Chapter 10
Chaperones in the Endoplasmic Reticulum (ER): Function and Interaction Network

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Abstract The directional entry, oxidative folding, and quality control of proteins that enter the secretory pathway is mediated by chaperones and foldases in and adjacent to the endoplasmic reticulum (ER). Properly folded and assembled proteins continue along the secretory pathway while proteins that ultimately fail quality control are targeted to the proteasome by removal from the ER in a process called ER-associated degradation (ERAD). The protein folding machineries in the ER interact with each other to form functional complexes. Studies have revealed that abundant chaperones and foldases serve multiple functions in the ER through membership in diverse complexes that can target their activities to substrates at different stages of maturation. These findings are providing insight into how ER complexes combine various functions together to engage substrates and determine their fates.

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Abbreviations

AAT	α-1 antitrypsin
AGR	Anterior gradient
AP	Affinity purification
APEX	Ascorbate peroxidase
ATP	Adenosine triphosphate
BAP	BiP-associated protein
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CGHC	Cysteine-Glycine-Histidine-Cysteine
Co-IP	Co-immunoprecipitation
COP	Coat protein
CPHC	Cysteine-Proline-Histidine-Cysteine
CSMC	Cysteine-Serine-Methionine-Cysteine
EDEM	ER degradation enhancing mannosidase-like protein
EGF	Epidermal growth factor
ENaC	Epithelial sodium channel
ER	Endoplasmic reticulum
ER-MYTHS	Endoplasmic reticulum-membrane yeast two hybrid system
ERAD	Endoplasmic reticulum-associated degradation
FAD	Flavine adenine dinucleotide
FKPB	FK506 binding protein
GFP	Green fluorescent protein
Glc	Glucose
GlcNAc	N-acetylglucosamine
GPI	Glycophosphatidylinositol
HPD	Histidine-proline-aspartate
Hsp	Heat shock protein
ITC	Isothermal titration calorimetry
LDL	Low density lipoprotein
Man	Mannose
MAP	Membrane Yeast Two Hybrid System, Affinity Purification, NMR
MHC	Major Histocompatability Complex
MS	Mass spectrometry
NEF	Nucleotide exchange factor
NHK	Null Hong Kong
NMR	Nuclear magnetic resonance
NOX	Nicotinamide adenine dinucleotide oxidase
OST	Oligosaccharyltransferase
PCA	Protein complementation assay
PDB	Protein Data Bank
PDI	Protein disulfide isomerase
PDIr	Protein Disulfide Isomerase-related protein
PERK	PKR-like endoplasmic reticulum kinase
PPI	Peptidyl-prolyl *cis-trans* isomerase
PPI	Protein-protein Interaction
PRIME	Probe incorporation mediated by enzymes

Prx	Peroxiredoxin
RAMP	Ribosome associated membrane protein
shRNA	Short hairpin ribonucleic acid (RNA)
SIL 1	Suppressor of the Ire1/Lhs1 double mutant 1
SRP	Signal recognition particle
TRAP	Translocon-associated protein
UGGT	UDP-glucose:glycoprotein-glucosyltransferase
UPR	Unfolded protein response
Y2H	Yeast two-hybrid

1 Introduction

Recent studies have uncovered many novel interactions between proteins within the endoplasmic reticulum (ER). These advances have come through application of interaction detection methods in both yeast and mammalian studies. While studies of yeast ER protein-protein interactions (PPIs) provides a rational starting point to understand interactions of homologous mammalian ER proteins, it is not surprising that there are many more specialized mammalian ER chaperones and folding enzymes that do not have specific yeast counterparts, limiting the extent to which comparisons can be made. However, expression of mammalian proteins in yeast systems allows for simpler genetic manipulations and can uncover binary interactions. The yeast two-hybrid (Y2H) methods can be combined with affinity purification and mass spectrometry (AP-MS) methods and nuclear magnetic resonance (NMR) using mammalian proteins, and ideally, short hairpin ribonucleic acid (shRNA) methods to provide broader perspectives and functional insight. We discuss some of the recently identified interactions between the mammalian ER proteins with a focus on proteins involved in initial entry into the ER, protein folding, sorting, and ER-associated degradation (ERAD). Significant advances have been made in understanding ER membrane architecture [1], the proteins, and complexes involved in determining reticular shape [2] and fusion [3] and cytosolic components involved in ERAD [4]. We will focus on luminal chaperone interactions and their functional consequences.

