first evidence for a specific molecular function of Grp170 in the ER was provided by a recent study with Lhs1p, showing that ERAD of the α-subunit of the epithelial sodium channel (ENaC) is dependent on Lhs1p, while BiP was dispensable (Buck et al, 2013). Exciting *in vitro* data provide evidence that cytosolic large and conventional Hsp70s and Hsp40 co-chaperones assemble into a powerful machine that can efficiently disassemble large protein aggregates (Shorter, 2011; Rampelt et al, 2012; Mattoo et al, 2013). Interestingly, in the ER Grp170 is part of the large chaperone complex centered around BiP and ERdj3, the Hsp40 orthologue, is also part of this complex when it is associated with free Ig heavy chains (Meunier et al, 2002). The function of this thesis is to gain a better understanding of Grp170´s function in the ER on a molecular level.

5 Objective of the PhD thesis

While the role of BiP in the ERQC process and its mechanism of action have been dissected in detail, the second member of the ER Hsp70 superfamily, Grp170, remains poorly understood in both regards. This raises several central questions: (i) does Grp170 function as a chaperone in the absence of BiP, and if so, is its interaction with substrates regulated *via* the same mechanism as has been established for conventional Hsp70s or does Grp170 interact differently with substrates; (ii) do Grp170 and BiP recognize the same binding sites within substrates, hence do Grp170 and BiP compete for these sites, or do they recognize distinct sites on substrate proteins; and (iii) does Grp170 have a similar interaction network of co-chaperones as has been established for BiP and the ERdj proteins. The answers to these questions constitute the main objectives of this PhD thesis.

Central to the interaction of BiP with its substrates is its ATPase cycle, which is regulated by its co-factors (Figure 4). Similar to other large Hsp70s, Grp170 can serve as a NEF for BiP during this cycle. The binding of ATP to Grp170 regulates its association with BiP, leading to the exchange of ADP to ATP in BiP. In addition to serving as a NEF for BiP, Grp170 is also found in complex with a number of BiP substrates (Lin et al, 1993; Bando et al, 2000; Schmidt & Perlmutter, 2005). This raises the question as to whether Grp170 could bind to substrates independent of BiP or only *via* its association with BiP. Thus the initial phase of the PhD thesis was aimed at dissecting whether the release of BiP from these chaperone:client complexes disrupted the association of Grp170 with theses substrates, or if Grp170 also bound to substrates in the absence of BiP. If so, this would raise the issue of whether there might be a different regulatory cycle for the binding of Grp170. Lastly, experiments were designed to determine whether Grp170s unique structural features, which distinguish large from conventional Hsp70s, play a role in its chaperone activity or its regulation.

There are no studies to demonstrate which binding sites are recognized *in vivo* within substrates not only by Grp170 and BiP, but also by any other ER chaperone. *In vitro*, it is possible to use e.g. peptide libraries to determine possible binding motifs or hydrogen-deuterium (H-D) exchange assays on substrate proteins. However, these same approaches are not possible *in vivo*, since short peptides are rapidly degraded in the cell and thus not readily amenable to chaperone-peptide interaction studies and H-D exchange assays have not been developed for *in vivo* use. Using *in vitro* peptide and protein binding studies, it has been established that BiP recognizes sequences of

between 7-9 amino acids and that include primarily alternating hydrophobic amino acids (Blond-Elguindi et al, 1993a). A few *in vitro* and *in silico* studies have identified BiP binding sites on an amylogenic LC (Davis et al, 1999), the C_H1 domain of the γ_1 HC (Marcinowski et al, 2013) or both (Knarr et al, 1995). However, a C_H1 domain mutant that does not interact with BiP *in vitro* still binds this chaperone when expressed in cells, underscoring a need to conduct chaperone-binding screens *in vivo.* In the case of the large Hsp70s there is very limited data in regard to possible binding motifs, with only two peptides identified so far that bind a yeast or mammalian cytosolic large Hsp70 protein (Goeckeler et al, 2008; Xu et al, 2012). In both cases aromatic residues were enriched, and in the latter case, mutation of these residues to aliphatic or charged amino acids dramatically reduced the affinity of Hsp110 for this peptide. Determination of binding specificities of Grp170 and BiP within full-length proteins *in vivo* would provide an opportunity to determine if these two chaperones compete for binding to the same sites or if they recognize distinct regions. If unique sites are recognized by these two chaperones, it would reveal whether there are restrictions on the proximity of sites that are actually used *in vivo*. Taken together the second aim of the PhD project constitutes engineering and establishing an *in vivo* peptide expression library covering overlapping sequences of two substrates common to Grp170 and BiP and dissecting the binding pattern of these two chaperones.

The focus of the third part of the thesis was the question of how Grp170 functions in ERQC, i.e. to promote folding or degradation. Although a number of DnaJ-like (ERdjs) co-chaperones have been identified that regulate the functions of BiP, it is not known whether Grp170 has interacting regulatory partners. A first hint toward major differences between Grp170 and BiP in that regard are provided by the fact that the NBD of Grp170 does not possess the well-conserved arginine residue that is crucial for the interaction of BiP and all other Hsp70 proteins with DnaJ-like co-factors (Awad et al, 2008), suggesting that Grp170 will not use the ERdj proteins as co-factors. In addition, Grp170 possesses a particularly high number of glycosylation sites, while BiP has none. As recent studies have suggested that glycan recognition plays a critical role in the interaction of components of the ERAD machinery and a very recent study demonstrated a role for yeast Grp170, Lhs1p, in ERAD (Buck et al, 2013), it is possible that Grp170 is connected to the ER resident ERAD machineries *via* its glycosylation sites. To address these possibilities will be the focus of this last part of the thesis.

6 Results

6.1 Identification of Grp170 as a chaperone that binds to unfolded protein substrates in vivo with a regulation distinct from the conventional Hsp70 BiP[1]

6.1.1 Grp170 can bind to unfolded Ig substrates *in vivo* in the absence of BiP

Antibodies and their subunits, which are composed of series of Ig domains, are one of the best-characterized secretory pathway proteins in terms of their folding status, interaction with chaperones and their fate in cells. Therefore, we chose several Ig proteins with well-defined folding characteristics to study Grp170's substrate binding *in vivo* (Figure 7). These included two substrates that do not require subunit assembly for their secretion, the well-folded constant domain of the Ig lambda light chain (λ C_L) and a secreted lambda light chain (λ LC) that transiently interacts with BiP during its maturation (Hellman et al, 1999). In addition, three substrates were used that possess unfolded domains: the NS-1 LC, whose V_L domain does not fold (Skowronek et al, 1998); an Ig gamma heavy chain (γHC) in which the first constant domain (C_H1) remains unfolded in the absence of its partner, the LC (Hendershot et al, 1987; Feige et al, 2009); and a truncated, HA-tagged version of the γHC that is devoid of the second and third constant domain (mHC^{HA}) (Lee et al, 1999).

[1] This chapter was previously published in a modified form: Behnke J, Hendershot LM (2014) The large Hsp70 Grp170 binds to unfolded protein substrates in vivo with a regulation distinct from conventional Hsp70s. *J Biol Chem* **289:** 2899-2907

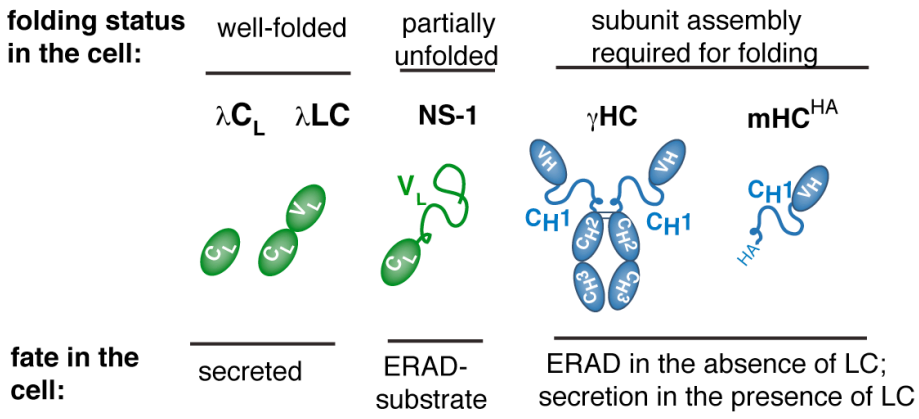

Figure 7. Schematic of substrates used to analyze substrate binding properties of Grp170 *in vivo*.

Color-filled ovals represent folded Ig domains and lines show unfolded regions. The folding status of the substrate and their fate within the ERQC process are indicated. Adapted and modified from (Behnke & Hendershot, 2014).

In addition to direct substrate binding, we also wished to determine how ATP influences association of Grp170 with its substrates compared to the established nucleotide-dependent BiP-substrate interaction (Figure 4). To this end COS-1 cells were co-transfected with BiP, Grp170 and the various Ig constructs and metabolically labeled 24 hrs later (see Material and Methods for experimental details). We exploited the ATP regulation of the BiP-substrate interaction, which is stabilized in the absence of ATP, whereas ATP addition releases BiP-bound substrates (Hendershot et al, 1996; Kampinga & Craig, 2010; Braakman & Bulleid, 2011), and prepared cell lysates with either Mg-ATP or with apyrase that hydrolyzes ATP. The substrates were subsequently immunoprecipitated with substrate-specific immune reagents, which allowed us to analyze under which conditions would Grp170 or BiP, respectively, co-precipitate with their substrate (Figure 8*A*). Upon hydrolysis of ATP by apyrase, a band migrating at the size of BiP co-precipitated with all of the Ig proteins, except the well-folded λCL domain, and as expected was released from these substrates by ATP (Figure 8*A*). Intriguingly, an additional band migrating at the expected size of Grp170 similarly bound to the substrates with permanently unfolded domains in the presence of apyrase: NS-1 LC, γHC, and mHCHA.

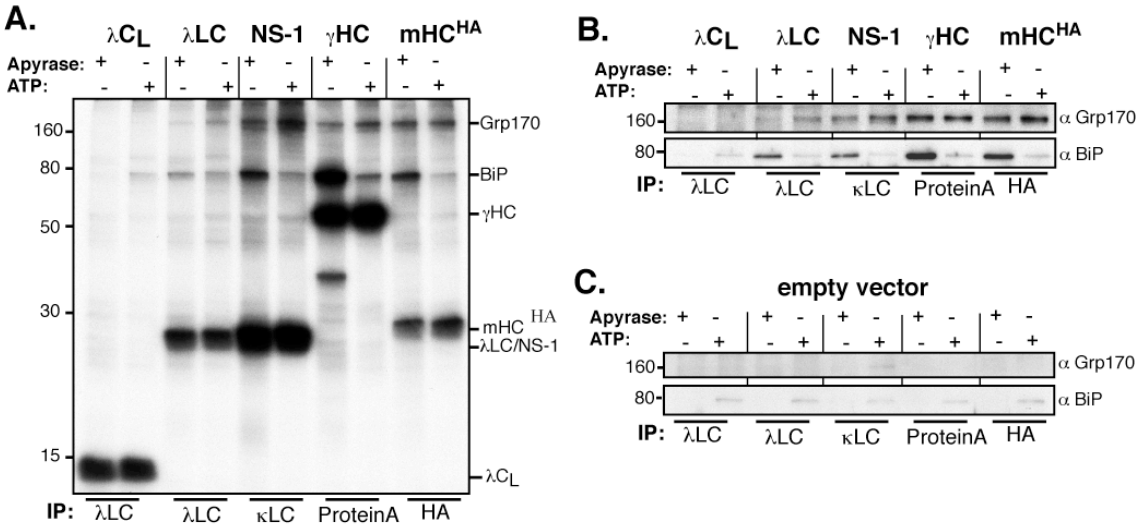

Figure 8. Grp170 directly binds to unfolded Ig substrates *in vivo* and remains bound in the presence of ATP in contrast to BiP.

(A) COS-1 cells were transfected with Ig substrates with defined folding characteristics along with Grp170 and BiP. Cells were pulse-labeled with [^{35}S]cysteine/methionine for 1 hr and chased for 1 hr to allow maturation of the chaperones before lysing either in the presence of ATP or apyrase. Lysates were immunoprecipitated with the indicated reagents and separated by 12% SDS-PAGE followed by autoradiography. (B) Lysates from cells transfected as in (A) were immunoprecipitated with substrate specific antibodies, separated by SDS-PAGE and transferred to membranes that were blotted with either anti-Grp170 or anti-BiP antisera followed by species-specific secondary reagents. (C) COS-1 cells were transfected and analyzed as in (B) except an empty vector was used instead of the substrates. Adapted and modified from (Behnke & Hendershot, 2014).

The well-folded λLC domain co-precipitated only trace amounts of this band, whereas no binding was observed to the well-folded λC_L domain, similar to BiP (Figure 8*A*). To confirm the co-precipitating bands as BiP and Grp170, immunoprecipitation coupled with western blotting was performed and indeed both bands at 80kDa and 170kDa were identified as BiP and Grp170, respectively (Figure 8*B*). In addition, to exclude non-specific binding of the different substrate-targeted antibodies to Grp170 or BiP, the same experiments were carried out transfecting cells with BiP, Grp170, and instead of a substrate, an empty vector was co-transfected, confirming substrate-dependent immunoprecipitation of BiP and Grp170 (Figure 8*C*). However, because Grp170 and BiP bound to the same substrates, and at the same time are known to form a complex *in vivo* (Meunier et al, 2002), the binding of Grp170 to these substrates in the presence of apyrase could be mediated *via* its interaction with BiP. Therefore we added ATP to

release BiP from its substrates. Despite the dissociation of BiP, no decrease in Grp170 association with any of these substrates was observed (Figure 8*A* and *B*). Taken together these results demonstrate two characteristic aspects of the interaction between Grp170 and its substrates: Grp170 binds substrates independent of the presence of BiP *in vivo* and in contrast to BiP it remains bound to its substrates in the presence of ATP.

6.1.2 Grp170 and BiP recognize the same molecular species of different substrates

Two protein modifications are characteristic to the ER: the formation of disulfide bonds and N-linked glycosylation (Figure 2). Accordingly, proteins modified in this manner can populate different species on their way to the native state: having either one, several or all of disulfides formed or N-linked sugars attached. Since BiP and Grp170 co-precipitated with the same set of substrates (Figure 8*A* and *B*), we investigated if they recognized different molecular species of two substrates, representing either different disulfide-linked or N-linked glycosylation forms. One established substrate that exists in two different oxidization states in the cell is NS-1 LC; one in which only the C_L domain has formed its internal disulfide bridge (ox1) and one where the V_L and C_L domains are both oxidized (ox2) (Skowronek et al, 1998; Okuda-Shimizu & Hendershot, 2007). We wished to analyze the interaction of BiP and Grp170 with this substrate in a native setting and thus we used the myeloma cell-line P3U.1, which naturally produces the NS-1 LC (Skowronek et al, 1998). We performed immunoprecipitation-coupled western blotting assays in a similar way as in Figure 8*B*, but under non-reducing conditions to preserve the ox1 and ox2 form of NS-1 and besides the substrate, Grp170 and BiP were immunoprecipitated individually to identify NS-1 species bound to one or the other chaperone (Figure 9).

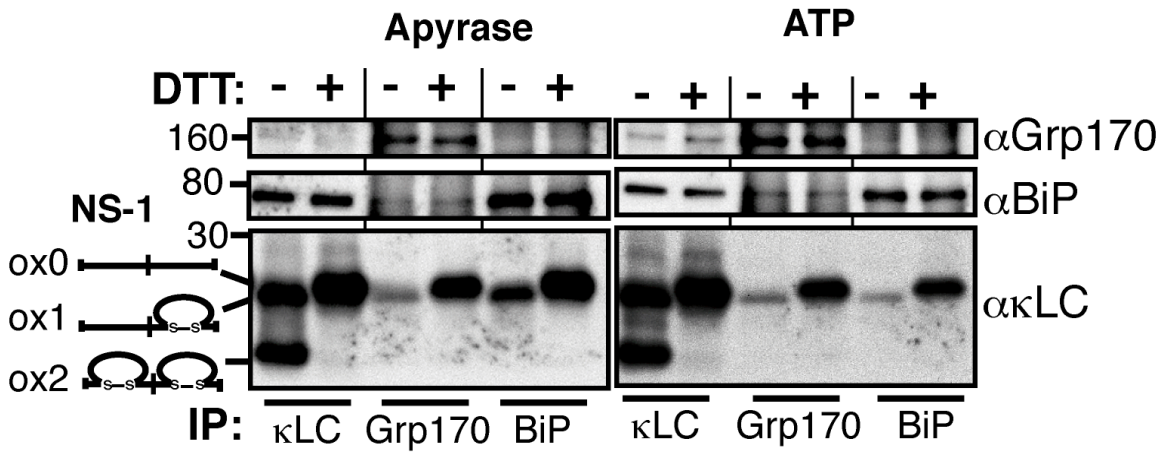

Figure 9. Grp170 and BiP bind to the same oxidation species of NS-1 LC.

P3U.1 murine myeloma cells were lysed either in the presence of apyrase or ATP, immunoprecipitated with the indicated reagents and analyzed under non-reducing conditions. As BiP is ~10-fold more abundant than Grp170 in the cell (Weitzmann et al, 2007), five times more lysate was used for Grp170 immunoprecipitations than for κLC or BiP. Isolated proteins were separated on 13 % SDS-PAGE gels and transferred for blotting with the indicated reagents. Adapted and modified from (Behnke & Hendershot, 2014).

As previously reported (Skowronek et al, 1998; Okuda-Shimizu & Hendershot, 2007), BiP co-immunoprecipitated only the ox1 form of the NS-1 LC under non-reducing conditions (Figure 9). Reduction of the NS-1 LC disulfide bonds in cells with DTT resulted in a slight reduction in NS-1 LC mobility (ox0 form), as expected, due to a more extended conformation when all disulfide bonds are reduced, and dramatically enhanced co-precipitation with BiP (Figure 9). Of note, Grp170 also followed the same binding pattern as BiP and only bound to the partially oxidized (ox1) species of NS-1 LC in non-treated cells and showed increased binding to the fully reduced form (ox0) when cells were treated with DTT (Figure 9). Thus, in terms of oxidation status, our data show that BiP and Grp170 recognize the same molecular form of the NS-1 LC in a native setting and both exhibit increased binding to the fully reduced ox0 form.

In addition to disulfide bond formation, N-linked glycosylation is another typical modification occurring on proteins synthesized in the ER (Braakman & Bulleid, 2011; Hebert & Molinari, 2012). It has been reported that the T cell receptor β-chain (TCRβ) populates two isoforms, either a fully glycosylated species with oligosaccharides attached to both of its glycosylation sites or just one of its two sites being glycosylated. Both species are readily distinguished by SDS-PAGE (Lee, 1998; Feige & Hendershot,

2013). Although the majority of TCRβ is co-translationally glycosylated at its two sites, a species can be detected that is only glycosylated on a single site (Feige & Hendershot, 2013). To analyze which species bound to Grp170 or BiP, respectively, COS-1 cells were co-transfected with vectors encoding TCRβ, BiP, and Grp170 and metabolically labeled. Immunoprecipitated BiP bound both species in a ratio approximately 1:1, although the concentration of the fully glycosylated form in the cell was significantly higher than of the singly glycosylated species. Accordingly, a smaller fraction of the fully glycosylated form compared to the less glycosylated form was bound by BiP (Figure 10).