2 Overview

Relatively few PPIs between ER resident chaperones were known until the discovery of the unfolded protein response and the link between ER protein folding and human disease. Historically, George Palade's pioneering work on this organelle demonstrated its role as the entry point for secreted proteins. We aim to trace, from the perspective of ER-protein complexes encountered, the maturation of a newly synthesized secretory protein from nascent chain entry into the ER to exit from the ER as either a correctly folded polypeptide, or a terminally misfolded protein

targeted for retrotranslocation and proteasomal degradation. Along the way, we will highlight the latest complexes identified, and describe their functional contributions to the process of protein folding.

It is not currently clear how ER protein folding machineries may be spatially arranged for sequential protein maturation; many of the protein complexes we will discuss act simultaneously, so our journey from translocon to exit sites is heuristic, but we hope this narrative will offer food for thought to develop testable models of ER function. Furthermore, while it is not possible to understand the functions of all identified interactions, and it has been argued that non-functional interactions may be more common than appreciated [5], we will focus on interactions validated through various means that have been implicated both directly and indirectly in ER functions through additional evidences. We will also address less well-characterized interactions with a more philosophical discussion of possible functions for further investigation. We do not attempt to describe in detail all that is known regarding nascent protein entry until exit from the ER, as these have been extensively reviewed elsewhere (see a recent special issue on ER structure and function in Biochemica et Biophysica Acta, Vol. 1833 Issue 11), but rather highlight the novel interactions recently identified and discuss their place within the context of ER protein folding, quality control, and secretion. Particular emphasis is placed on interactions between chaperones and foldases of different functional annotations. We begin with a discussion of the methods used to identify interactions in the ER.

3 Methods for Studying Interactions

Protein interactions maps for cytosolic and nuclear proteins have been useful for the elucidation of the function of proteins and of protein complexes. These maps have relied on two hybrid systems that establish binary interactions and methods such as tandem affinity purification (TAP)-tagging and mass spectrometric identification of protein complexes. Interaction maps of membrane proteins have been more of a technical challenge, but robust methods are being developed. What is lacking is a toolbox of methods to study interactions of proteins of cellular compartments that compose organelles such as the ER.

Most ER proteins are membrane proteins that require both specific posttranslational modifications and the specific environmental control of the ER to be able to fold and function properly. These specific requirements have hindered many attempts using conventional methods to interrogate the properties of ER-localized proteins and their physical and functional interactions.

Tailoring existing approaches to the necessities of ER proteins has been essential to capture physical associations on the level of complexes and defined binary interactions. Table 10.1 summarizes some of the methods that have been used to identify protein interactions of ER proteins. Traditionally, ER proteins were first defined in complexes using co-immunoprecipitation (IP) with either unfolded substrate baits or functional interaction partners. Binding partners were identified with using available antibodies [6–8], and more recently, in high-throughput studies using affinity puri-