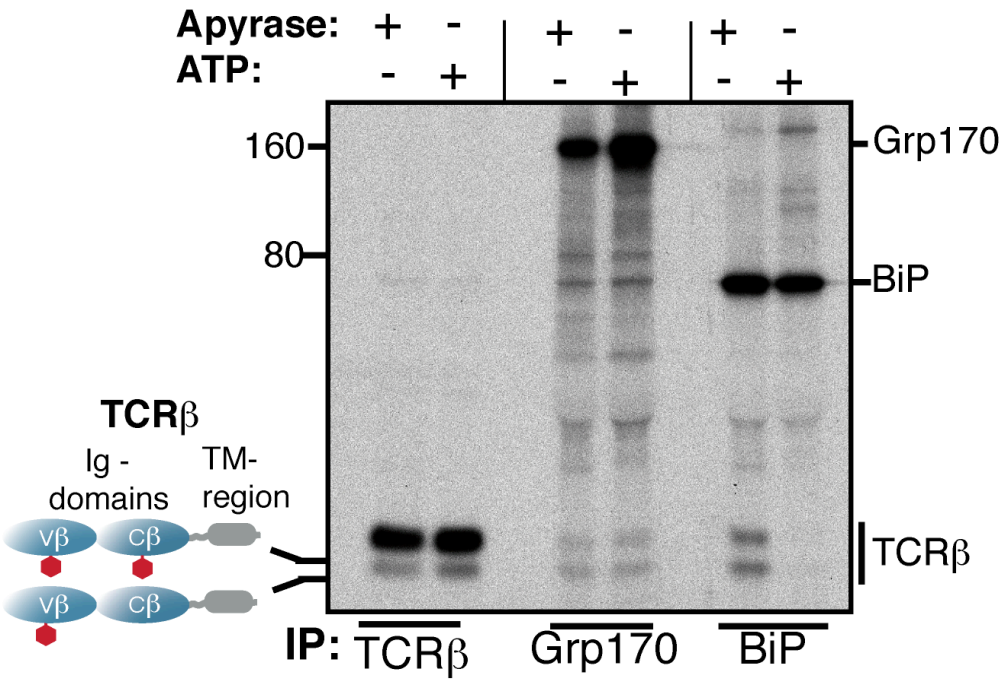

Figure 10. Grp170 and BiP bind to the same glycosylation species of TCRβ.

COS-1 cells were transfected with vectors encoding TCRβ, Grp170 and BiP, pulse-labeled with [^{35}S]cysteine/methionine for 0.5 hr, chased for 1 hr and lysed either in the presence of apyrase or ATP. Interactions between TCRβ, Grp170 and BiP were analyzed *via* immunoprecipitation with the indicated antisera and separation on 10% SDS gels. Four times less lysate was used for the TCRβ immunoprecipitation than for Grp170 or BiP to make both species of TCRβ clearly visible. The mobility of the two TCRβ glycoforms is indicated. Adapted and modified from (Behnke & Hendershot, 2014).

When Grp170 association with TCRβ was examined, we found that although it bound to less of the TCRβ than BiP, its pattern of binding to the two glycosylation species was very similar (Figure 10). Thus, our data show for two different substrates and two

different types of ER-specific protein modifications that Grp170 and BiP bind similar molecular species *in vivo.*

6.1.3 Release from BiP is not essential for substrate binding to Grp170

Our data revealed that as expected for *bona fide* chaperones both Grp170 and BiP associated with incompletely folded proteins (Figure 8). Furthermore, they both also recognized the same molecular forms of two substrates with different modifications (Figure 9 and Figure 10). As the NEF function of Grp170 could induce BiP release from substrates leading to a subsequent transfer to Grp170, we wished to determine if Grp170 acted downstream of BiP. To address this, we performed a pulse-chase experiment using P3U.1 cells so that interactions between endogenous proteins in a native setting could be examined. In this setup cells were metabolically labeled and after the [^{35}S]methione/cysteine mix was washed out, cell aliquots were taken at different time points after the pulse label and the cell lysates were divided for immunoprecipitations against the substrate, Grp170 and BiP. This allowed us to follow the time course of the degradation of NS-1 LC and the kinetics of its association with BiP or Grp170.

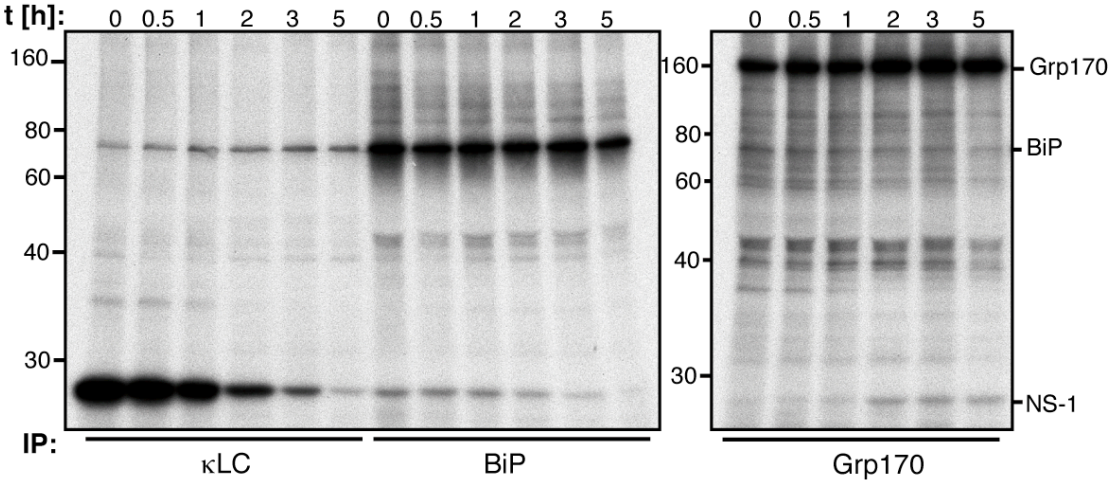

Figure 11. Grp170 and BiP exhibit different binding kinetics to NS-1 LC.

P3U.1 murine plasmacytoma cells were pulse-labeled with [^{35}S]cysteine/methionine for 30 min followed by the indicated chase times. After lysing in the presence of apyrase, the clarified lysate was divided for immunoprecipitation with the indicated antisera. Due to different expression levels of Grp170 and BiP (Weitzmann et al, 2007), five times more lysate was used for Grp170 immunoprecipitations. Proteins were separated by 10% SDS-PAGE and visualized by autoradiography. Adapted and modified from (Behnke & Hendershot, 2014).

Consistent with a previous report (Knittler & Haas, 1992), the NS-1 LC could be co-immunoprecipitated with BiP immediately after labeling, and the amount bound to BiP decreased over time as the LC was degraded (Figure 11, left panel). When the binding of NS-1 LC to Grp170 was examined, no association was detected initially, but in contrast to its binding to BiP, we observed a slight increase of NS-1 LC binding to Grp170 over time (Figure 11, right panel).

To better understand what appeared to be a temporal relationship between the binding of these two chaperones to the NS-1 LC, we asked if BiP must be released from the substrate for Grp170 to bind. The expression of a BiP "trap" mutant that is defective in ATP-induced substrate release (BiP^{T37G}) (Wei et al, 1995) (Figure 12*A*) allowed us to dissect whether Grp170 has to take over NS-1 LC after its release from BiP. If Grp170 binding to its substrates would entirely depend on direct substrate transfer from BiP, then the amount of NS-1 LC bound to Grp170 in the presence of BiP^{T37G} should be reduced. As expected, significantly more NS-1 LC remained bound to BiP^{T37G} after the addition of ATP as compared to wild-type BiP. Nonetheless the association of NS-1 LC with Grp170 under these conditions was not significantly affected (Figure 12*A*). When cell extracts were prepared in the presence of apyrase, the percentage of NS-1 LC associated with BiP^{T37G} was slightly increased (Figure 12*B*). Although the increased binding to BiP^{T37G} was not dramatic, there was no indication that this resulted in a decrease in the amount of NS-1 LC bound to Grp170 (Figure 12*B*), suggesting that Grp170 binding to a substrate does not necessarily depend on its release from BiP.

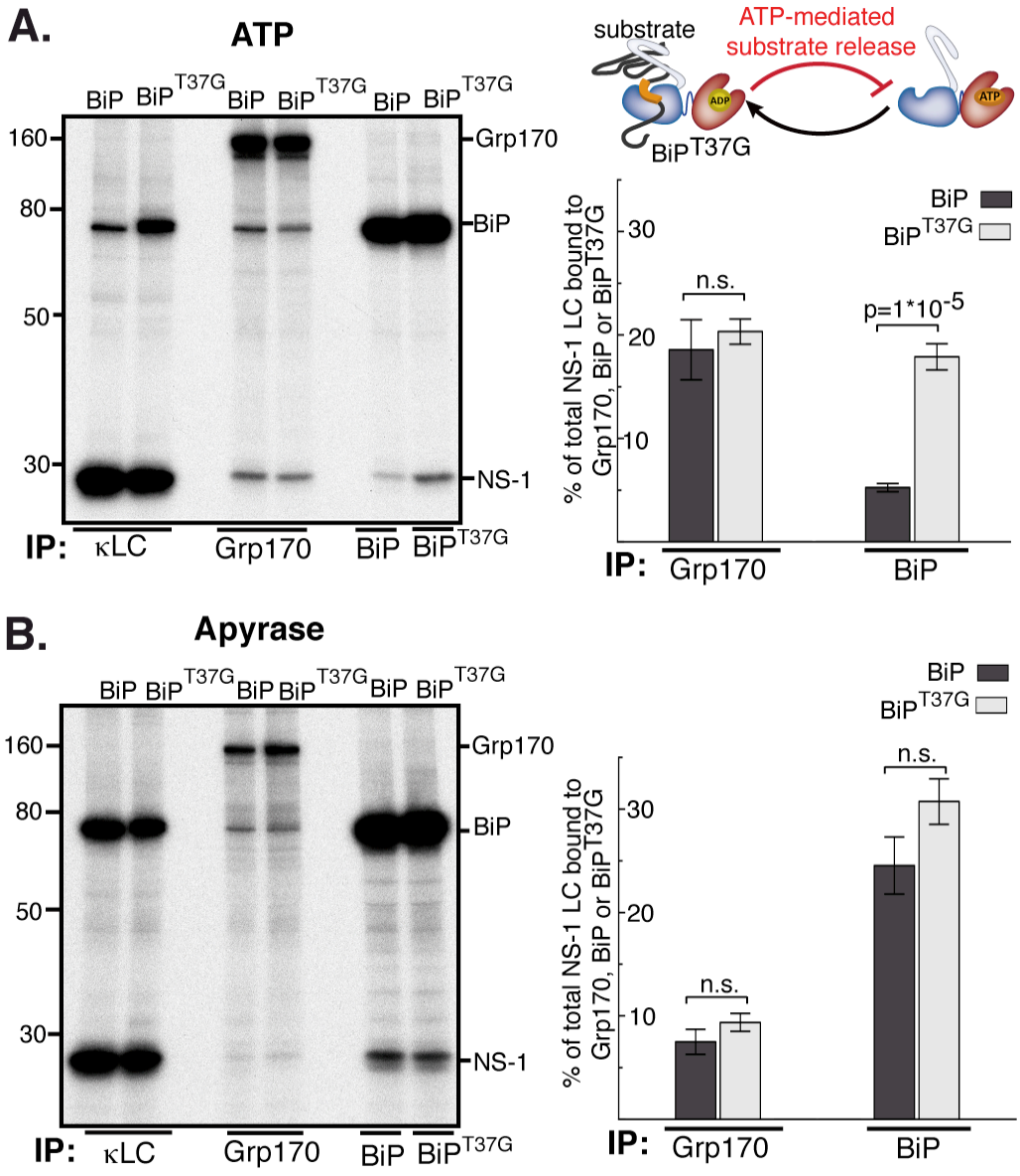

Figure 12. Release from BiP is not required for substrate binding to Grp170.

COS-1 cells were transfected with NS-1 LC, Grp170 and BiP^{WT} or BiP^{T37G} as indicated. Subsequently cells were pulse-labeled with [^{35}S]cysteine/methionine for 0.5 hr, chased for 1 hr and lysed in the presence of ATP (*A*) or apyrase (*B*). The lysate was equally divided and immunoprecipitated with the indicated immune reagents. For a quantitative analysis the signal of NS-1 LC bound to Grp170, BiP or BiP^{T37G} was divided by the value for NS-1 LC obtained with κLC immunoprecipitations (n=4 ± S.E., wild-type BiP, and n=8 ± S.E., BiP^{T37G}; n.s.: not significant). Adapted and modified from (Behnke & Hendershot, 2014).

6.1.4 Grp170's characteristic structural elements modulate substrate binding

The fact that ATP did not have as profound of an effect on substrate release from Grp170, as has been established for the conventional Hsp70 BiP (Hendershot et al, 1996; Vanhove et al, 2001), led us to explore how substrate binding to Grp170 might be regulated. All large Hsp70s possess unique structural features, which set them apart from conventional Hsp70s: an unstructured loop insertion within their β-sheet domain and an extended α-helical domain at their C-terminus. Although uncharacterized in this regard, we hypothesized that these structural elements might play a role in substrate binding *in vivo*. Thus, we modeled Grp170 based on the crystal structures of its cytosolic orthologue Sse1p (Liu & Hendrickson, 2007; Polier et al, 2008; Schuermann et al, 2008) (Figure 13).

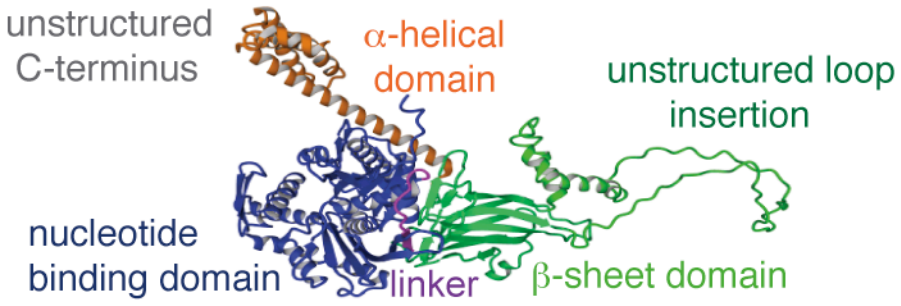

Figure 13. Structural model of Grp170 and its domain organization.

The structure of human Grp170 (shown in ribbon) was modeled using Yasara Structure (www.yasara.org) based on the crystal structures of its cytosolic yeast orthologue Sse1p (Liu & Hendrickson, 2007; Polier et al, 2008; Schuermann et al, 2008) (blue: NBD, magenta: linker, green: β-sheet domain and unstructured loop insertion, orange: α-helical domain). Adapted and modified from (Behnke & Hendershot, 2014).

The model allowed us to construct a number of domain deletions mutants (Figure 14*A*) with a FLAG-tag for specific isolation of the mutants *vs*. endogenous Grp170.

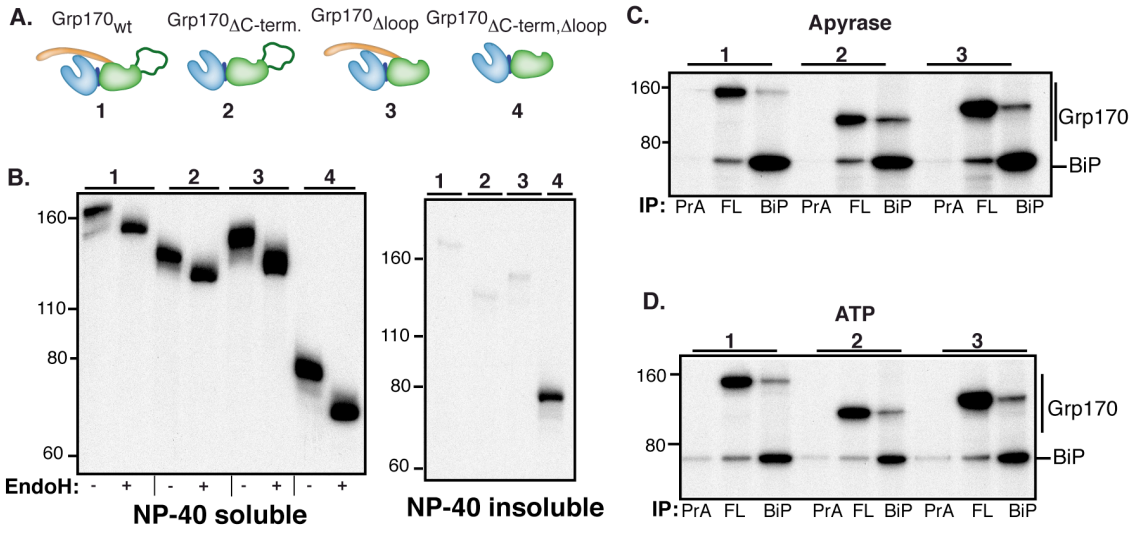

Figure 14. Construction and characterization of Grp170 domain deletion mutants.

(A) The structural model of Grp170 was used to design FLAG-tagged Grp170 whole domain deletion mutants, which are numbered. (B) COS-1 cells were transfected with empty pSVL vector, BiP, and the indicated Grp170 constructs. After a 1 hr pulse-label with [^{35}S]cysteine/methionine and a 1 hr chase, cells were lysed in the presence of ATP. After centrifugation, samples were divided into NP-40 soluble and NP-40 insoluble fraction and immunoprecipitated with antiserum against the FLAG-tag. The eluted protein was divided and left undigested or treated with Endo H. Samples were analyzed by 10% SDS-PAGE, followed by autoradiography. The numbers above each group correspond to the deletion mutants shown in (A). (C) and (D), COS-1 cells were transfected with BiP and the indicated Grp170 constructs. After labeling with [^{35}S]cysteine/methionine for 5 hrs and a chase period of 16 hrs, cells were lysed in the presence of apyrase (C) or ATP (D). Cell lysates were divided equally and immunoprecipitated with an antiserum against either the FLAG-tag (FL), BiP, or Protein A (PrA) only. The proteins were separated on 10% SDS-PAGE, followed by autoradiography. Deletion mutants are indicated by the number above each group. Adapted and modified from (Behnke & Hendershot, 2014).

To analyze whether the generated mutants have major structural deficiencies, which might result in aggregation in the cell, we investigated the intracellular solubility of the mutants. We found that all mutants, except for a double mutant of Grp170 in which both the unstructured loop insertion and extended α-helical domain were deleted ($Grp170_{\Delta C\text{-}term,\ \Delta loop}$), where the structural integrity was likely perturbed, remained soluble (Figure 14*B*) and none were secreted (data not shown). The EndoH digest, which results in the removal of N-linked glycans, demonstrated that all domain deletion mutants were glycosylated (Figure 14*B*), consistent with nine predicted glycosylation sites of Grp170 and indicative of glycosylation occurring at multiple sites throughout the protein. This finding is in contrast to a previous study on mouse Grp170 expressed in insect cells

where only the two predicted glycosylation sites in the NBD of Grp170 were found to be glycosylated (Park et al, 2003).