Table 10.1 Methods for studying interactions

ID	Details	References
CoIP/AP	Affinity purification	[6–8]
TAP-MS	Tandem-tag purification and mass spectrometry	[9, 10]
AP-cross	Affinity purification with cross-linker	[4, 6, 11]
Assembly	In vitro complex assembly	[13]
PCA	Protein complementation assay	[12]
Y2H	Yeast two hybrid	[13, 14]
OP-MS	Organelle purification and mass spectrometry	[15, 16]
Enzyme-shRNA	Functional assay and knockdown	[17]
APEX	Affinity purification, mass spectrometry, microscopy	[18]
ER-MAP	Affinity purification, ER-MYTHS, NMR, functional assay	[13]
Integrative mapping	Affinity purification, mass spectrometry, knockdown	[4]
Real time homeostasis	Microscopy, affinity purification	[19]
–	High-resolution electron microscopy, knockdown	[20, 21]
–	In vivo complex tagging	[22, 23]

fication with tandem tags in combination with mass spectrometry [9, 10]. The more static complexes can be readily identified, but the discovery of other more transient ones must be aided by stabilizing agents such as cross-linkers [4, 6, 11]. These newly identified complexes are then dissected into binary interactions using in vitro assembly studies with purified proteins [12], or different protein complementation assay (PCA) and two-hybrid technologies [12–14]. Where applicable, protein complexes are subjected to enzymatic characterization. In the proteomics area of ER research, mass spectrometry coupled to pull-down and knockdown approaches is being used to monitor changes in complex compositions and their functional consequences on ER processes such as ERAD [4]. In contrast to top-down approaches like affinity purification (AP) and Y2H, mass spectrometry of purified whole ER is used in a bottom-up approach to identify novel ER components [15, 16].

More recent developments in technologies for genetic manipulation have opened the research for the in-depth characterization of the mammalian ER function as it is known for model organisms like *Saccharomyces cerevisiae*. Next to transcription activator-like effector nucleases (TALENS) and clustered, regularly interspersed, short palindromic repeats (CRISPR) associated (Cas) manipulations to introduce very specific genetic changes in genomic DNA, shRNA has become a valuable tool in ER-Omics that allows the directed inactivation of single genes in mammalian genomes. These mutations can then be subjected to a multitude of functional tests. Rutkevitch et al. for example have used shRNA technology to study the functional relationship between different protein disulfide isomerases illustrating overlap in substrate specificities [17].

However it has become more and more evident that no single method is sufficient to study the complex organization of the ER, and research groups have now moved on to using combinatorial experimental approaches like ascrobate peroxidase (APEX)-protein fusions for electron microscopy, ER-MAP (ER-Membrane yeast two hybrid system and Affinity Purification, NMR), integrative mapping (ERAD using AP; mass spectronomy, MS; shRNA), and real-time homeostasis (ERdj3 using green fluorescent protein (GFP)-fusion microscopy, AP) [4, 13, 18, 19].

There have been several exciting recent methodological advances that have allowed more detailed study of the secretory pathway and relevant interactions. Coupling high-throughput high resolution light microscopy with electron microscopy and shRNA has allowed unprecedented details to be revealed including network structures [20, 21]. These approaches not only offer promising ways to validate hypotheses from less direct observations (AP-MS and Y2H) but also provide ways to visualize how multi-chaperone complexes may be organized within the ER in a single cell. Similarly, recent advances in nanotechnology have allowed microscopic visualization of molecular assemblies in living cells independent of fluorescence, opening up new avenues to study organelle architecture and organization [22]. Finally, a recently developed methodology that relies on probe incorporation mediated by enzymes (PRIME) has been adapted for the secretory pathway in yeast, and interaction-dependent coumarin probes have been developed to allow specific labeling of proteins of interest [23]. This technology requires genetic addition of a 13-mer peptide acceptor and expression of a ligase that couples the probe specifically to acceptor peptides, and has been applied to extra-cellular protein interactions. While these technologies have not yet been applied to protein-protein interactions in the ER, they provide additional tools to enhance the clarity of previously used combinatorial approaches in the future.