We next determined the effect of the domain deletions on the ability of Grp170 to interact with BiP, since structural data obtained for the yeast large Hsp70s argued that the α-helical domain at the C-terminus reaches out to embrace the NBD of its partner Hsp70 when acting as a NEF (Polier et al, 2008; Schuermann et al, 2008; Andreasson et al, 2010). A very minor underglycosylated species was detected for all mutants (Figure 14*B*), which was less stable than the fully glycosylated form (data not shown). As we were concerned that this underglycosylated form might interact with BiP as an unfolded substrate instead of a NEF, we performed metabolic labeling experiments with an extended chase period of 16 hrs to allow the underglycosylated species to be degraded due to its shorter half-life compared to the fully glycosylated form (Figure 14*C* and *D*). The effects of the two deletion mutants on association with BiP was determined both in the presence of apyrase (*C*) and ATP (*D*). Under these conditions, both Grp170 deletion mutants bound to BiP as well as the full-length protein, and this association was not disrupted by the presence of ATP (Figure 14*D*) arguing that neither the extended α-helical domain nor the unstructured loop insertion were essential for the BiP-Grp170 interaction *in vivo.* Besides, since the binding of BiP with Grp170 was not regulated by ATP, as expected for a regular substrate interaction, it also indicates that none of these associations represented a chaperone:client interaction.

To assess the effect of the domain deletions on substrate binding, COS-1 cells were co-transfected with the indicated Grp170 constructs along with BiP and the various Ig substrates. Cells were lysed in the presence of ATP to release BiP. To exclude unspecific binding of substrate specific antisera to the Grp170 unstructured loop and α–helical domain deletion mutants, same set of experiments was performed, but instead of the substrates empty pSVL vector was transfected (Figure 15).

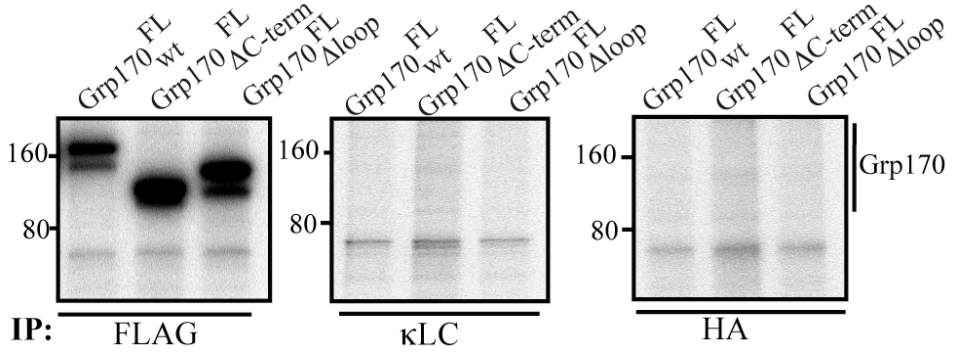

Figure 15. Test of substrate specific antisera for unspecific binding to Grp170 unstructured loop and C-terminal α-helical domain deletion mutants.

COS-1 cells were transfected with FLAG-tagged Grp170 constructs, BiP and empty pSVL vector. Following a 1 hr pulse-label with [^{35}S]cysteine/methionine and 1 hr chase, cells were lysed in the presence of ATP and the cell lysate was equally divided for immunoprecipitation with substrate-specific antisera or FLAG-tag as indicated. Proteins were separated on 10% SDS-gels, followed by autoradiography.

None of the substrate targeting antibodies used to immunoprecipitate substrates bound any of the Grp170 constructs (Figure 15) and thus all detected interactions were specific. When the same set of experiments was performed with substrates analogous data as in Figure 8 was obtained for the interaction of the substrates with wild-type Grp170 (Figure 8A and B) and also neither of the Grp170 mutants bound to the well-folded λCL domain (Figure 16). Interestingly, deletion of the α-helical domain at the C-terminus of Grp170 (Grp170 ΔC-term) appeared to decrease its binding to mHCHA, NS-1 LC and λ LC compared to wild-type Grp170 (Figure 16). A quantitative analysis, where it was feasible due to signal intensities, confirmed a significant reduction in the binding of Grp170ΔC-term to mHCHA and NS-1 LC (Figure 16) relative to wild-type Grp170. In striking contrast, absence of the unstructured loop visibly increased binding of Grp170Δloop to these same three substrates (Figure 16). Quantitative analyses showed that the binding of Grp170Δloop to mHCHA and NS-1 LC was ~2.5 fold greater than for Grp170WT (Figure 16). To rule out any influence from slight variations in the expression levels of the Grp170 mutants, the binding values were normalized to the amount of Grp170 present (see Materials and Methods for details).

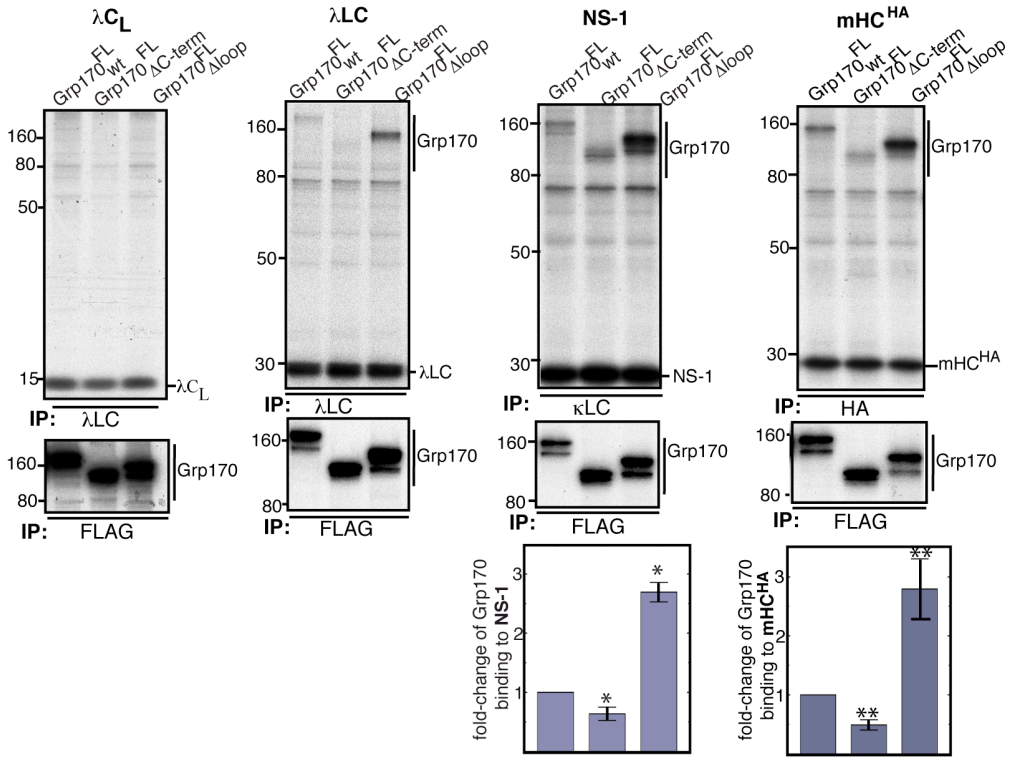

Figure 16. Grp170's unstructured loop and C-terminal α-helical domain modulate substrate binding.

COS-1 cells were transfected with FLAG-tagged Grp170 constructs, BiP and the indicated Ig proteins. Following a 1 hr pulse-label with [^{35}S]cysteine/methionine and 1 hr chase, cells were lysed in the presence of ATP and the cell lysate was equally divided for immunoprecipitation with substrate-specific antisera or FLAG-tag as indicated. Proteins were separated on 10% SDS-PAGE gels, followed by autoradiography. The signals of Grp170 constructs bound to NS-1 and mHC^{HA} were quantified and corrected for the Grp170 and substrate expression levels. The value obtained for Grp170$_{wt}^{FL}$ was set to 1 (NS-1 LC: n=7 ± S.E., *p≤0.007; mHC^{HA}: n=3 ± S.E., **p≤0.02). Adapted and modified from (Behnke & Hendershot, 2014).

These results demonstrate an important role for both of the large Hsp70-specific domains in substrate binding *in vivo*: the α-helical domain at the C-terminus increases substrate binding, whereas the unstructured loop negatively regulates Grp170 binding to substrates. These insights, together with the data demonstrating that Grp170 remains bound to its substrates in the presence of ATP in contrast to BiP, point towards a different regulation of substrate binding to large Hsp70s compared to conventional Hsp70 chaperones.

6.2 Grp170 and BiP show different binding specificities despite common substrates *in vivo*

6.2.1 Engineering of an antibody-derived peptide library to analyze chaperone binding sequences *in vivo*

In spite of the critical importance of ER quality control, many questions remain unanswered. In particular, it is not known how nascent incompletely folded proteins, which will ultimately fold, are distinguished from perpetually unfolded proteins that must be degraded, as many of the same structural features are present on both types of proteins. This is even more striking as some chaperones such as BiP fulfill opposing functions (e.g. ERAD *vs.* folding/secretion) for the same protein. In addition, since Grp170 and BiP bind to the same set of substrates and can even recognize the same molecular forms of some proteins (Chapter 6.1), it remains unclear if and how, these two chaperones distinguish between their set of substrates. Thus, at the heart of ER quality control, specific sequences on nascent proteins must be detected by the various components of the chaperone system to either protect unfolded proteins and allow them to fold or to identify proteins that cannot fold and target them for degradation.

However, almost nothing is known about recognition sequences that dictate these critical and opposing functions. To gain the ability to address these questions, we engineered an *in vivo* peptide display library. It is comprised of overlapping sequences from the V_H and C_H1 domains of an immunoglobulin γ heavy chain (mHC) and the complete non-secreted light chain NS-1 LC; substrates that were previously established to directly interact with both ER Hsp70 superfamily members (see Chapter 6.1). To determine which sequences within these two proteins are recognized by Grp170 and BiP, we divided the mHC and the NS-1 LC into 25 amino acid, overlapping segments. The estimated size of a recognition sequence for conventional Hsp70s based on peptide binding, *in silico*, and H/D exchange studies is ~7-13 amino acids (Blond-Elguindi et al, 1993a; Knarr et al, 1995; Rodriguez et al, 2008; Marcinowski et al, 2013). Therefore, the 25 amino acid stretches are long enough to harbor binding sites for BiP. In order to avoid missing potential binding sites at peptide boundaries, each peptide overlaps by six amino acids on each side (Figure 17*A*).

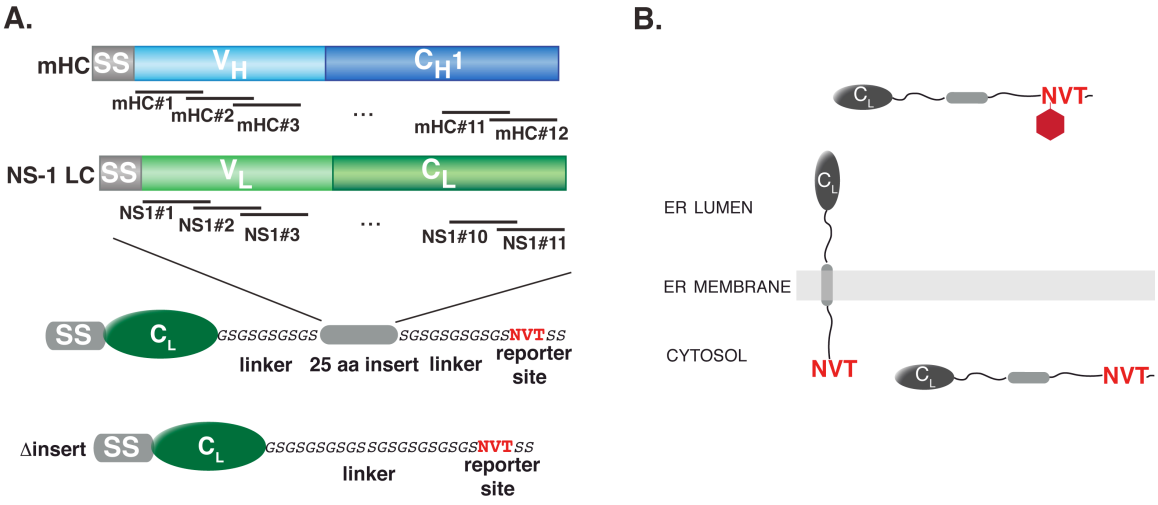

Figure 17. In vivo peptide library to dissect chaperone binding sites in the cell.

(A) mHC and NS-1 LC were divided into 25 amino acid stretches with six amino acid overlaps on each end, resulting in 12 and 11 peptides, respectively. These peptides were inserted into a reporter construct (Feige & Hendershot, 2013), which includes an ER import signal sequence (SS), a C_L domain and an NVT-reporter site which becomes N-linked glycosylated upon entry into the ER lumen (B) and thus allows monitoring the ER entry of the construct.

In contrast to an *in vitro* peptide screen, it is not feasible to use short peptides on their own *in vivo*, since it would be impossible to detect each unique peptide without specific immune reagents and peptides generated by the proteasome in the cell are either loaded onto MHC complexes for immunopresentation or rapidly degraded. To overcome this limitation we inserted the peptides into a recently developed reporter construct, which was constructed to monitor the insertion of the T-cell receptor α (TCRα) transmembrane region into the ER membrane (Feige & Hendershot, 2013). This construct is composed of a well-folded, ER-targeted λ LC constant domain (ER-λC_L), which importantly does not bind BiP (Hellman et al, 1999) or Grp170 (Chapter 6.1), tethered to a flexible GS-linker, followed by the 25 amino acid insert, and ending in a consensus N-linked glycosylation site (NVT), separated by another flexible linker, to monitor translocation into the ER (Figure 17*B*). A construct lacking the 25 amino acid insert (=Δinsert) acts as a negative control to monitor possible unspecific interactions of chaperones with the reporter construct itself.

It is critical that the constructs fully enter the ER, so they can potentially interact with the various lumenal chaperones. This is readily monitored by glycosylation of the

reporter site, as neither the ER-λC_L domain nor any of the mHC or NS-1 LC sequences contains a consensus N-glycosylation site. Coverage of the complete mHC and NS-1 LC resulted in 12 and 11 peptides, respectively (Figure 18). Signal sequences were not included in the peptide library, since these do not enter the ER and hence do not interact with the ER resident soluble chaperones.

A.

	protein	amino acid sequence of the insert	ΔG predicted	# Met/Cys
	mHC			
	mHC_SS	MGWSYIILFLVATATDVHS	1.9	
	mHC_1	QVQLQQPGAELVKPGASVK***LSCKAS***	10.4	-/1
	mHC_2	***LSCKAS***GYTFTSYWMHWVK***QRPGQG***	8.8	1/1
V_H	mHC_3	***QRPGQG***LDWIGEINPSNGR***TNYNEK***	12.1	-/-
V_H	mHC_4	***TNYNEK***FKSKATLTVDKSS***STAYMQ***	11.8	1/-
V_H	mHC_5	***STAYMQ***LSSLTSEDSAVYY***CASYDY***	6.6	1/1
V_H	mHC_6	***CASYDY***DWFAYWGQGTLVT***VSSAST***	6.1	-/1
V_H	mHC_7	***VSSAST***KGPSVFPLAPSSK***STSGGT***	9.8	-/-
V_H	mHC_8	***STSGGT***AALGCLVKDYFPE***PVTVSW***	7.4	-/1
C_H1	mHC_9	***PVTVSW***NSGALTSGVHTFP***AVLQSS***	6.6	-/-
C_H1	mHC_10	***AVLQSS***GLYSLSSVVTVPS***SSLGTQ***	5.6	-/-
C_H1	mHC_11	***SSLGTQ***TYICNVNHK***PSNTKVDKK***	11.6	-/1
C_H1	mHC_12	***PSNTKVDKK***VGQ	12.6	-/-

B.

	protein	amino acid sequence of the insert	ΔG predicted	# Met/Cys
	NS-1 LC			
	NS1_SS	MHQTSMGIKMESQTLVFISILLWLYGADG	3.6	
	NS1_1	NIVMTQSPKSMSMSVGERV***TLTCKA***	8.2	3/1
	NS1_2	***TLTCKA***SENVVTYVSWYQQ***KPEQSP***	11.2	-/1
V_L	NS1_3	***KPEQSP***KLLIYGASNRYTG***VPDRFT***	9.9	-/-
V_L	NS1_4	***VPDRFT***GSGSATDFTLTIS***SVQAED***	7.9	-/-
V_L	NS1_5	***SVQAED***LADYHCGQGYSYP***YTFGGG***	10.6	-/1
V_L	NS1_6	***YTFGGG***TKLEIKRADAAPT***VSIFPP***	9.4	-/-
V_L	NS1_7	***VSIFPP***SSEQLTSGGASVV***CFLNNF***	8.0	-/1
V_L	NS1_8	***CFLNNF***YPKDINVKWKIDG***SERQNG***	13.2	-/1
C_L	NS1_9	***SERQNG***VLNSWTDQDSKDS***TYSMSS***	14.8	1/-
C_L	NS1_10	***TYSMSS***TLTLTKDEYERHN***SYTCEA***	12.3	1/1
C_L	NS1_11	***SYTCEA***THKTSTSPIVKSFNRNEC	10.7	-/2

Figure 18. Peptide sequences of the mHC and the NS-1 LC divided into 25 amino acid stretches.
Amino acid sequences of peptides derived from the mHC (A) and the NS-1 LC (B), divided into 25 amino acid stretches. Amino acids highlighted in bold and underlined indicate the six amino acids overlap between the peptides. Amino acids in red mark the V_H-C_H1 (A) or V_L-C_L (B) domain boundaries. The predicted apparent free energy of membrane insertion (ΔG_{aa}^{pred}), calculated according to (Hessa et al, 2007), is indicated for each peptide. The number of methionines and cysteines is indicated for each peptide since [^{35}S]cysteine/methionine was used in metabolic labeling experiments.

It was possible that the reporter constructs could integrate into the ER membrane instead of entering the ER lumen where they could associate with the molecular chaperones, if the hydrophobicity of the insert was sufficiently high (Hessa et al, 2007). To estimate the chance of this scenario, we calculated the predicted ΔG_{app}^{pred} for membrane insertion for each of the 25 amino acid sequences according to (Hessa et al, 2007) and found all of them to be >5.6, indicating they are highly unlikely to integrate into the ER membrane instead of entering the ER. Consistent with their hydrophobic nature in general, both signal sequences, which were not part of the peptide set, had a relatively low ΔG_{app}^{pred} compared to the rest of the sequences (Figure 18).

To specifically monitor translocation of the peptides into the ER, an N-linked glycosylation consensus sequence at the C-terminus was added. As the purpose of the engineered NVT site was only to monitor full translocation of the sequence into the ER lumen and could divert peptides to the lectin chaperone system, we generated a second set of the same constructs with the NVT site mutated to QVT to prevent glycosylation. The N→Q substitution does not influence the ΔG_{app}^{pred} in the insert region of the reporter construct and thus should not alter its entry into the ER. For this reason, after the initial characterization of the constructs in regard to their ER entry, the peptide library with the N→Q substitution was used to identify binding motifs of Grp170 and BiP, thereby excluding a competition with CNX and CRT.

6.2.2 Characterization of the peptide library in the cellular environment

A crucial test for the feasibility of the *in vivo* peptide library was the demonstration of ER entry for each of the reporter constructs. To this end, constructs with the NVT glycosylation site and inserts ranging from mHC_1 - 12^{NVT} or NS1_1 - 12^{NVT}, respectively, as well as Δinsert were transfected into COS-1 cells, which were metabolically labeled and lysed. After immunoprecipitation of the lysates with antisera targeting the C_L-domain of the reporter constructs, the eluates were divided and half of each was digested with EndoH before separating the proteins on SDS-PAGE gels (Figure 19).

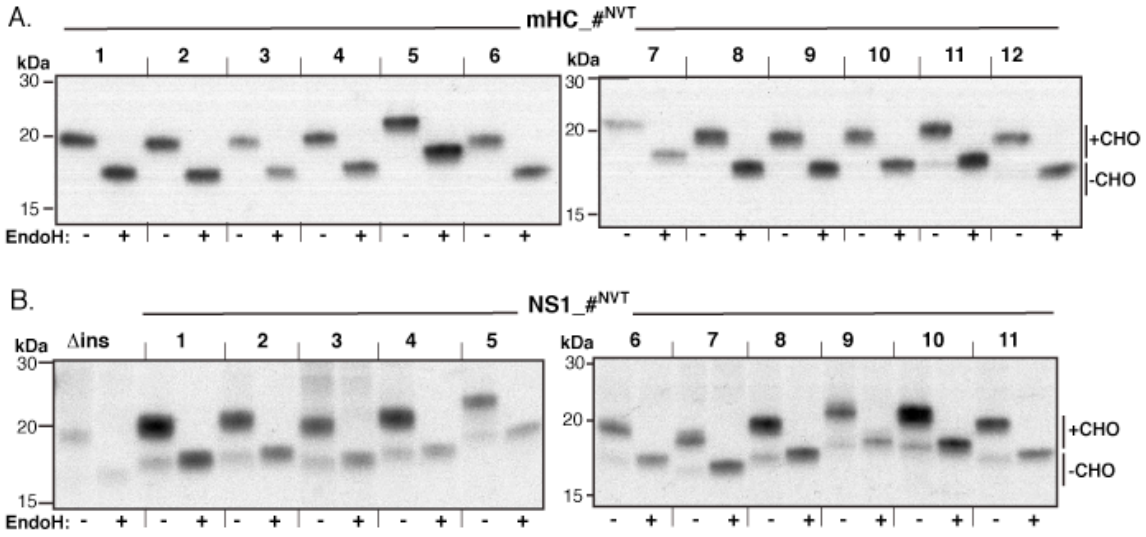

Figure 19. Reporter constructs with mHC_1 – 12^{NVT}, NS1_1 – 11^{NVT} and Δinsert are imported into the ER.

(A) COS-1 cells were transfected with reporter constructs mHC_1 – 12^{NVT}, NS1_1 - 11^{NVT} or Δinsert. After 24 hrs the cells were pulse-labeled with [^{35}S]cysteine/methionine for 1 hr, lysed with RIPA buffer and the cell lysate was immunoprecipitated with antisera against the C_L domain. Half of the eluate was treated with EndoH to remove N-linked sugars and the other half was left untreated. Proteins were separated on 13% SDS-PAGE gels, followed by autoradiography.

Importantly, all constructs migrated more rapidly on the SDS gels upon EndoH digestion, demonstrating that they were N-linked glycosylated and thus imported into the ER (Figure 19). Consequently the peptides are exposed to the chaperones in the ER lumen for potential interaction. Besides, all constructs were expressed well, providing sufficient levels for the analysis of the interaction with other proteins. All were glycosylated with ~80-90% efficiency, which is excellent for a C-terminal NVT site that in general cannot be expected to be glycosylated at 100% due to its C-terminal location (Shrimal et al, 2013), further underscoring the efficiency of their ER entry. To test the properties of the reporter constructs, we analyzed their half-life in the cells and tested their tendency for either secretion or aggregation. Since the constructs with the N-terminal QVT site were ultimately used to analyze their interaction with the different chaperones, we used the reporter constructs with the QVT site at the C-terminus in this set of experiments.

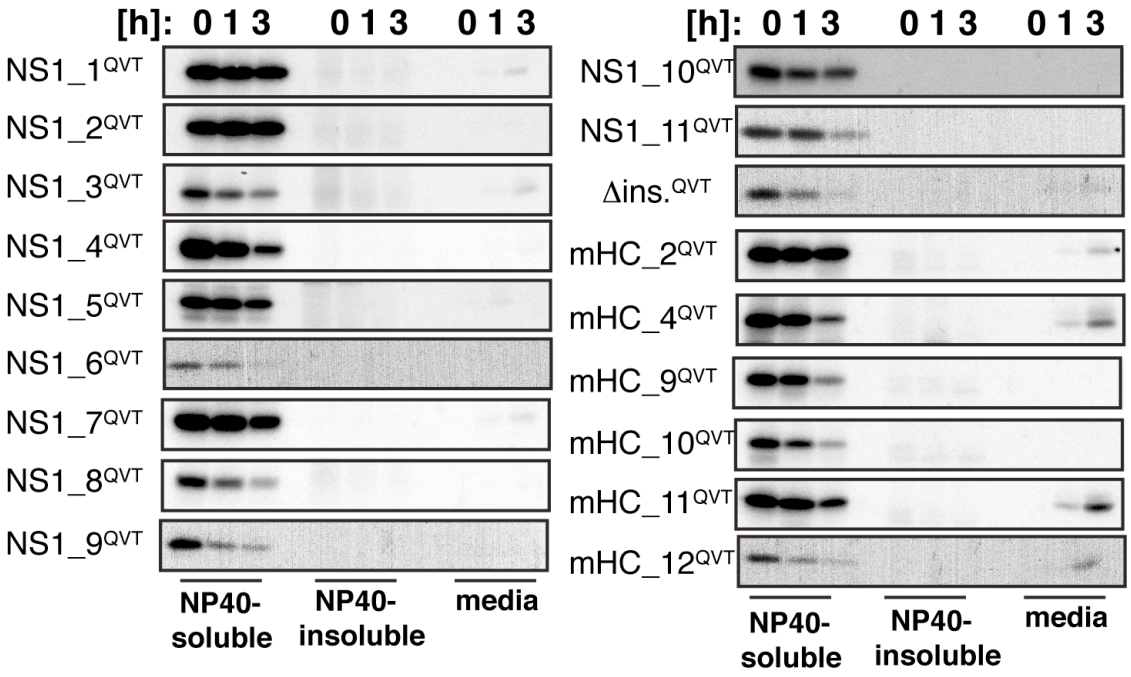

Figure 20. Properties of the QVT-reporter constructs in the cell.

COS-1 cells were transfected with reporter constructs as indicated. After 24 hrs cells were pulse-labeled with [^{35}S]cysteine/methionine for 1 hr and were either lysed directly, or a chase period of 1 hr or 3 hrs was included. The cell lysates (NP40-soluble), pellets (NP40-insoluble) and media were immunoprecipitated with antisera against the C_L domain. Proteins were separated on 13% SDS-PAGE gels, followed by autoradiography.

The analysis of the almost complete set of constructs revealed that none of the analyzed constructs exhibited a tendency to aggregate as monitored by the protein levels in the NP40-insoluble pellets after cell lysis. Interestingly, some constructs were secreted to varying extents (e.g. mHC_2^{QVT} or mHC_11^{QVT}), while other constructs were fully retained in the cell (e.g. NS_4^{QVT} and NS_2^{QVT}) (Figure 20). Also the intracellular half-lives differed somewhat between the constructs: some were relatively stable over time (e.g. mHC_2^{QVT} and NS1_2^{QVT}), while others exhibited a shorter half-life (e.g. mHC_10^{QVT} and NS1_3^{QVT}). Since chaperones orchestrate the degradation and secretion of proteins originating in the ER, the differences in the cellular properties of the various constructs, which are only distinguished by a 25 amino acid insert, could suggest a different engagement of the chaperone machinery determined by the 25 amino acid insert. Nonetheless, all of the tested reporter constructs efficiently entered the ER lumen (Figure 19) and were present at sufficient intracellular levels for analysis of their

interaction with the various ER chaperones (Figure 20). Combined, these features make the reporter constructs a perfect tool to determine the binding sites of Grp170 and BiP *in vivo*.

6.2.3 Grp170 and BiP show different binding patterns within the mHC and the NS-1 LC

The *in vivo* expressed peptide library allowed us to dissect the precise binding sites of Grp170 and BiP within their endogenous substrates mHC and NS-1 LC (Chapter 6.1). Therefore, we transiently expressed mHC_1–12^{QVT} or NS1_1–11^{QVT} peptide constructs in COS-1 cells along with BiP and Grp170. To exclude possible non-specific interactions with the reporter construct itself, we also included the Δinsert^{QVT} control construct. The cells were lysed either in the presence of apyrase or ATP. The ATP-dependent BiP release would provide an additional proof that any observed binding represented normal BiP:client interactions, which are readily disrupted by ATP (Chapter 6.1); whereas the interaction of Grp170 with clients is either not affected or in some cases appears even enhanced in the presence of ATP (Chapter 6.1). To indicate the precise mobility of Grp170 and BiP on the gel, both were immunoprecipitated in addition to the reporter constructs and ran in separate outermost lanes as indicated.

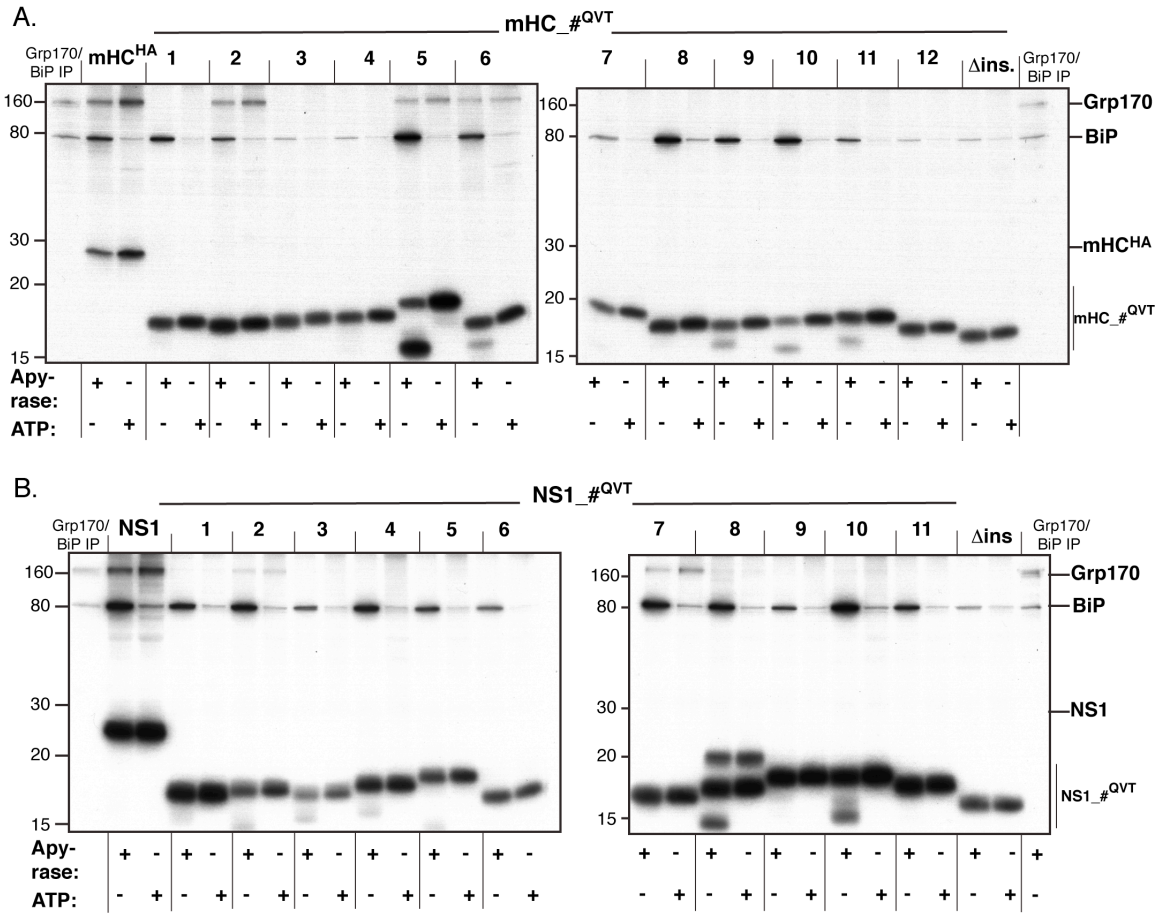

Figure 21. Grp170 and BiP exhibit different binding patterns within their substrates.

COS-1 cells were transfected with reporter constructs mHC_1 – 12^{QVT} (A), NS1_1 – 11^{QVT} , Δinsert or empty vector (B) along with Grp170 and BiP. After 24 hrs cells were pulse-labeled with [^{35}S]cysteine/methionine for 2.5 hrs and lysed in the presence of apyrase or ATP. Cell lysates were immunoprecipitated with antisera against the C_L-domain of the reporter construct, κLC (NS1), HA-tag (mHC^{HA}), Grp170 or BiP (Grp170/BiP IP). Proteins were separated on 13% SDS-PAGE gels, followed by autoradiography. Some constructs show an additional faster migrating band exclusively in the presence of apyrase, which is thus most likely a post-lysis cleavage event of the C_L-domain from the reporter construct, as the same band is absent in all immunoprecipitations in the presence of ATP and can still be recognized by the anti-C_L antibody. In the NS_8QVT immunoprecipitation an additional unspecific band was observed at a slightly higher molecular weight.

The results for the peptide constructs covering the complete mHC and NS1-LC provide several intriguing results. First, in spite of the fact that BiP binds to Ig heavy chains *in vivo* in an approximately 1:1 ratio (Haas & Wabl, 1983; Bole et al, 1986) and that this interaction is restricted to the C_H1 domain (Hendershot et al, 1987), there are many BiP

binding sequences that occur throughout the first two domains of the mHC. This finding is consistent with early *in vitro* peptide binding studies that predicted BiP binding sites were likely to occur ca. every 16 amino acids in a protein (Flynn et al, 1991) and studies suggesting that IgG heavy chains are likely to have many potential BiP binding sites (Knarr et al, 1995) based on an algorithm generated by a bacterial phage display study (Blond-Elguindi et al, 1993a). This argues that the peptides identified using *in vitro* and *in silico* studies are also recognized within the context of the ER lumen, but that many of them are likely to represent transient interactions in the context of the full-length protein and disappear once the domain folds or, in the case of the C_H1 domain, are perhaps mutually excluded due to steric hindrance.

Second, we find that the mHC_7^{QVT} and mHC_9^{QVT} peptides, which include the BiP binding peptides characterized in a recent *in vitro* study of the C_H1 domain (SVFPLAP and HTFPAVL) (Marcinowski et al, 2013) also bind to BiP in our *in vivo* assay. However, BiP binds significantly better to the mHC_8^{QVT} and mHC_10^{QVT} inserts (Figure 21*A*) thus identifying new high affinity BiP binding sites within the C_H1 domain. Third, we detected only few but very distinct Grp170 binding peptides. These appear to be restricted to the V_H region of the mHC, and once again show different strengths of binding, with the mHC_5^{QVT} and mHC_6^{QVT} inserts showing the strongest binding. It is presently unclear if this represents one site that is present on the overlapping portions of these two peptides or if we are detecting two separate sites. Interestingly, both peptides are rich in aromatic residues in keeping with the limited data available on *in vitro* peptide binding for two other cytosolic large Hsp70 (Goeckeler et al, 2008; Xu et al, 2012). Although all of the Grp170 binding peptides also bind to BiP, the converse is not true and in fact the best BiP binding sequences do not interact with Grp170. This demonstrates that sequence preferences between Grp170 and BiP are likely to be distinct. Similar to the mHC, Grp170 associated with only two peptides derived from the NS-1 LC, NS1_2^{QVT} and NS_7^{QVT} (Figure 21B). Again BiP bound throughout the whole sequence with different apparent affinities. Lastly, BiP binding to the peptide inserts is sensitive to ATP addition, whereas that of Grp170 is not, which is in keeping with data obtained on these full-length substrates (see also Figure 8) (Behnke & Hendershot, 2014), further validating the relevance of the peptide library to analyze *in vivo* substrate:chaperone interactions. The data presented in Figure 21 is summarized in Figure 22, where the apparent affinities of peptide inserts for BiP were ranked relative to each other and where the identified Grp170 binding sites are marked.

A.

mHC_#^{QVT}	BiP binding	Grp170 binding
1	++	no
2	+	**yes**
3	-	no
4	-	no
5	+++	**yes**
6	++	**yes**
7	+	no
8	+++	no
9	++	no
10	+++	no
11	+	no
12	-	no

B.

NS1_#^{QVT}	BiP binding	Grp170 binding
1	+	no
2	++	**yes**
3	-	no
4	++	no
5	+	no
6	-	no
7	+	**yes**
8	+	no
9	-	no
10	++	no
11	-	no

Figure 22. Comparison of Grp170 and BiP binding sites.

Apparent BiP binding affinities to peptides in mHC_1-12^{QVT} (A) and NS1_1-11^{QVT} (B) are shown relative to each other. Observed Grp170 binding to the peptides is marked accordingly.

Taken together, an overlapping peptide expression library covering two BiP and Grp170 substrates was engineered and expressed in mammalian cells. This library provided for the first time an *in* vivo tool for the identification of specific peptide sequences that interacted with each of these chaperones. For Grp170 only a few distinct binding peptides were identified, which were restricted to the V_H domain of mHC and one was found in each domain of the NS-1 LC. In contrast, BiP-bound peptides were detected throughout the whole substrate sequence and showed clear differences in binding affinities. This strongly suggests an intriguing difference between these two Hsp70 superfamily members in the recognition of their substrates. Further subdivision and mutation of these sequences will be performed to identify minimal binding sequences and biological consequences of the presence or absence of specific chaperone binding.

6.3 Glycosylation of Grp170 influences its interaction with the ER-associated degradation (ERAD) machinery

6.3.1 Grp170 is a fully glycosylated protein in the ER

The emerging evidence that Grp170 possesses dual functions within the ER, as a NEF and a chaperone, poses the question of how it functions in ERQC. Data from the preceding chapters demonstrated that Grp170 binds to similar substrates as BiP, but that its binding is regulated differently than BiP and perhaps occurs later (Chapter 6.1) and both exhibit different binding patterns within their substrates (Chapter 6.2). These data hint towards a different function of Grp170 compared to BiP, which might require unique networks of interaction partners within the ER for these two chaperones. While a co-chaperone network has been established for BiP composed of seven identified ERdj proteins so far (Otero et al, 2010), similar insights into interaction partners of Grp170 are missing. When analyzing the sequence of Grp170, as well as those of other ER-localized large Hsp70s from many different organisms, we discovered that these are missing the key arginine residue in their NBD, which was shown to be important for the interaction of BiP with ERdj proteins (Awad et al, 2008). Thus, Grp170 is unlikely to interact productively with the ERdj proteins in a BiP-like manner and may instead interact with a distinct set of partners *via* a different mechanism. We hypothesized that identifying interaction partners might help establish Grp170s function in ERQC. One hint towards possible interaction partners was provided by the comparison of the predicted N-linked glycosylation sites of the cytosolic and ER resident human Hsp70 superfamily (Table 1; NPT: an N-linked glycosylation motif that harbors a proline and is thus unlikely to get glycosylated).