4 Protein Complexes of Nascent Chain Entry

4.1 SRP-Dependent Entry

Perhaps one of the most well studied steps in the process of protein folding and secretion through the ER is the entry of nascent chains into the ER [24–29], but this process has recently been updated with fresh data pertaining to alternative entry mechanisms [30, 31], and is far from completely understood. Nascent chain entry into the mammalian ER occurs primarily through recognition of signal peptides on ribosome-bound proteins destined for secretion by the signal recognition particle (SRP) [28] followed by recruitment of the proteins either co or posttranslationally to the SRP receptor (SR) docked at the translocation channel (translocon). The translocon is comprised of Sec61α, β, and γ subunits, and depending on its association with other proteins, it can function in either co-translational translocation [28], posttranslational translocation [32], and perhaps retro-translocation of proteins for ERAD [33, 34] although other proteins such as Derlins have been implicated and may be more suitable for this process [35] (ERAD will be revisited later in this chapter). The prokaryotic SecY has been posited to have a similar structure to that of eukaryotic Sec61, and structural insights into this pore indicate that during translocation, a small molecule barrier is maintained around the translocating peptide by amino acids lining the pore that form a “gasket-like” seal [36]. When the channel is not translocating peptides, it is plugged on the cytosolic side by a helix of the channel that shifts into a blocking position [36]. In eukaryotes, chaperones includ-

ing the ER-resident Hsp70-like protein BiP aid the directional translocation of the nascent chain, and further translocation after cleavage of the signal sequence by the translocon-associated signal peptidase [37].

The Sec61 translocon is part of a much larger ribosome-anchored membrane protein (RAMP) complex that resolves by high resolution native electrophoresis into three distinct multicomponent complexes that at their core include the oligosaccharyltransferase complex (OST), glucosidase I, the microtubule tethering protein CLIMP63, and on the luminal side, the J-domain co-chaperone ERdj3 [11]. The larger two complexes contain the kinesin-motor-component-binding protein p180 and Sec61 while the largest complex contains the translocon associated protein (TRAP) complex and Bap31 (apoptotic regulator protein), as determined using proteomics and high-resolution native electrophoresis [11]. Interaction of ERdj3 with the Sec61α subunit of the translocon has been confirmed in separate Co-IP, native gel, and mobility studies [19]. The translocon complex also interacts in a less stable manner with the signal peptidase complex.

Importantly, in addition to ERdj3, there are several other translocon-associated J-domain co-chaperones of BiP that interact with BiP through hydroxyphenylpyruvate dioxygenase (HPD) motifs in their J-domains, and/or through other, still poorly understood mechanisms. These other J-domain containing co-chaperones include ERdj1, ERdj2/Sec63, and the Sec63 interacting protein Sec62. ERdj1 is a membrane protein that recruits BiP to ribosomes and also regulates translation [38, 39]. Sec63 contains three transmembrane domains with a luminal J domain and is found in stoichiometric amounts with the Sec61 alpha subunit in dog pancreatic microsomes [40]. While loss of function mutations of mammalian Sec63 are not lethal (as observed for Sec63p in yeast), they are associated with polycystic liver disease [41]. Sec62, a double-spanning membrane protein, associates with ribosomes and with Sec63, and has been recently shown to play a role in mediating membrane insertion and orientation of moderately hydrophobic signal anchor proteins in the ER [42]. Sec62 is also critical for SRP-independent translocation of short peptides (~160 amino acids) into the ER [43]. Although the precise functions of each mammalian translocon-associated co-chaperone are still being worked out, studies in yeast indicate that their ability to recruit BiP defines the specific roles of BiP in translocation [44].

New studies have shed light on the functions of the translocon and associated J-domain co-chaperones. A recent study in human cells examined gene silencing of Sec61α, Sec62, and Sec63 and its effects on growth and trafficking of proteins of different topologies [45]. Sec61α was found to be essential for growth (also lethal as expected), but analysis of time-points prior to cell death showed no defects in tail-anchored protein insertion while signal-peptide-dependent translocation was inhibited [45]. Silencing of Sec62 inhibited posttranslational transport of small presecretory proteins into the ER while silencing of Sec63 only affected a subset of signal-peptide containing precursor proteins, including Prion protein [45]. A subsequent gene silencing and overexpression study also in human cells found that Sec63 has a negative feedback role in multi-spanning membrane protein insertion that is independent of its interaction with Sec62 [46]. This suggested that Sec63 may play a quantity control function in transmembrane protein biosynthesis, and inter-