Table 1: Number of predicted N-linked glycosylation sites in the mammalian Hsp70 superfamily

localization	conventional and large Hsp70 (UniProt ID)	# of predicted glycosylation sites
ER	BiP (P11021)	0
	Grp170 (Q9Y4L1)	9
Cytosol	Hsp70-1a/b (P08107)	5
	Hsp70-1t (P34931)	5
	Hsp70-2 (P54652)	6 (7 incl. NPT)
	Hsp70-6 (P17066)	4
	Hsc70 (P11142)	6 (7 incl. NPT)
	Hsp110 (Q92598)	7
	Apg-1 (O95757)	4
	Apg-2 (P34932)	4

Although the cytosolic Hsp70 superfamily members (including conventional and large Hsp70s) all have N-glycosylation sequons, none of them is used in the cytosol as the glycosylation machinery exists only inside the ER lumen. In contrast, the ER resident conventional Hsp70 BiP has no consensus glycosylation sites and Grp170 possesses nine. Thus, it is likely that BiP specifically deleted its glycosylation sites in the course of eukaryotic evolution, since both cytosolic and ER resident conventional Hsp70s most likely originated from a gene duplication (Lin et al, 2001). Interestingly, although the ER large Hsp70s are also likely to have evolved from their cytosolic orthologues, they have retained and often even expanded the number of consensus glycosylation sites as compared to their cytosolic orthologues. The absence of glycosylation sites in the conventional ER-localized Hsp70 and often a high number of sites in the large Hsp70 is true not only for the mammalian ER Hsp70 superfamily (Table 1), but also for *S. cerevisiae*, *Drosophila melanogaster, Caenorhabditis elegans and Arabidopsis thaliana* (Table 2; SS: signal sequence). We therefore wanted to address whether the

glycosylation sites in Grp170 play a role in its ER functions and/or its interactions with other ER resident proteins that might help in understanding its biological functions.

Table 2: Number of predicted N-linked glycosylation sites of ER resident Hsp70 superfamily members in different species

species	conventional and large Hsp70 (UniProt ID)	# of predicted glycosylation sites
Saccharomyces cerevisiae	Kar2p (E7KEB7)	0
	Lhs1p (P36016)	7 (8 incl. NPT)
Drosophila melanogaster	Hsc-3 (P29844)	0
	HSP110-3 (O46067)	4
Caenorhabditis elegans	Hsp70-3 (P27420)	0
	Hsp70-4 (P20163)	0
	Hsp110-2 (Q22758)	9
	Hsp110-3 (Q22515)	3
Arabidopsis thaliana	Athsp70-13 (Q8H1B3)	0
	Athsp70-12 (Q39043)	0 (1 within SS)
	Athsp70-11 (Q9LKR3)	0 (1 within SS)
	Athsp70-17 (F4JMJ1)	11

To begin to address this question, an analysis of Grp170´s actual glycosylation status in the cell was required first, because recombinant human Grp170 isolated from insect cells (Park et al, 2003) was only modified on the two sites in its NBD, making it unclear whether Grp170 is indeed a fully glycosylated protein in the ER. We surmised that glycosylation of only two (within the NBD) out of the nine predicted glycosylation sites, which occurred when Grp170 was expressed in insect cells (Park et al, 2003), might be due to its expression in a non-native host. Therefore we first determined the glycosylation status of full-length Grp170 when it was expressed in its authentic environment of the mammalian ER (Figure 23).

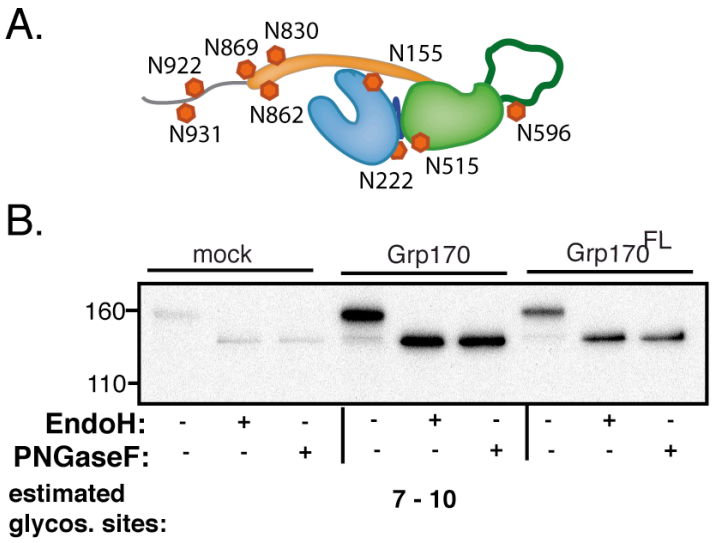

Figure 23. Predicted glycosylation sites and glycosylation status of Grp170 in cells.

(A) Schematic of the structural organization of Grp170 (blue: NBD, magenta: linker, green: β-sheet domain and unstructured loop insertion, orange: α-helical domain, orange hexagons: predicted N-linked glycosylation sites). (B) COS-1 cells were transiently transfected with BiP, Grp170 either with or without a FLAG-tag, as indicated, along with empty pSVL vector. Cells of the mock sample were transfected with an empty pcDNA3.1(+) vector only. After a pulse-label with [^{35}S]cysteine/methionine and a chase of 1 hr each, cells were lysed in the presence of ATP and immunoprecipitated against Grp170 (mock and Grp170) or the FLAG-tag (Grp170^{FL}). The eluted protein was equally divided for undigested control, EndoH and PNGaseF digest. The proteins were separated on 10% SDS-PAGE gels, followed by autoradiography. The number of glycosylation sites was estimated based on the molecular weight shifts of the EndoH/PNGaseF digested samples compared to undigested control.

When FLAG-tagged and untagged human full-length Grp170 was over-expressed in transfected COS-1 cells, we detected a doublet for both the untagged and FLAG-tagged protein in the untreated samples, one band migrating with the endogenous protein and one that migrated faster (Figure 23*B*) as described previously (Figure 14). Digestions of the samples were carried out with the glycosidases EndoH, which removes glycans that were not modified in the Golgi, and PNGaseF, which cleaves at the innermost GlcNAc and the asparagine residue, also in the case of sugars that have been modified in the Golgi. The data revealed that the major upper band corresponds to the fully glycosylated, ER localized form and the minor band represents an unglycosylated form, which corresponded to the fully glycosylated and unglycosylated form of endogenous Grp170 (Figure 23*B*), respectively. The addition of a FLAG-tag allowed us to distinguish

between transfected and endogenous Grp170 and to subsequently analyze deletion mutants. The small gel shift of 2-3kDa per glycosylation site compared to the large molecular weight of Grp170 made it difficult to assess whether all of the Grp170 sites were glycosylated, although the molecular weight change upon deglycosylation suggested that it were likely to be many more than two sites. Therefore we used the same experimental methodology on Grp170 domain deletion mutants that would reveal, which domains were modified by glycosylation and their smaller size would make 2-3 kDa changes in molecular mass easier to detect (Figure 24).

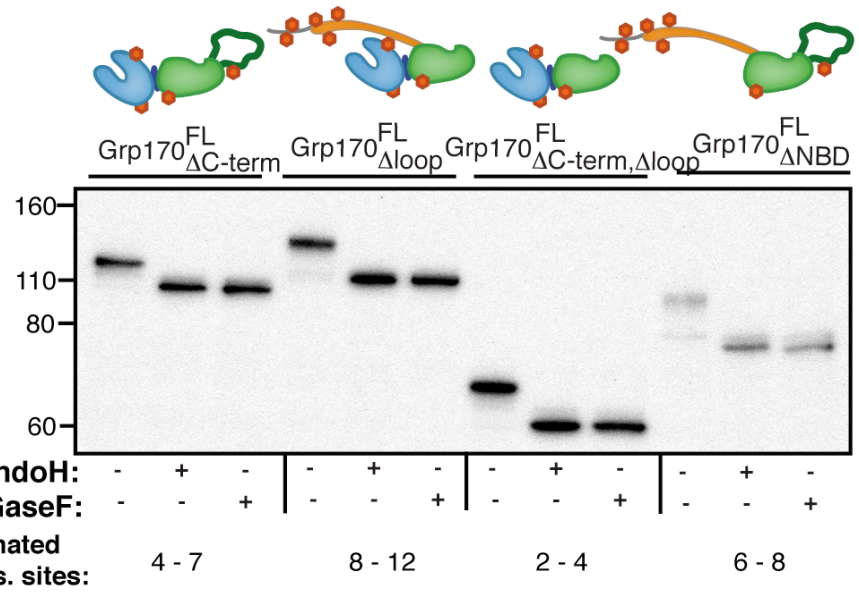

Figure 24. Grp170 domain deletion mutants show that Grp170 is glycosylated throughout all domains.

COS-1 cells were transiently transfected with BiP, Grp170 domain deletion mutants, along with empty pSVL vector. After a pulse-label with [^{35}S]cysteine/methionine and a chase of 1 hr each, cells were lysed in the presence of ATP and immunoprecipitated against the FLAG-tag. The eluted proteins were equally divided for an undigested control, EndoH and PNGaseF digests. The proteins were separated by 10% SDS-PAGE, followed by autoradiography. The number of glycosylation sites was estimated based on the molecular weight shifts of the EndoH/PNGaseF digested samples as compared to undigested controls.

These data confirmed that Grp170 expressed in mammalian cells is modified at multiple sites throughout the protein, and that the C-terminus is particularly heavily glycosylated (Figure 24). We found that all constructs exhibited small amounts of a faster migrating band, which was not affected by EndoH or PNGaseF digestion. In the case of the

deletion mutants with a smaller molecular mass, where separation by SDS-PAGE performs better, this band ran slightly slower than the deglycosylated form (Figure 24) suggesting it might represent a species with an uncleaved signal sequence that was most likely to be cytosolic. In conclusion, our data suggest that Grp170 is a fully glycosylated chaperone in the ER with most, if not all, predicted glycosylation sites being modified. The fact that all glycosylation is EndoH sensitive and that PNGaseF digestions produces the same mobility pattern (Figure 24) demonstrates that the individual mutants and full-length Grp170 remained in the ER as opposed to being processed in the Golgi for eventual secretion. Establishing Grp170 as a fully glycosylated protein enabled us to generate a set of Grp170 glycosylation mutants to address the question of whether these sites play a role in its interaction with other members of the ER resident chaperone machinery.

6.3.2 Specific glycosylation sites in Grp170 modulate its interaction with the ERAD machinery

To analyze a possible role for Grp170s glycans in its interaction with other resident ER proteins, an initial set of Grp170 glycosylation mutants was produced that included all nine glycosylation sites individually removed by an N to Q point mutation within the predicted glycosylation sites and one mutant with all nine sites simultaneously mutated ($Grp170_{\Delta glyco}$). Since Grp170 is a NEF for BiP and both proteins interact with the same set of substrates (Figure 8), we first investigated whether the removal of any specific glycosylation site would interfere with the ability of Grp170 to interact with BiP. To analyze this, COS-1 cells were transfected with BiP along with the Grp170 glycosylation mutants, metabolically labeled and subjected to co-immunoprecipitation (Figure 25).

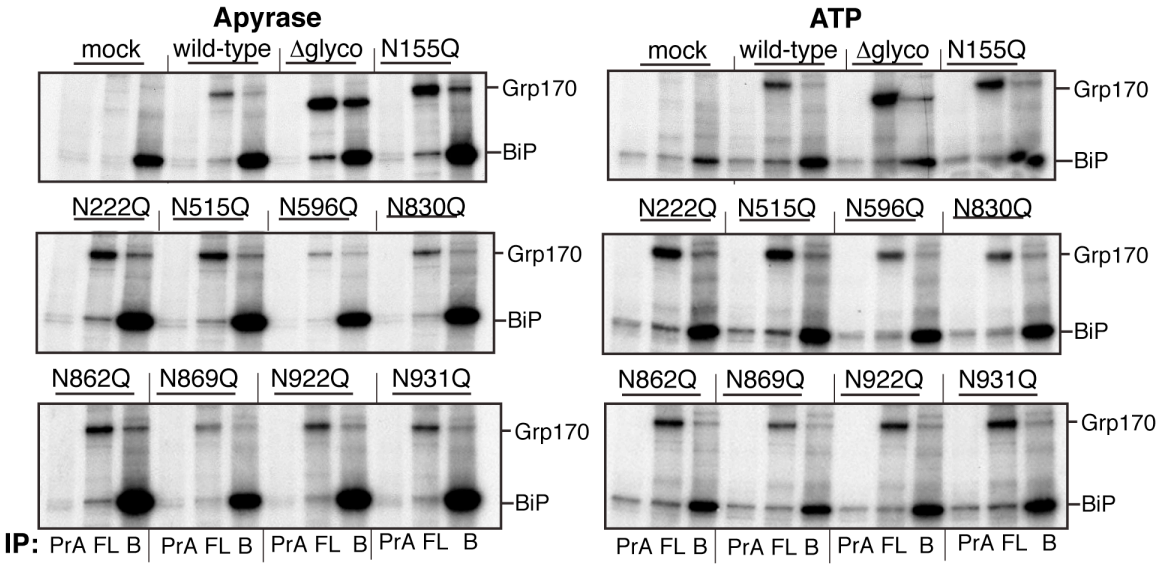

Figure 25. Deletion of Grp170 glycosylation sites does not disrupt binding to BiP.

COS-1 cells were transiently transfected with BiP and Grp170 glycosylation mutants. After a pulse-label with [^{35}S]cysteine/methionine for 5 hrs and a chase for 16 hrs, cells were lysed in the presence of apyrase or ATP. The cell lysates were equally divided for immunoprecipitations with ProteinA only (PrA), against the FLAG-tag (FL) or BiP (B). The proteins were separated by 10% SDS-PAGE, followed by autoradiography.

All of the Grp170 glycosylation mutants could be co-immunoprecipitated with BiP and *vice versa* in the presence of apyrase and ATP (Figure 25). Some minor variations in the levels of co-immunoprecipitations were detected for several of the constructs, which are likely to be due to different expression levels. Importantly, the binding of all the Grp170 constructs, except perhaps that of Δglyco, with BiP was not disrupted with ATP arguing that they represented normal chaperone:chaperone interactions and not client:chaperone interactions. The presence of BiP in the ProteinA control when cells were lysed with ATP occurs in all samples including mock transfectants, and thus likely represents a non-specific binding of BiP to ProteinA. For this reason, in the ATP-supplemented samples, the co-immunoprecipitation of Grp170 in the anti-BiP lanes is more informative.

A recent study on Lhs1p, the yeast homologue of Grp170, revealed that the chaperone but not the NEF function of this large Hsp70 was important for the degradation of αENaC, the α–subunit of the epithelial sodium channel (Buck et al, 2013), suggesting a

role in ERAD. Additionally, two ERAD-specific proteins OS-9 and XTPB-3 have MRH domains that bind high mannose containing glycans on ERAD substrate proteins and which are important for their interaction with the ERAD adaptor protein Sel1L (Cormier et al, 2009; Christianson et al, 2012; Sun et al, 2014). As Grp170 possesses numerous high mannose N-linked glycans, this led us to investigate whether Grp170 associates with any of these ERAD associated proteins, and whether the glycosylation status of Grp170 plays a role in this interaction. COS-1 cells were transfected with the individual FLAG-tagged Grp170 mutants and after cell lysis were subjected to immunoprecipitation coupled to immunoblot experiments. We began by investigating the interaction of the various glycosylation-mutants with endogenous Sel1L, which is a central component of the ERAD complex.

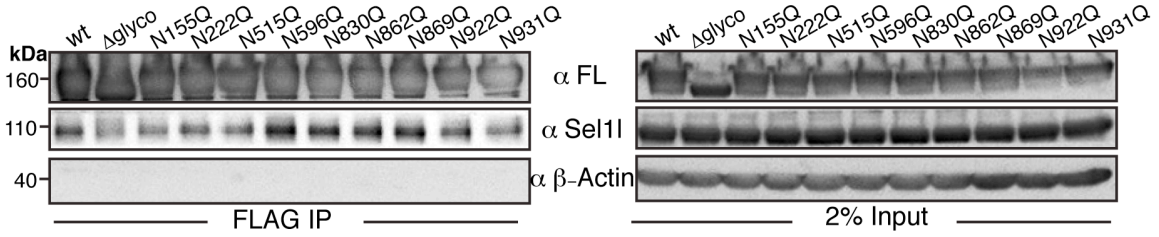

Figure 26. Grp170 interacts with endogenous Sel1l and simultaneous deletion of all Grp170 glycosylation sites disrupts this interaction.

COS-1 cells were transiently transfected with FLAG-tagged Grp170 constructs as indicated. After cell lysis, the cell lysates were immunoprecipitated against FLAG to analyze co-immunoprecipitation of endogenous Sel1L with the various Grp170 constructs. The proteins were separated on 10% SDS-PAGE and subsequently subjected to immunoblotting.

The co-immunoprecipitation of endogenous Sel1L with significant amounts of transfected wild-type Grp170 suggested that this could represent a functional interaction as opposed to a small pool of misfolded Grp170 within the over-expressed pool (Figure 26). This possibility is further supported by the finding the several of the glycosylation mutants appear to reduce the interaction with Sel1L and thus might implicate glycan interactions in this binding. We next designed a second set of Grp170 glycosylation mutants (Figure 27), which included the deletion of its C-terminal cluster of glycosylation sites, as well as several mutants focusing on dissecting the role of glycosylation sites on the NBD, since only these sites were glycosylated when Grp170 was produced in insect cells (Park et al, 2003).

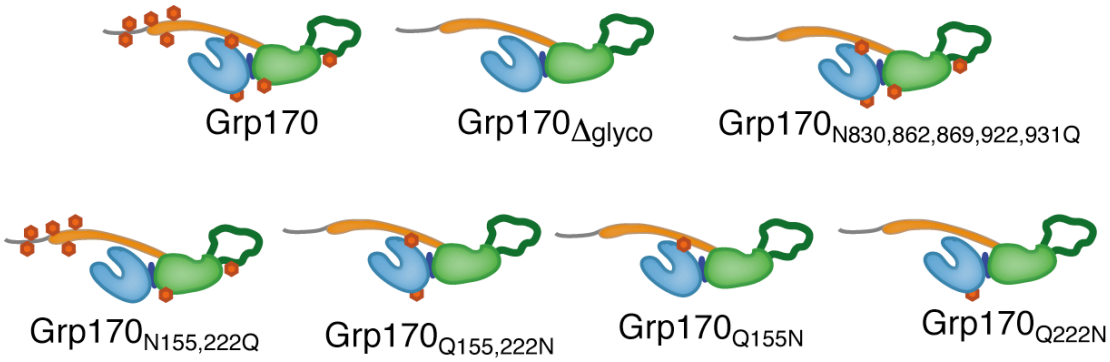

Figure 27. Overview of Grp170 multi-site glycosylation mutants.

Grp170 is depicted in schematic representation with orange hexagons showing the nine predicted glycosylation sites. In $Grp170_{\Delta glyco}$ the N in all of these sites were mutated to Q using FLAG-tagged wild-type Grp170 construct as a template. The $Grp170_{N830,862,869,922,931Q}$ and $Grp170_{N155,222Q}$ were generated *via* the same method where only the indicated amino acids were mutated. $Grp170_{Q155,222N}$, $Grp170_{Q155N}$, $Grp170_{Q222N}$ were generated using $Grp170_{\Delta glyco}$ as a template and only the indicated glycosylation sites were restored.

These Grp170 mutants enabled us to analyze whether glycosylation sites in specific areas within Grp170 are important for its association with Sel1L as opposed to single sites. As before (Figure 26), the interaction of Sel1L with Grp170 multi-site glycosylation mutants was investigated by immunoprecipitation with anti-FLAG antibody coupled to immunoblotting with either anti-FLAG combined with anti-Grp170 or anti-Sel1L.