estingly, this down-regulating function relied on HPD-mediated interaction with BiP. Perhaps the Sec63-BiP interaction functions to slow translocation of polytopic membrane proteins to prevent overload of the possibly complex chaperone-mediated folding of these proteins on both the ER and cytosolic side. The interaction may provide a feedback to slow translation to a rate that complies with chaperone availability. The role for BiP in protein translocation as a molecular ratchet through binding and release [37, 47] may be not only to maintain inward directional flow but also to control the rate, and perhaps in concert with Sec63 to slow it for more complex proteins. The J-domain co-chaperone and BiP interactions located at the translocon may represent one of the first places where chaperone interactions function to assure quality of products in the secretory pathway.

4.2 SRP Independent Entry

While SRP-independent mechanisms for nascent chain entry into the ER have been known to exist for some time [25, 29] there is now strong evidence for some of the machineries involved and possible mechanisms used, particularly in yeast [30–32]. These alternative entry methods utilize cytosolic factors and require chaperoning of the proteins prior to entry, efficient targeting to the ER, and directional entry of the nascent chains (reviewed in [48]). It has been suggested that alternative entry methods function to facilitate specific clients with signal sequences that are not optimal for SRP recruitment (lacking adequate hydrophobicity) and are unable or poorly able to bind the SRP receptor. Alternative entry methods may direct certain nascent chains to particular fates on the luminal side better suited for their particular folding needs; for example near the nucleus [30].

Regardless of entry point, even before the nascent protein fully enters the ER lumen, oxidative protein folding involving interactions with co-chaperones, chaperones, and foldases (some of which are translocon-associated) begins.

5 Early Chaperone Encounters

5.1 BiP and Its J-Domain Co-chaperones

Probably the first chaperone encountered in the ER lumen by most nascent chains is BiP, given its direct role in translocation. BiP is the ER-resident Hsp70 that functions to bind and release substrates through cycles of adenosine triphosphate (ATP) hydrolysis and nucleotide exchange. A family of J-domain co-chaperones (Erdj1–7) stimulate hydrolysis of ATP bound to BiP, and several nucleotide exchange factors (NEFs) including BiP-associated protein (BAP)/SIL1, and glucose regulated protein GRp170 help exchange adenosine diphosphate (ADP) to ATP, crucial for substrate release. Importantly, a rapid regulatory modification of BiP has been uncov-

ered where ADP-ribosylation of BiP by an unknown ADP-ribosyltransferase acts to destabilize substrate binding, acting as a rapid brake to inhibit BiP-substrate interactions [49]. This rapid regulation of the substrate binding of BiP could be crucial to prevent aggregation of substrates at lower concentrations, and also allow more rapid folding without overzealous chaperone binding of less abundant substrates. ADP-ribosylation (of Arg470 and Arg492) of BiP can be rapidly reversed upon increased protein load [49], presumably by an as yet unidentified ADP-ribosylhydrolase. The future identification of the ER located ADP-ribosyltransferase and ribosylhydrolase will provide further insight into rapid temporal regulation of chaperone networks in the ER, which may be critical for optimal protein folding quality control in the ER.

As described above, ERdj1 and Erdj2/Sec63 are closely associated with the translocon, and, along with ERdj3, serve to recruit BiP to nascent chains as they emerge. Available evidence suggests that the other J-domain co-chaperones ERdj4, ERdj5, and ERdj6/DnajC3 (p58 IPK), and ERdj7 likely function as specialized adapter proteins to target BiP to specific substrates and/or functions in the ER. While ERdj3 can recruit BiP to newly synthesized proteins through direct interaction with unfolded protein regions [50], ERdj5 functions as a reductase to prepare substrates for ERAD in concert with ER degradation enhancing mannosidase like protein (EDEM)1 and BiP [51], and as a specific chaperone/foldase for the low-density lipoprotein (LDL) receptor [52]. The crystal structure and in vitro functional analysis of ERdj5 [53] revealed how it interacts with EDEM and BiP, and utilizes two highly reductive thioredoxin domains to facilitate reduction of disulfide bonds in proteins destined for ERAD. Structural insight into DnaJc3/ERdj6 (P58/IPK), that is strongly induced by the unfolded protein response, revealed that the putative nascent chain or unfolded protein-binding site is situated 100 Å away from the HPD motif that interacts with BiP, suggesting that handoff of substrates to BiP may involve structural re-arrangements of the J-domain [54].

It is also possible that co-chaperones with hydrophobic substrate-binding sites provide a distant alternative “safe-holding” site for exposed hydrophobic regions of substrate proteins in between cycles of binding and release by BiP. It is important to note that for at least one J-domain co-chaperone (ERdj3), and likely others, release of ERdj3 from substrates is in turn regulated by BiP, and only occurs in the presence of ATP [55]. This suggests that ERdjs could be interacting with clients and chaperoning them until they recruit BiP to engage in protein binding and release cycles that result in back and forth handoff of the protein until correctly folded. This relationship is more than a simple binding and release, as the J-domain co-chaperone ERdj3 can help open the lid of BiP to facilitate peptide binding in a nucleotide dependent manner [56]. The different domain architectures of the other co-chaperones might provide versatile adapters to facilitate productive binding and release of substrates with different folds.

Importantly, of the seven J-domain containing co-chaperones in the ER, 4 are induced by the unfolded protein response (UPR; ERdj3, ERdj4, ERdj5, and DNAJc3/ERdj6), suggesting that these have important roles in coping with unfolded protein stress. The function of ERdj7 is not yet known, but its unresponsiveness to the UPR suggests it may be involved in constitutive processes like translocation or