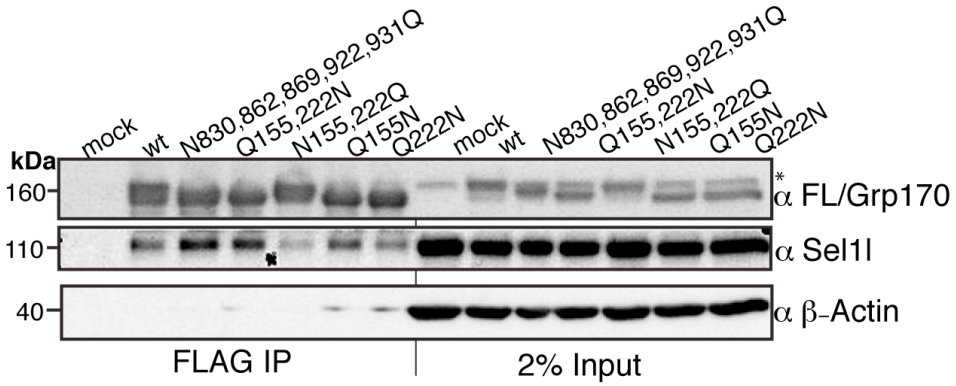

Figure 28. Mutation of both glycosylation sites in the NBD of Grp170 affects its association with Sel1L.

COS-1 cells were transiently transfected with FLAG-tagged Grp170 constructs as indicated. After cell lysis, the cell lysates were immunoprecipitated against FLAG. The proteins were separated on 10% SDS-PAGE and subsequently subjected to immunoblotting as indicated. The asterisk marks the running height of endogenous Grp170.

Mutation of the five glycosylation sites clustered at the C-terminus, $Grp170_{N830,862,869,922,931Q}$, did not inhibit interaction with Sel1L but actually enhanced it slightly as did mutation of all sites except the two in the NBD, $Grp170_{Q155,222N}$ (Figure 28). In keeping with the importance of glycosylation of these two residues in Sel1L interaction, when only these two sites were mutated together, $Grp170_{N155,222Q}$, the co-immunoprecipitation of Sel1L was significantly reduced (Figure 28). Interestingly, the presence of either of the glycosylation sites on the NBD appears to restore the interaction with Sel1L. This evidence combined with the fact that Sel1L does not possess glycan-recognition domains, opened up an intriguing question of whether a glycan-binding factor might serve as a scaffold connecting Grp170 and Sel1L. Either OS-9 or XTPB-3 are good candidates (Christianson et al, 2008) and will be examined in further studies. Taken together, an intriguing relationship between the glycosylation sites on the NBD of Grp170 and Sel1L interaction was established, and might help to explain why ER resident conventional Hsp70s deleted their glycosylation sites, while large Hsp70s maintained and often even expanded the number of these sites.

7 Discussion

7.1 Function of Grp170 as a chaperone and its regulation

In the first part of the thesis, studies were undertaken to explore the role of Grp170 as a chaperone *in vivo*. Using transient transfection experiments and immune cell lines, Grp170 was found to interact with a number of partially folded BiP substrates *in vivo*. To determine if Grp170 was binding to these substrates *via* its interaction with BiP or independently, we exploited the ability of ATP to induce release of BiP from isolated complexes. Upon release of BiP, Grp170 remained associated with its substrates. This led us to conclude two things; that (i) Grp170 is not always interacting with substrates *via* BiP; and (ii) that unlike BiP, Grp170s association with substrates is not regulated by adenosine nucleotides as Grp170 bound to substrates both in the presence of apyrase, which hydrolyzes ATP to AMP and upon addition of ATP. These data are compatible with studies showing that recombinant yeast Grp170, Lhs1p, could suppress the aggregation of denatured luciferase regardless of whether it was in the apo-form or had ADP or ATP present in its NBD (de Keyzer et al, 2009). In support of this, unlike conventional Hsp70s, the ADP- versus ATP-bound form of Lhs1p did not give distinct protease protection patterns, which in the case of Hsp70s reflects changes in the interaction between the NBD and SBD that in turn affect substrate binding (de Keyzer et al, 2009), although there may be some species-specific differences (Raviol et al, 2006a).

The fact that Grp170's binding is not dependent on BiP argues for direct substrate binding, which is consistent with a number of *in vitro* studies conducted with purified recombinant mammalian Grp170 (Park et al, 2003), yeast Lhs1p (de Keyzer et al, 2009), and other cytosolic large Hsp70s (Oh et al, 1999; Goeckeler et al, 2002; Polier et al, 2008; Polier et al, 2010), which showed that these large Hsp70s prevented the aggregation of denatured luciferase. Thus we next began to explore how the interaction of Grp170 with substrates might be regulated and turned our attention to the unique features of the large Hsp70s, which include an unstructured loop that is inserted into the NBD of all large Hsp70s, an α-helical domain, which follows their β-sheet domain (Polier et al, 2008), and an unstructured C-terminal domain. Previously, based on structural data, the α-helical domain was shown to form a major interaction site with the Hsp70

NBD and to be important for Sse1p's nucleotide exchange activity (Polier et al, 2008; Schuermann et al, 2008). A similar interaction between ER resident large and conventional Hsp70s was also demonstrated in cross-linking studies (Andreasson et al, 2010). However, we found that deletion of the α-helical domain of Grp170 did not disrupt Grp170´s interaction with BiP *in vivo* (Figure 14), but instead significantly reduced its interaction with substrates (Figure 16). In support of a role of this C-terminal α-helical domain in substrate interaction another *in vitro* study demonstrated that a purified protein construct corresponding to only this C-terminal region of murine Grp170 prevented luciferase aggregation (Park et al, 2003). Thus, it is possible that this α-helical domain at the C-terminus could exist in different conformations. One in which it interacts with the NBD of BiP to function as a NEF and another where it forms a lid of some sort over the bound substrate to act as a chaperone. Importantly, the available crystal structure was obtained for a complex of Sse1 with the NBD of Hsp70, but there are no structural data for a large Hsp70 bound to a substrate or peptide to address what happens to the α-helical bundle under this circumstance.

The second domain characteristic for large Hsp70s is the acidic unstructured loop insertion in the β-sheet domain whose function has remained completely unclear. Human Hsp110 deficient in this domain exhibited an increased ability to maintain luciferase in a soluble form but decreased its ability to refold it in the presence of reticulocyte lysates which contains conventional Hsp70s (Oh et al, 1999). In keeping with this finding, when this NBD insertion was deleted in yeast Sse1p, its ability to support luciferase refolding *in vitro* in conjunction with Hsp70 was also diminished (Polier et al., 2008). This data might suggest enhanced binding of Hsp110 and Sse1p to luciferase but reduced release, which would be consistent with our finding that deletion of this domain from Grp170 resulted in strongly enhanced binding to all of the analyzed Ig substrates (Figure 16). It is conceivable that the unstructured loop occludes the SBD (*e.g.,* when Grp170 is functioning as a NEF for BiP), and that the removal of this region exposes the SBD thereby increasing its affinity for substrates. Since the mechanism for substrate release remains unknown for all large Hsp70 family members, it is also possible that the unstructured loop plays a critical role in substrate release. To provide an answer to these alternatives further structural studies with a large Hsp70 bound to either a peptide or a substrate as well as identification of potential co-factors for these large chaperones will be required.

7.2 Identification of substrate binding sites of the ER resident Hsp70 superfamily members

The fact that our substrates bound to both BiP and Grp170, an that both chaperones even recognized the same molecular forms of the analyzed substrates (Figure 9 and Figure 10), led us to ask if the binding of these two chaperones might be sequential leading to different fates for the substrate or if they each interacted with distinct sites on the substrates or a combination of both. To begin to answer these questions, it was necessary to identify the substrate binding sites for these chaperones. Previously, a number of studies were designed to define the sequence binding specificity of several molecular chaperones. All involve either the *in vitro* binding of chaperones to peptides (Flynn et al, 1991; Blond-Elguindi et al, 1993a; Rudiger et al, 1997; Davis et al, 1999; Rudiger et al, 2001) or proteins (Rodriguez et al, 2008; Kota et al, 2009; Marcinowski et al, 2011) or the *in silico* analysis of peptide interactions with chaperones (Srinivasan et al, 2012; Marcinowski et al, 2013). However, given the high concentration of unfolded proteins in the ER and its unique chemical environment, *in vivo* binding studies are even more pertinent for determining the specificity of resident ER chaperones and co-factors. In addition, the biological consequence of sequence binding specificity, the presence or absence of binding sites for specific chaperones and its role in ER quality control can only be determined *in vivo.*

Thus, we established an *in vivo* peptide expression library that covered the V_H and C_H1 domains of the heavy chain (mini-HC) and the entire NS-1 LC and used it to determine the *in vivo* binding patterns of both BiP and Grp170 for the first time. Even before identifying actual minimal binding sequences, data obtained with our *in vivo* peptide expression system already allowed us to draw a number of conclusions. First, we found that when these two proteins were deconstructed, BiP bound to multiple peptides throughout the sequences, including the C_L and V_H domains, which are well-folded and oxidized *in vivo* in the context of the full-length proteins. The fact that binding of BiP to these peptides was sensitive to ATP addition argues that this represented a physiological interaction. While the peptides recognized in the V_H domain might reflect sequences that transiently interact with BiP prior to folding, those in the C_L domain are not likely to be used *in vivo* in a full-length LC that folds too rapidly and stably for BiP to bind (Hellman et al, 1999). The large number of possible binding sites for BiP throughout these proteins is in keeping with a previous study, which used a BiP-binding algorithm to

predict possible binding sequences in antibody molecules (Knarr et al, 1995). Importantly, it appears that BiP binds to the C_H1 domain in a 1:1 ratio both *in vivo* and *in vitro,* arguing that although multiple BiP binding sequences may exist, only one is used at a time in a given molecule.

Second, the apparent affinities for BiP binding varied significantly, which in several cases were even stronger compared to the complete mini-HC. Of particular interest are those in the C_H1 domain, which is known to comprise the BiP binding site in the full-length HC (Hendershot et al, 1987), as this domain remains unfolded in the absence of LC assembly (Lee et al, 1999; Feige et al, 2009). In preceding studies, two BiP binding sites were identified on the C_H1 domain of a γ_1 HC using biophysical binding studies, and mutation of a single amino acid in both of these sites resulted in a recombinant C_H1 protein that no longer bound to BiP *in vitro* (Marcinowski et al, 2013). These two sequences are encoded in mHC_#7 and mHC_#9, respectively, demonstrating that our *in vivo* BiP binding assay is detecting binding when it was also observed *in vitro*. However, when we introduced the same mutations into the mini-HC and analyzed it in mammalian cells, it bound BiP as well as the wild-type mini-HC and was retained in the ER. This suggested that additional BiP binding sites might exist that were masked on the C_H1 domain used in the *in vitro* experiments, which contained the intradomain disulfide bond, unlike the C_H1 domain of the unassembled mini-HC that remains reduced *in vivo* (Lee et al, 1999). Consistent with this observation, we found two additional BiP binding sites within the C_H1 domain (mHC_#8 and mHC_#10) that exhibited a significantly stronger relative BiP binding affinity than the two sites detected *in vitro*. Of note, the first of these peptides contains the cysteine residue that forms the intradomain disulfide, and thus a potential site might have been masked by disulfide bond formation in the *in vitro* study.

The identification of strong BiP binding sites in the V_H domain is also noteworthy. The variable domains of both the HC and the LC are formed by genetic rearrangements and undergo addition of non-templated bases and hypermutation, which increase the likelihood of producing a domain that will be unable to fold readily. The presence of a BiP binding site in mHC_#1 and mHC_#2 correspond to framework regions that are not subject to mutation and thus could serve to monitor folding of the VH domain. Similarly, a strong BiP binding site was identified in mHC_#5 and mHC_#6, which also correspond to the framework of the V_H domain and include in their overlapping region the cysteine residue that will form the intradomain disulfide bond upon folding. Thus the peptides

detected in the V_H region are well positioned to monitor folding of the variable domain of the HC.

Unlike conventional Hsp70s for which a large amount of *in vitro* data were available on size of binding sites and specificity, very little is understood about the size or composition of binding sites detected by any large Hsp70. The only evidence in that regard so far is based on two *in vitro* studies with cytosolic large Hsp70s using a very limited number of peptides that demonstrated a preference of large Hsp70s for aromatic residues (Goeckeler et al, 2008; Xu et al, 2012). Third, we found that in contrast to BiP, only a few defined Grp170 binding sites were detected in each of our substrates using the *in vivo* peptide expression panel. Whether some of the adjacent binding sites are actually distinct Grp170 binding sites or are located in the overlapping region would require further fragmentation of the peptides into shorter sequences as the size of a large Hsp70 binding site has not been determined for any family member. Fourth, although BiP also bound to all of the constructs that co-immunoprecipitated Grp170, the apparent affinity of BiP binding did not correlate with the ability of Grp170 to bind, as the strongest BiP binding peptides did not interact with Grp170, arguing for different sequence specificities for these two chaperones. Consistent with the limited data that are available on large Hsp70 sequence preferences (Goeckeler et al, 2008; Xu et al, 2012), we found that the three Grp170 binding peptides identified in the V_H domain were particularly enriched in aromatic residues and both the V_L (NS1_#2) and C_L (NS_#7) domain peptides included at least 3 aromatic residues.

Perhaps the most interesting finding is the location of the Grp170 binding sequences in these two proteins. Intriguingly, Grp170 did not bind to any sequence located in the C_H1 domain, which is known to remain unfolded in the context of the full-length unassembled HC. This domain has evolved to be the center of ERQC for immunoglobulin assembly (Hendershot et al, 1987; Lee et al, 1999; Feige et al, 2009), and perhaps surprisingly, even though this represents a partially unfolded protein, unassembled γ HCs have a very long half-life in cells (> 12hr). Since the chaperone function of Grp170 has recently been implicated in the ERAD of the α-ENAC subunit (Buck et al, 2013), this raises the very interesting possibility that the C_H1 domain specifically evolved not to contain Grp170 interaction sites to provide it with a longer time frame for finding and combining with a LC. Instead, Grp170 interaction sites were found in the VH domain, which folds independent of LC assembly for this particular HC. As we detected *in vivo* association of Grp170 with the mini-HC (Behnke & Hendershot, 2014), it

is likely that the V_H domain had to unfold to be recognized. Similarly, we found that full reduction of the NS-1 LC significantly increased the binding of Grp170 to this substrate. Thus in both cases, unfolding of the "well-folded" domain is likely to be required for significant Grp170 binding, which may then target the substrate for ERAD.

7.3 Detecting interaction partners of Grp170 that might serve to specify its functions within the ER

Establishing Grp170 as a chaperone together with striking differences in the regulation of this function compared to conventional Hsp70s and the absence of a conserved arginine in the NBD for interaction with ER localized DnaJ-like proteins poses the intriguing question, which lumenal proteins interact with Grp170 to help specify its function. Intriguingly, when we compared the glycosylation status of ER resident large Hsp70s to the conventional Hsp70, it revealed that all conventional Hsp70s got rid of their glycosylation sites during the evolution, whereas large Hsp70s retained or even expanded the number of their glycosylation sites. This pattern was conserved from *A. thaliana*, *S. cerevisiae*, *D. melanogaster*, *C. elegans* (Table 2) to humans (Table 1). Because glycan recognition plays a critical role in ERAD and because Grp170 was demonstrated to play a role in ERAD (Buck et al, 2013), we wanted to address the question of whether the heavy glycosylation of Grp170 plays a role in its interaction with other proteins involved in the ERQC. To this end we generated a series of Grp170 glycosylation mutants. The selection of possible candidates was guided by recent studies demonstrating that the glycosylation status of Sel1L was important for its association with EDEM1, OS-9 and XTP3-B, which are all involved in the ERQC process (Christianson et al., 2008; Cormier et al., 2009). Our initial set of experiments focused on demonstrating the interaction of Sel1L with Grp170. Consistent with recent evidence that Grp170 is involved in ERAD (Buck et al., 2013), in our preliminary experiments we found that Grp170 interacted with Sel1L, and that this interaction appeared to be influenced by the presence of glycosylation sites in the NBD of Grp170 (Chapter 6.3). Clearly many more experiments are required to confirm and expand this possibility. However, this preliminary, but intriguing finding, raises the possibility that glycosylation of Grp170 allows it to connect with the glycan recognition machinery to target non-glycosylated

substrates for degradation, further underscoring the importance of the different substrate binding specificity of BiP and Grp170.

7.4 Towards an understanding of the role of Grp170's structural features in its functions.

In order to obtain a comprehensive overview of Grp170 functions *in vivo*, the expression of whole domain deletion mutants, as well as nucleotide binding and glycosylation mutants in cells should provide helpful insights. However, due to the presence of endogenous Grp170, any possible functional deficiencies of Grp170 mutants are likely to be masked by endogenous Grp170. To decrease endogenous levels of Grp170 but at the same time to be able to introduce various Grp170 mutants, we designed several siRNAs targeting the 3´untranslated region (3´ UTR) of Grp170, which did not affect the open reading frame (ORF) of the Grp170 gene. After validation and selection of the most efficient siRNA, stable COS-1 cell clones expressing doxycycline-inducible shRNA specific for the 3' UTR of Grp170 mRNA were established (see Materials and Methods for details) and 3 single cell clones were selected in which a ~90% reduction in Grp170 expression can be sustained for several days (Figure 29*A*). These clones give us a unique tool to begin to dissect Grp170s roles as a chaperone *vs.* its function as a NEF for BiP in protein folding, maintenance of substrate solubility, and targeting proteins for degradation.

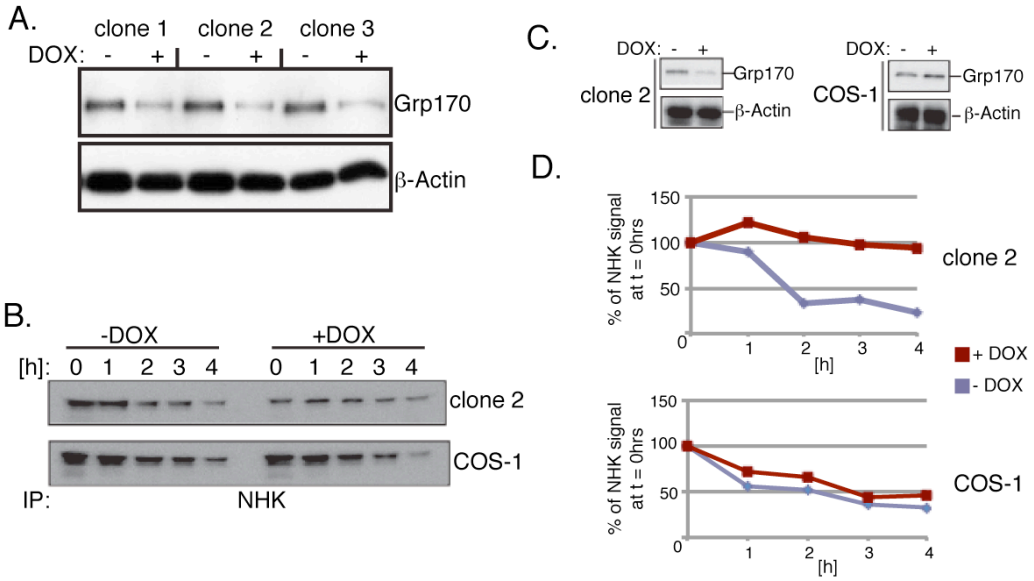

Figure 29. Grp170 knock-down induced by DOX increases the half-life of NHK.

(A) Stable COS-1 cell lines expressing the Dox-inducible shRNA targeting the 3´UTR of the Grp170 gene were plated and kept in 5 µg/ml doxycycline for 5 days. Subsequently, cells were lysed and 5 µg total protein of the lysates were loaded for the immunoblot analysis of Grp170 levels. (B) Stable Grp170 knock-down cell lines (clone 2) or COS-1 cells were kept in DOX (+DOX) or normal media (-DOX) for 5 days and transfected with NHK on day 4. After a pulse-label for 30min, cells were chased as indicated. After cells lysis in NP40 lysis buffer, NHK was immunoprecipitated, separated on 10% SDS-PAGE followed by autoradiography. (C) Grp170 levels were determined via immunoblotting on day 5 of the pulse-chase experiments in cells with or without DOX. (D) The half-life of NHK in cells with or without DOX was determined by quantifying the autoradiography signal in (B) with ImageQuantTL and setting the NHK value at time point 0 to 100%.

Taken together, within this Thesis Grp170 could be established as a chaperone, that binds directly to unfolded proteins *in vivo*. This interaction is modulated by intramolecular mechanisms, which involve domains of Grp170 that set large Hsp70s apart from conventional Hsp70s and occurs both in the presence and absence of ATP. Intriguing evidence was collected that Grp170 may associate with the central ERAD factor Sel1L and that the glycosylation sites of Grp170 in the NBD may play a role in this interaction. A novel *in vivo* peptide expression library was engineered that was based on two proteins that are substrates of both Grp170 and BiP and which are both poised at the juncture of protein folding and degradation. This allowed the identification of peptides

that bound to Grp170 and BiP, which provided insights into their frequency, their locations with domains that are known to be folded *vs.* unfolded within full-length substrates, and lastly has indicated the presence of new binding sites for BiP within the C_H1 domain. These findings are summarized in the figure below (Figure 30). The development of COS-1 cell clones that can be used to express a variety of Grp170 mutants in the absence of endogenous protein, and which can be readily transfected with a variety of secretory proteins and ERAD substrates, provides an important tool to further extend our understanding of Grp170 in mammalian cells.

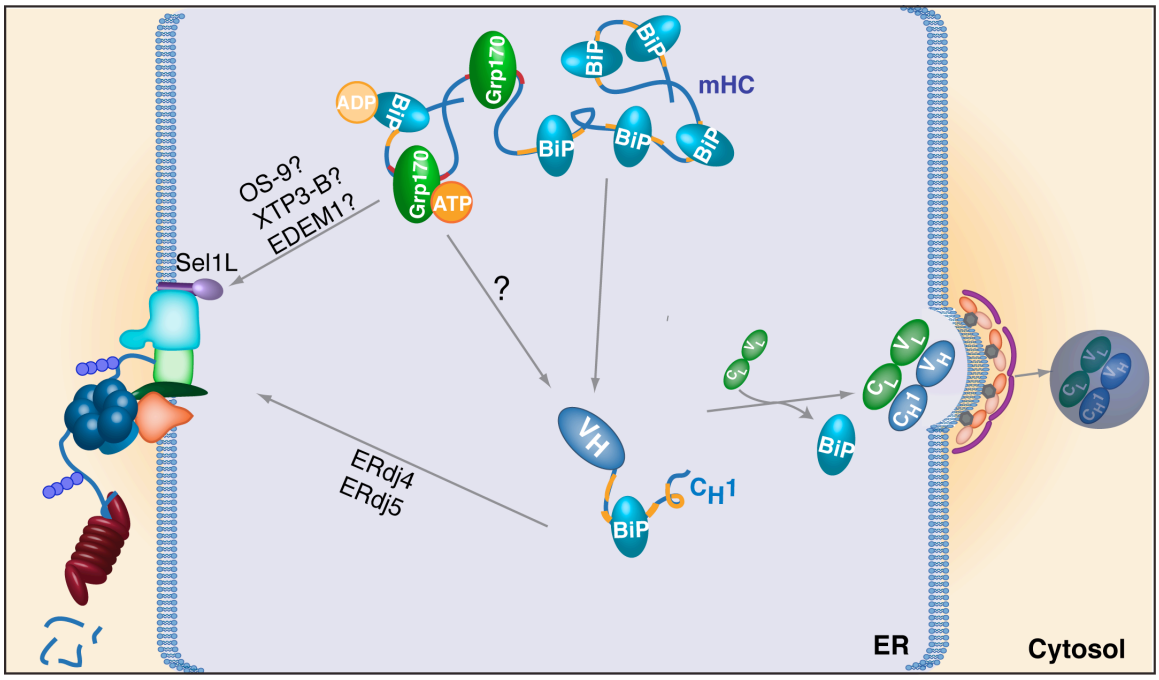

Figure 30. Role of Grp170 within the ERQC network.

8 Materials and Methods[2]

8.1 Cell lines and antibodies

COS-1 monkey fibroblasts were cultured in DMEM (Cellgro, USA) supplemented with 10% FBS, 2 mM L-Glutamine (Cellgro, USA) and 100 U/ml penicillin-streptomycin (Cellgro, USA) (=complete DMEM medium) at 37°C in 3% CO_2. P3U.1 mouse myeloma cells, which naturally produce NS-1 LC, were cultured in RPMI (Cellgro, USA) supplemented with 15% FBS, 2 mM L-Glutamine (Cellgro, USA), 100 U/ml penicillin-streptomycin (Cellgro, USA) and 55 nM 2-Mercaptoethanol (Gibco, USA) at 37°C in 5% CO_2.

The anti-Grp170 antiserum was produced by Rockland Immunochemicals Inc., USA by immunization of rabbits with the peptides STGQKRPLKNDEL and SAGQKRPSKNDEL, corresponding to the human and mouse Grp170 C-terminus, respectively, along with the purified NBD (aa 36-427) of human Grp170. All other antibodies were obtained commercially: goat anti-mouse κ LC (1050-01) and goat anti-mouse λ LC (1060-01) (SouthernBiotech, USA); mouse anti-β Actin (clone AC-15) (Sigma-Aldrich, USA); rabbit anti-FLAG (F7425) (Sigma-Aldrich, USA); rabbit anti-Sel1L (S3699) (Sigma-Aldrich, USA); mouse anti-TCRβ (TCR1151) (Thermo Fisher Scientific, USA); HRP-conjugated goat anti-rabbit (sc-2054), HRP-conjugated donkey anti-goat (sc-2020) and HRP-conjugated goat anti-mouse (sc-2031) (Santa Cruz Biotechnology, USA).

8.2 DNA Expression vectors

8.2.1 DNA Constructs

The pSVL expression vectors encoding the truncated γHC with a double HA- tag, mHC^{HA} (Lee et al, 1999), NS-1 LC (Skowronek et al, 1998), murine λ LC (Skowronek et al, 1998), λC_L (Hellman et al, 1999), and TCRβ (Feige & Hendershot, 2013) have been

[2] Parts of this chapter were previously published in a modified form: Behnke J, Hendershot LM (2014) The large Hsp70 Grp170 binds to unfolded protein substrates in vivo with a regulation distinct from conventional Hsp70s. *J Biol Chem* **289:** 2899-2907

described previously, as have the pMT vectors expressing wild-type BiP and the BiP^{T37G} mutant (Gaut & Hendershot, 1993; Hendershot et al, 1996). A human Grp170 cDNA clone in the pcDNA3.1(+) vector was a generous gift of Dr. Osamu Hori (Kanazawa University, Japan), into which we introduced a Kozak sequence (GCCACC) before the initiating methionine to increase expression.

8.2.2 Generation of Grp170 mutants

All Grp170 mutants were generated using human Grp170 in pcDNA3.1(+) with the Kozak sequence ($Grp170_{wt}$) as a template. Two different mutagenesis methods were used to introduce various Grp170 mutants as outlined in Table 3: either the restriction free cloning (RFC) method was used as described in (van den Ent & Lowe, 2006) or the QuickChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies, USA) (Multi) following the manufacturer´s protocol. The following table lists all generated Grp170 constructs, the corresponding primers and the method used:

Table 3: Overview of generated Grp170 mutants

Grp170 mutant	deletion/insertion/replacement of aa (based on human Grp170 sequence UniProt ID Q9Y4L1)	template used to generate the mutant	aim	primer	mutagenesis method
Grp170$_{wt}^{FL}$	amino acids GSDYKDDDDKGS were introduced between aa 994 and 995 in Grp170	Grp170$_{wt}$	insertion of a C-terminal FLAG-tag before the KNDEL ER retention sequence	fwd: 5´-cgacaggacagaagcggcctttgggcagcgactacaaggacgacgacgacaagggcagcaagaacgacgaactataagaattctgcagatatccagcaca-3´; rev: 5´-tgtgctggatatctgcagaattcttatagttcgtcgttcttgctgccctttgtcgtcgtcgtccttgtagtcgctgcccaaaggccgcttctgtcctgtcg-3´	restriction free cloning
Grp170$_{\Delta NBD}^{FL}$	deletion of aa 38-439	Grp170$_{wt}^{FL}$	deltion of the Grp170 NBD including linker	fwd: 5´-gcactgagtgatacactggcagtgatgtctgcagtggtctaccccatcctggtg-3´; rev: 5´-caccaggatggggtagaccactgcagacatcactgccagtgtatcactcagtgc-3´	restriction free cloning
Grp170$_{\Delta loop}^{FL}$	replacement of aa 591-696 with a GS-linker to connect the flanking β-sheets	Grp170$_{wt}^{FL}$	deletion of the unstructured loop insertion in the β-sheet domain	fwd: 5´-atttccagcctgtttggaggcggtaccacaggcagcgaggagatcggggtggagctggttgttc–3´; rev: 5´-gaacaaccagctccacccgatctcctcgctgcctgtggtaccgcctccaaacaggctggaaat-3´	restriction free cloning
Grp170$_{\Delta C\text{-}term}^{FL}$	deletion of aa 712-994	Grp170$_{wt}^{FL}$	deletion of the predicted α-helical domain	fwd: 5´-ggttgttctggacctgcctgacttgggcagcgactacaaggacgacgac-3´; rev: 5´-gtcgtcgtccttgtagtcgctgcccaagtcaggcaggtccagaacaacc-3´	restriction free cloning
Grp170$_{\Delta C\text{-}term,\Delta loop}^{FL}$	combination of the mutants Grp170ΔC-termFL and Grp170ΔloopFL	Grp170$_{\Delta loop}^{FL}$	simultaneous deletion of α-hecial domain and unstructured loop	same as used for Grp170$_{\Delta C\text{-}term}^{FL}$	restriction free cloning
Grp170$_{N155Q}^{FL}$	point mutation N155Q	Grp170$_{wt}^{FL}$	point mutation of N155 glycosylation site	fwd: 5´-gaagtgttgggcatggttctccagtattctcgttctctagctgaag-3´; rev: 5´-cttcagctagagaacgagaatactggagaaccatgcccaacacttc-3´	restriction free cloning
Grp170$_{N222Q}^{FL}$	point mutation N222Q	Grp170$_{wt}^{FL}$	point mutation of N222 glycosylation site	fwd: 5´-ccggaaagatattcagaccactgcccag-3´; rev: 5´-ctgggcagtggtctgaatatctttccgg-3´	restriction free cloning
Grp170$_{N515Q}^{FL}$	point mutation N515Q	Grp170$_{wt}^{FL}$	point mutation of N515 glycosylation site	fwd: 5´-gtatttggctcccagcagctgaccacagtg-3´; rev: 5´-cactgtggtcagctgctgggagccaaatac-3´	restriction free cloning
Grp170$_{N596Q}^{FL}$	point mutation N596Q	Grp170$_{wt}^{FL}$	point mutation of N596 glycosylation site	fwd: 5´-gatgccaaggagcagggtactgatactg-3´; rev: 5´-cagtatcagtaccctgctccttggcatc-3´	restriction free cloning
Grp170$_{N830Q}^{FL}$	point mutation N830Q	Grp170$_{wt}^{FL}$	point mutation of N830 glycosylation site	fwd: 5´-gccctcgataatctcctccagcattccagcatgttcctc-3´; rev: 5´-gaggaacatgctggaatgctggaggagattatcgagggc-3´	restriction free cloning
Grp170$_{N862Q}^{FL}$	point mutation N862Q	Grp170$_{wt}^{FL}$	point mutation of N862 glycosylation site	fwd: 5´-caacgttagagaaagtcatccaggagacctgggcctggaagaatg-3´; rev: 5´-cattcttccaggcccaggtctcctggatgactttctctaacgttg-3´	restriction free cloning
Grp170$_{N869Q}^{FL}$	point mutation N869Q	Grp170$_{wt}^{FL}$	point mutation of N869 glycosylation site	fwd: 5´-cctgggcctggaagcaggcaactctggccg-3´; rev: 5´-cggccagagttgcctgcttccaggcccagg-3´	restriction free cloning
Grp170$_{N922Q}^{FL}$	point mutation N922Q	Grp170$_{wt}^{FL}$	point mutation of N922 glycosylation site	fwd: 5´-cctaaggacaagcaggggacccgggcagagc-3´; rev: 5´-gctctgcccgggtcccctgcttgtcctagg-3´	restriction free cloning
Grp170$_{N931Q}^{FL}$	point mutation N931Q	Grp170$_{wt}^{FL}$	point mutation of N931 glycosylation site	fwd: 5´-gagccacccctccaggccagtgccagtgac-3´; rev: 5´-gtcactggcactggcctggaggggtggctc-3´	restriction free cloning
Grp170$_{\Delta glyco}^{FL}$	point mutations N155,222,515,596,830,862, 869,922,931Q	Grp170$_{wt}^{FL}$	simultaneous deletion of all N-linked glycosylation sites	5´-cttcagctagagaacgagaatactggagaaccatgcccaacacttc-3´; 5´-catgatattctgggcagtggtctgaatatctttccggcggaagac-3´; 5-ccttttagcttcactgtggtcagctgctgggagccaaataccc-3´; 5´-cctggacagtatcagtaccctgctccttggcatctggtg-3´; 5´-gaggaacatgctggaatgctggaggagattatcgagggc-3´; 5´-cggccagagttgcctgcttccaggcccaggtctcctggatgaccaaataccc-3´; 5´-gctctgcccgggtcccctgcttgtcctagg-3´; 5´-gtcactggcactggcctggaggggtggctc-3´	multisite mutagenesis kit
Grp170$_{N830,862,869,922,931Q}^{FL}$	point mutations N830,862,869,922,931Q	Grp170$_{wt}^{FL}$	simultaneous deletion of N-linked glycosylation sites at the C-terminus	5´-gaggaacatgctggaatgctggaggagattatcgagggc-3´; 5´-cggccagagttgcctgcttccaggcccaggtctcctggatgactttctctaacg-3´; 5´-gctctgcccgggtcccctgcttgtcctagg-3´; 5´-gtcactggcactggcctggaggggtggctc-3´	multisite mutagenesis kit
Grp170$_{N155,222Q}^{FL}$	point mutations N155,222Q	Grp170$_{wt}^{FL}$	simultaneous deletion of N-linked glycosylation sites at the NBD	5´-cttcagctagagaacgagaatactggagaaccatgcccaacacttc-3´; 5´-catgatattctgggcagtggtctgaatatctttccggcggaagac-3´;	multisite mutagenesis kit
Grp170$_{Q155N}^{FL}$	point mutations Q155N	Grp170$_{\Delta glyco}^{FL}$	restoration of N-linked glycosylation site N155 at the NBD	fwd: 5´-gggcatggttctcaattattctcgttctc-3´; rev: 5´-gagaacgagaataattgagaaccatgccc-3´	restriction free cloning
Grp170$_{Q222N}^{FL}$	point mutations Q222N	Grp170$_{\Delta glyco}^{FL}$	restoration of N-linked glycosylation site N222 at the NBD	fwd: 5´-ccggaaagatattaacaccactgcccag-3´; rev: 5´-ctgggcagtggtgttaatatctttccgg-3´	restriction free cloning
Grp170$_{Q155,222N}^{FL}$	point mutations Q155,222N	Grp170$_{Q222N}^{FL}$	restoration of both N-linked glycosylation sites at the NBD	same as used for Grp170$_{Q155N}^{FL}$	restriction free cloning

8.2.3 Cloning of the in vivo peptide library constructs

The reporter construct composed the C_L-domain of the λLC, two GS-linkers, the TM-region of TCRα followed by an NVT-glycosylation site was previously established and described (Feige & Hendershot, 2013). This construct was used as template to generate the constructs covering the mHC or the NS-1 LC as described in the text resulting in 12

constructs mHC_1-12^{NVT} for the mHC and 11 constructs NS1_1-11^{NVT} for the NS-1 LC. The constructs were generated *via* restriction free cloning (RFC) or, where not feasible, full-length constructs were synthesized by Life Technologies (LT), USA. The amino acid sequences are listed in Figure (Figure 18) in the main text and the following table summarizes the primers used to generate the mHC_1-12^{NVT} constructs, if the construct was obtained *via* RFC or whether the construct was ordered from LT instead.

Table 4: primer sequences used to generate the mHC_1-12^{NVT} constructs

construct	primer used to generate the construct	obtained via
mHC_1^{NVT}	fwd: 5´-gtggtagtggcagccaggtccaattgcagcagcctggggctgaactggtgaaacctggggcttcagtgaagctgtcctgcaaggcctctggcagcggcag-3´; rev: 5´-ctgccgctgccagaggccttgcaggacagcttcactgaagccccaggttcaccagttcagccccaggctgctgcaattggacctggctgccactaccac-3´	RFC
mHC_2^{NVT}	fwd: 5´-gtggtagtggcagcctgtcctgcaaggcctctggctacaccttcaccagctactggatgcactgggtgaagcagaggcctggacaaggcggcagcggcag-3´; rev: 5´-ctgccgctgccgccttgtccaggcctctgcttcacccagtgcatccagtagctggtgaaggtgtagccagaggccttgcaggacaggctgccactaccac-3´	RFC
mHC_3^{NVT}	-	LT
mHC_4^{NVT}	fwd: 5´-gtggtagtggcagcactaattacaatgagaagttcaagagcaaggccacactgactgtagacaaatcctccagcacagcctacatgcaaggcagcggcag-3´; rev: 5´-ctgccgctgccttgcatgtaggctgtgctggaggatttgtctacagtcagtgtggccttgctcttgaacttctcattgtaattagtgctgccactaccac-3´	RFC
mHC_5^{NVT}	-	LT
mHC_6^{NVT}	-	LT
mHC_7^{NVT}	-	LT
mHC_8^{NVT}	-	LT
mHC_9^{NVT}	fwd: 5´-gtggtagtggcagcccggtgacggtgtcgtggaactcaggcgccctgaccagcggcgtgcacaccttccccgctgtcctacagtcctcaggcagcggcag-3´; rev: 5´-ctgccgctgcctgaggactgtaggacagcggggaaggtgtgcacgccgctggtcagggcgcctgagttccacgacaccgtcaccgggctgccactaccac-3´	RFC
mHC_10^{NVT}	fwd: 5´-gtggtagtggcagcgctgtcctacagtcctcaggactctactccctcagcagcgtggtgaccgtgccctccagcagcttgggcacccagggcagcggcag-3´; rev: 5´-ctgccgctgccctgggtgcccaagctgctggagggcacggtcaccacgctgctgagggagtagagtcctgaggactgtaggacagcgctgccactaccac-3´	RFC
mHC_11^{NVT}	fwd: 5´-gtggtagtggcagcagcagcttgggcacccagacctacatctgcaacgtgaatcacaagcccagcaacaccaaggtggacaagaaaggcagcggcag-3´; rev: 5´-ctgccgctgcctttcttgtccaccttggtgttgctgggcttgtgattcacgttgcagatgtaggtctgggtgcccaagctgctgctgccactaccac-3´	RFC
mHC_12^{NVT}	fwd: 5´-gtggtagtggcagccccagcaacaccaaggtggacaagaaagttggccaaggcagcggcag-3´; rev: 5´-ctgccgctgccttggccaactttcttgtccaccttggtgttgctggggctgccactaccac-3´	RFC

The following table contains primers that were used to generate the constructs NS1_1-11^{NVT} and the Δinsert^{NVT} negative control. It is also annotated whether constructs were

generated via RFC or were ordered from Life Technologies (LT). In order to mutate the NVT glycosylation site to QVT the same primer pair was used for all constructs and all constructs were generated *via* RFC.

Table 5: primer sequences used to generate the NS_1-11^{NVT} , Δinsert^{NVT} constructs as well as the NVT to QVT point mutation in all constructs.

construct	primer used to generate the construct	obtained via
NS1_1^{NVT}	fwd: 5´-gtggtagtggcagcaacattgtaatgacccaatctcccaaatccatgtccatgtcagtaggagagagggtcaccttgacctgcaaggccggcagcggcag-3´; rev: 5´-ctgccgctgccggccttgcaggtcaaggtgaccctctctcctactgacatggacatggatttgggagattgggtcattacaatgttgctgccactaccac-3´	RFC
NS1_2^{NVT}	fwd: 5´-gtggtagtggcagcaccttgacctgcaaggccagtgagaatgtggttacttatgtttcctggtatcaacagaaaccagagcagtctcctggcagcggcag-3´; rev: 5´-ctgccgctgccaggagactgctctggtttctgttgataccaggaaacataagtaaccacattctcactggccttgcaggtcaaggtgctgccactaccac-3´	RFC
NS1_3^{NVT}	fwd: 5´-gtggtagtggcagcaaaccagagcagtctcctaaactgctgatatatggggcatccaaccggtacactggggtccccgatcgcttcacaggcagcggcag-3´; rev: 5´-ctgccgctgcctgtgaagcgatcggggaccccagtgtaccggttggatgccccatatatcagcagtttaggagactgctctggtttgctgccactaccac-3´	RFC
NS1_4^{NVT}	fwd: 5´-gtggtagtggcagcgtccccgatcgcttcacaggcagtggatctgcaacagatttcactctgaccatcagcagtgtgcaggctgaagacggcagcggcag-3´; rev: 5´-ctgccgctgccgtcttcagcctgcacactgctgatggtcagagtgaaatctgttgcagatccactgcctgtgaagcgatcggggacgctgccactaccac-3´	RFC
NS1_5^{NVT}	fwd: 5´-gtggtagtggcagcagtgtgcaggctgaagaccttgcagattatcactgtggacagggttacagctatccgtacacgttcggaggggggggcagcggcag-3´; rev: 5´-ctgccgctgccccccccctccgaacgtgtacggatagctgtaaccctgtccacagtgataatctgcaaggtcttcagcctgcacactgctgccactaccac-3´	RFC
NS1_6^{NVT}	-	LT
NS1_7^{NVT}	fwd: 5´-gtggtagtggcagcgtatccatcttcccaccatccagtgagcagttaacatctggaggtgcctcagtcgtgtgcttcttgaacaacttcggcagcggcag-3´, rev: 5´-ctgccgctgccgaagttgttcaagaagcacacgactgaggcacctccagatgttaactgctcactggatggtgggaagatggatacgctgccactaccac-3´	RFC
NS1_8^{NVT}	fwd: 5´-gtggtagtggcagctgcttcttgaacaacttctaccccaaagacatcaatgtcaagtggaagattgatggcagtgaacgacaaaatggcggcagcggcag-3´; rev: 5´-ctgccgctgccgccatttttgtcgttcactgccatcaatcttccacttgacattgatgtctttggggtagaagttgttcaagaagcagctgccactaccac-3´	RFC
NS1_9^{NVT}	-	LT
NS1_10^{NVT}	fwd: 5´-gtggtagtggcagcacctacagcatgagcagcaccctcacgttgaccaaggacgagtatgaacgacataacagctatacctgtgaggccggcagcggcag-3´; rev: 5´-ctgccgctgccggcctcacaggtatagctgttatgtcgttcatactcgtccttggtcaacgtgagggtgctgctcatgctgtaggtgctgccactaccac-3´	RFC
NS1_11^{NVT}	-	LT
Δinsert^{NVT}	fwd: 5´-ggcagcggtagtggtagtggcagcggcagcggcagcggtagtggtag-3´; rev: 5´-ctaccactaccgctgccgctgccgctgccactaccactaccgctgcc-3´	RFC
NVT to QVT mutation	fwd: 5´-ggcagcggtagtggtagtggcagccaggtgaccagcagctagtaaggatcc-3´; rev: 5´-ggatccttactagctgctggtcacctggctgccactaccactaccgctgcc-3´	RFC

8.3 Transient transfections

8.3.1 Transient transfections to establish the chaperone function of Grp170

For transfections either 4.5 x 10^5 COS-1 cells were seeded in a p60 dish or 1.3 x 10^6 COS-1 cells in a p100 dish 24h prior to transfection with GeneCellin (BioCellChallenge, Toulon, France) according to the manufacturer's protocol. For the analysis of Grp170 binding to Ig-substrates (Figure 8) 0.4 µg of Grp170 in the pcDNA3.1(+) vector, 0.6 µg of BiP in the pMT vector and 2.5 µg of the indicated substrates in a pSVL vector were transfected in COS-1 cells in a p60 dish. For empty vector (mock) transfections in control experiments the same amounts of the corresponding vectors were used as in the experiment. For the analysis of substrate binding of Grp170 to NS-1 LC under oxidizing conditions (Figure 9), TCRβ (Figure 10) or NS-1 LC in the presence of BiP vs. BiP^{T37G} (Figure 12) the amount of Grp170 was titrated down, so that only the fully glycosylated form of Grp170 was expressed. Thus, 0.02 µg of Grp170, 0.6 µg BiP in pMT and 2.5 µg of NS-1 LC were transfected in COS-1 cells in a p60 dish. Due to lower affinity of Grp170 to TCRβ, the experiments were carried out in p100 dishes transfecting 0.06 µg Grp170, 1.8 µg BiP and 7.5 µg TCRβ. For experiments with Grp170 domain deletion mutants, in order to analyze their substrate binding (Figure 16), the same amounts of constructs as for the analysis of Grp170 binding to Ig-substrates (Figure 8) were transfected. For the experiments to determine the interaction between Grp170 mutants and BiP (Figure 14), 0.4 µg of the indicated Grp170 construct and 1.5 µg of BiP were transfected in a p60 dish.

8.3.2 Transient transfections in experiments using the Grp170 glycosylation mutants

For experiments with Grp170 domain deletion mutants to assess its glycosylation status (Figure 23 and Figure 24) the same amounts of constructs as for the analysis of Grp170 binding to Ig-substrates (Figure 8) were transfected in p60 dishes. For the experiments to determine the interaction between Grp170 glycosylation mutants and BiP (Figure 25),

0.4 µg of the indicated Grp170 construct and 1.5 µg of BiP were transfected in a p60 dish. To analyze the interaction of wild-type Grp170 and various Grp170 glycosylation mutants with endogenous Sel1L, 1.3 x 10^6 COS-1 cells were seeded in p100 dishes 24h prior to transfection with GeneCellin. Each p100 dish was transfected with 3 µg of the various Grp170 constructs.

8.3.3 Transient transfections for the in vivo peptide library experiments

For the analysis of the ER entry of NVT constructs (EndoH digestion experiments) 4.5 x 10^5 COS-1 cells were seeded in a p60 dish 24h prior to transfection with GeneCellin according to the manufacturer's protocol and 4 µg of each construct in the pSVL vector were transfected in each dish. Experiments analyzing the half-life, aggregation and secretion properties of the QVT constructs were done with the same amount of construct in p60 dishes as in the EndoH digest experiments of the NVT constructs. Experiments investigating the interaction of Grp170 and BiP with the QVT constructs were also carried out in p60 dishes transfecting 3 µg of each reporter construct or empty pSVL vector (BiP/Grp170 size standards), 1 µg $Grp170_{wt}$ in pcDNA3.1(+) vector and 1 µg BiP in pMT vector.

8.4 Metabolic labeling, Pulse-Chase experiments, Preparation of cell lysates and Immunoprecipitations

8.4.1 Metabolic Labeling and Pulse-Chase experiments

Twenty-four hrs post-transfection, cells were pre-incubated in complete cysteine/methionine-free DMEM labeling media (Cellgro, USA) supplemented with 10% dialyzed FBS for 30 min to starve cells for cysteine and methionine, and pulse-labeled with 100 µCi/p60 or 300 µCi/p100 of EasyTag™ EXPRESS35S Protein Labeling Mix (Perkin Elmer, USA) for the indicated periods of time. To analyze chaperone association with substrates, after labeling the cells were washed in PBS and chased in complete medium supplemented with 2 mM unlabeled Cys and Met for 1 hr to allow chaperones to mature properly and enter an active pool. For all experiments the pulse-labeling and chasing times are indicated in the corresponding figure legends. Cells were lysed in 1 ml of NP40 lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P40 substitute, 0.5% sodium deoxycholate, 0.1 mM PMSF, 1x Roche complete protease inhibitor tablets w/o EDTA) supplemented as indicated with either with 10 U/ml of apyrase (Sigma-Aldrich, USA), 2 mM Mg-ATP (Sigma-Aldrich, USA) and 25 mM KCl (Fisher Scientific, USA), or, if not indicated, then neither was added. After clearing the lysate at 20,000 g for 15 min at 4°C, the supernatant was divided and immunoprecipitated with the indicated antibodies, rotating overnight. Immune complexes were isolated with CaptivA™ PriMAB Protein A agarose slurry (RepliGen, USA), washed three times with NP-40 washing buffer (50 mM Tris/HCl, pH 7.5, 400 mM NaCl, 0.5% Nonidet P40 substitute, 0.5% sodium deoxycholate), eluted with 2x reducing Laemmli buffer and analyzed by SDS-PAGE. The resulting gels were incubated in Amplify (GE Healthcare, USA) supplemented with 3% glycerol for 30 min at RT before they were dried. To determine the kinetics of NS-1 LC binding to Grp170 and BiP, P3U.1 cells were split the day prior to the experiment, and 15 x10^6 cells were pulse-labeled with 1 mCi of EasyTag™ EXPRESS35S Protein Labeling Mix in 10 ml Cys/Met-free RPMI labeling media (Cellgro, USA) that was supplemented with 15% dialyzed FBS and chased for the indicated times. Cells were lysed in 1.5 ml NP-40 buffer in the presence of apyrase by rotating at 4°C for 1 hr. 150 µl of the clarified lysate were

immunoprecipitated with anti-mouse κ LC, 200 µl with anti-rodent BiP and 1100 µl with anti-Grp170. Further steps were carried out as described above.

To determine the solubility of the Grp170 single domain mutants, cells were lysed with NP-40 lysis buffer supplemented with ATP. After centrifugation, the NP-40 insoluble pellets were dissolved in 50 µl of Tris-SDS-buffer (50 mM Tris/HCl, pH 8.0, 2% SDS) and incubated at 95°C for 20 min with frequent agitation. Subsequently, 950 µl of NP40 lysis buffer with ATP was added to the solubilized pellets and proteins were analyzed *via* immunoprecipitation as described above. EndoH and PNGaseF (NEB, USA) digestions were carried out according to the manufacturer's instructions. Pulse-chase experiments analyzing the aggregation and secretion tendency of the reporter constructs with the QVT site were done analogously, but without adding ATP. For secretion analysis media was immunoprecipitated as described in immunoprecipitations of cellular lysates, by adding the antiserum directly into the media. Experiments analyzing the glycosylation status of the QVT reporter constructs were performed as described above, but lysing the cells in RIPA buffer (0.15MNaCl, 10mMTris-HCl,pH7.5, 1% Nonidet P-40 (NP-40), 0.1% SDS, 0.2% deoxycholate). EndoH digests following the immunoprecipitations were performed as described above. In experiments analyzing the interaction of Grp170 and BiP with the peptide reporter constructs cells were lysed 1ml in NP40lysis buffer, after centrifugation, the protein lysates were additionally precleared with 30 µl CaptivA™ PriMAB Protein A agarose slurry for 1hr rotating at 4°C. The pre-cleared supernatant was taken for further steps of the immunoprecipitations as described above.

8.4.2 Immunoprecipitations coupled to Immunoblot Experiments

To examine the molecular forms of NS-1 LC that Grp170 and BiP interact with, 2 x 10^6 P3U.1 murine plasmacytoma cells, which naturally synthesize the NS-1 LC, were used. 10 mM DTT was added to one half of the culture 30 min before lysis to reduce the LC while the other half remained untreated. In both cases, cells were washed with PBS supplemented with 20 mM NEM and lysed in the presence of apyrase or ATP as indicated with NP-40 lysis buffer supplemented with 20 mM NEM. Lysates were pre-cleared with CaptivA™ PriMAB Protein A agarose slurry for 30 min at 4°C, and immunoprecipitations were carried out with the indicated immune reagents as described above. After separating proteins on 13% non-reducing SDS-PAGE gels and transferring

them to a PVDF membrane, proteins were detected by blotting with the indicated antisera following a standard protocol using Gelatin Buffer (0.1% Gelatin, 15 mM Tris/HCl, pH7.5, 130 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.002% NaN_3). Primary antibodies were used at a dilution of 1:1,000, while secondary species-specific HRP-conjugated antibodies were diluted 1:10,000.

In the experiments confirming the identity of Grp170 and BiP bands in Figure 8 experiments were set up analogously as described in the corresponding metabolic labeling experiments leaving out the metabolic labeling steps and lysing the cells directly instead. The subsequent steps of immunoprecipitations were done the same way as described for the metabolic labeling experiments above and the western blotting was performed analogously as described in this section. Co-immunoprecipitations of Sel1L with various Grp170 glycosylation mutants were performed by lysing the cells in 1 ml of NP40 lysis buffer and clearing the lysate at 20,000 g for 15 min at 4°C. 900 µl of the supernatant were used for immunoprecipitation with anti-FLAG antisera rotating at 4°C over night. Immune complexes were isolated with CaptivA™ PriMAB Protein A agarose slurry, washed 3x with 1ml of NP-40 washing buffer, eluted with 2x reducing Laemmli buffer, separated by SDS-PAGE and transferred to a PVDF membrane as described above. 20 µl of the precleared supernatant were mixed with 5xLaemmli, boiled and used for the Input control blotting analogously as described for immunoprecipitations.

8.5 Quantification and analysis of the data

To quantify metabolically labeled proteins, a Storm 860 phosphorimager scanner (GE Healthcare, Pittsburgh, PA) was used, and the signals were analyzed with the ImageQuant TL software (GE Healthcare, Pittsburgh, PA). For the analysis of the percentage of NS-1 LC bound to Grp170, BiP or BiP^{T37G}, signal of NS-1 co-immunoprecipitating with the various chaperones was divided by the signal obtained by immunoprecipitating with anti-κ LC antibody. Association of Grp170 domain deletion mutants with mHC^{HA} or NS-1 LC was calculated as follows:

$$\frac{\mathbf{Grp170}^{FL}_{\text{co-IP with mHC}^{HA}}}{\mathbf{Grp170}^{FL}_{\text{IP with FLAG-antibody}}} \times \frac{\mathbf{mHC}^{HA}_{\text{IP with HA-antibody when Grp170}^{FL}_{wt}\text{ is co-expressed}}}{\mathbf{mHC}^{HA}_{\text{IP with HA-antibody when respective Grp170 construct is co-expressed}}}$$

Only the signal for the fully glycosylated form of Grp170 was included in the analysis, and the value of calculations in the presence of $Grp170_{wt}^{FL}$ was set to 1. The binding of Grp170 single domain deletion mutants to NS-1 LC was calculated analogously to mHC^{HA}. P values associated with all comparisons were derived from paired two-tailed Student's t-tests. Results are shown as means ± standard error (S.E.).

8.6 Structural Modeling of Grp170

The homology model of human Grp170 was based on the crystal structure of Sse1p bound to the Hsp70 NBD (Protein Data Bank code: 3D2F) and built with Yasara Structure (www.yasara.org) as previously described (Howes et al, 2012).

8.7 Establishing of a stable cell line expressing doxycycline inducible shRNA targeting endogenous Grp170

In order to establish a Grp170 knock-down cell line and have the ability to reintroduce various Grp170 mutants once the cell line is established, siRNAs targeting the 3´-untranslated region (UTR) of the Grp170 gene were designed using the siDESIGN tool of Dharmacon (http://dharmacon.gelifesciences.com/design-center/).

Three siRNA sequences (1.GGATGAAGGAGGAGGGTCA, 2. CTGGATTGGCGGAAGTAAA, 3. GGAAGTAAATCTGGAAGGA) were chosen and tested for their Grp170 knock-down efficiency in transient expression experiments. Sequence number 3 exhibited the highest efficiency and therefore shRNA based on this siRNA sequence was designed. The shRNA design and the subsequent cloning into the pSuperior.puro vector (Clontech, Invitrogen, USA) followed the recommendations of the manufacturer:

shRNA targeting 3´UTR of the Grp170 gene:

5'-TCGAGGGGAAGTAAATCTGGAAGGATTCAAGAGATCCTTCCAGATTTACTTCCTTTTTTTACGCGTA-3'

3´-CCCCTTCATTTAGACCTTCCTAAGTTCTCTAGGAAGGTCTAAATGAAGGAAAAAATGCGCATTCGA-5´

Design template from the manufacturer:

XhoI Target sense sequence Hairpin Loop Target antisense sequence Terminator Test RE site (MluI)

5'-TCGAGGNNNNNNNNNNNNNNNNNNTTCAAGAGANNNNNNNNNNNNNNNNNNTTTTTT-NNNNNN-A-3'

3'-CCNNNNNNNNNNNNNNNNNNAAGTTCTCTNNNNNNNNNNNNNNNNNNAAAAAA-NNNNNN-TTCGA-5'

HindIII

The pSuperior.puro (anti-Grp170-shRNA) was transfected along with pcDNA6/TR (Life Technologies, USA), which encodes a Tet-transcription repressor that is inhibited upon DOX addition, into COS-1 cells following the instructions of the manufacturer. Transfected COS-1 cells were selected with puromycin and blasticidin, for pSuperior.puro and pcDNA6/TR selection, respectively, with 2 µg/ml each for 2 weeks. Thereby only cells having both vectors were selected and subsequently subjected to single cell sorting. The single clones were expanded in media containing 0.5 µg/ml puromycin as well as blasticidin. After expansion, clones were characterized regarding the DOX-inducible Grp170 knock-down over time.

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10 Declaration

I, Julia Behnke, hereby declare that this thesis was prepared by me independently and using only the references and resources stated here. The work has so far not been submitted to any audit commission. Parts of this work have been published in scientific journals.

Hiermit erkläre ich, Julia Behnke, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die hier angegebenen Quellen und Hilfsmittel verwendet habe. Die Arbeit wurde bisher keiner Prüfungskommission vorgelegt. Teile dieser Arbeit wurden in wissenschaftlichen Journalen veröffentlicht.

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Julia Behnke

11 Publications

Feige MJ, Gräwert MA, Marcinowski M, Hennig J, Behnke J, Ausländer D, Herold EM, Peschek J, Castro CD, Flajnik M, Hendershot LM, Sattler M, Groll M, Buchner J. (2014). The structural analysis of shark IgNAR antibodies reveals evolutionary principles of immunoglobulins.
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