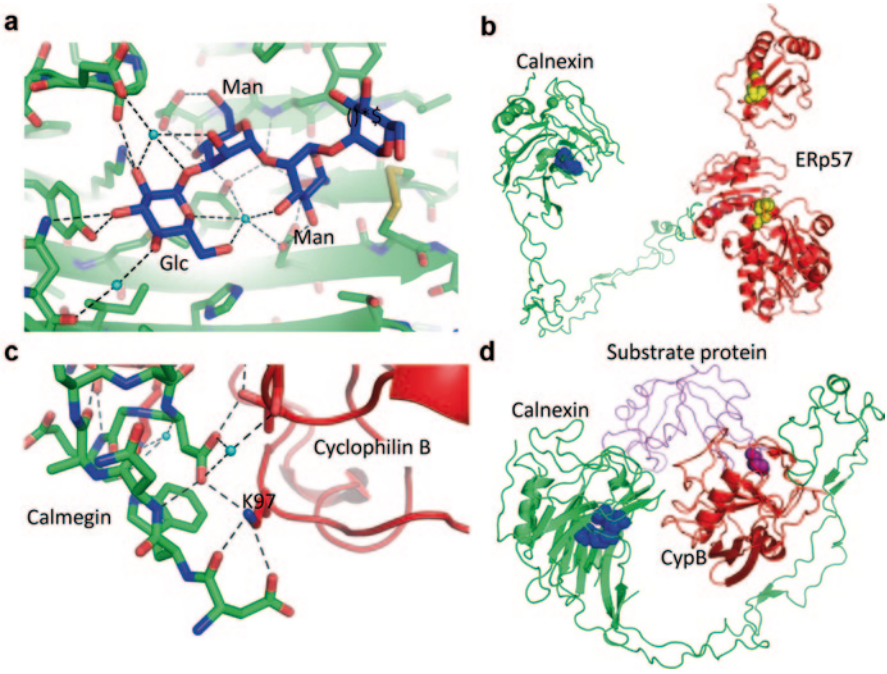

Fig. 10.1 Structural basis of ER interactions. **a** Structure of the tetrasaccharide Glc_1Man_3 (*blue*) bound to calreticulin (PDB code 3O0W). **b** Model of the complex between calnexin (PDB code 1JHN) and ERp57 (*red*; PDB code 3F8U). The glycan-binding site in calnexin is marked *blue* and the catalytic cysteines in ERp57 are highlighted in *yellow*. **c** Detailed view of hydrogen bonds and electrostatic interactions at the interface between the lectin chaperone, calmegin and cyclophilin B (PDB code 3ICI). K97 of cyclophilin B plays a key role in orchestrating the interaction. **d** Model of calnexin (*green*) and cyclophilin B (*red*; PDB code 3ICI) with a substrate protein (RNase B; *magenta*; PDB code 1Z6 S). The substrate is positioned so that proline 93 occupies the cyclophilin B active site.

membrane insertion as observed for ERdj1 and ERdj2/Sec63. While the specific functions of each ERdj co-chaperone are not fully understood, the emerging pattern is that they specify spatial targeting of BiP to specific ER functions through interactions with BiP and the machineries involved in these functions.

Recent insight into J-domain interactions with other ER luminal proteins has provided additional clues regarding their targeting to specific ER processes (Fig. 10.1 and Table 10.1). These interactions were identified mostly using an ER-specific membrane yeast two-hybrid system (ER-MYTHS) [13]. Interactions between the J-domain co-chaperones tested (ERdj3, ERdj4, ERdj5, DnaJc3) and BiP were confirmed, in addition to several novel interactions. Surprisingly, ERdj3, ERdj4, and ERdj5 all exhibited binary interactions with the protein disulfide isomerase (PDI) family member P5 that is known to interact with BiP. P5 is comprised of two active site containing thioredoxin domains and a single inactive thioredoxin-like domain that resembles the b domain of PDI [57]. The b domain of PDI is not sufficient for

binding peptides alone [58]. Similarly, the inactive b-like domain of P5 is unable to bind peptides directly (our unpublished observations). Perhaps these three J-domain co-chaperones act to recruit substrates to both BiP and P5, providing recruitment services to BiP through either or both proteins. Interaction of both ERdj3 and ERdj4 with the FK506 binding protein (FKBP) family member FKBP60, an ER-resident peptidyl-prolyl *cis-trans* isomerase (PPI) that is poorly characterized but contains four PPI domains [59] is a novel way to recruit PPI activity to nascent substrates. Interestingly, ERdj4 was found to interact with calnexin, and ERdj3 was found to interact with UDP-glucose:glycoprotein glucosyltransferase (UGGT), providing connections between the BiP and lectin chaperone systems (to be discussed below). Interaction between ERdj3 and UGGT, a protein strongly implicated in misfolding recognition and quality control of N-glycosylated proteins, suggests a previously unappreciated cooperation between the lectin and BiP chaperone systems. While ERdj3 has been most associated with folding of nascent chains, both ERdj3 and ERdj4 have been recently implicated in BiP-independent selection of substrates for ERAD [60], perhaps analogous to misfolding recognition by UGGT.

5.2 Nucleotide Exchange Factor Interactions of BiP

The NEFs BAP and GRp170 are important for release of substrates from BiP. GRp170 is unique in that it is also a large Hsp70 with capacity to bind unfolded/misfolded proteins. Recent studies have shown that GRp170 remains bound to unfolded substrates after BiP release [61], and functions in ERAD of the unglycosylated form of the epithelial sodium channel (ENaC) [62]. It is interesting to note that GRp170 interacts with ERdj5 [13], a J-domain co-chaperone with a key role in reducing disulfides of misfolded proteins prior to ERAD. GRp170 might be a glycan-independent chaperone of misfolded proteins, taking the place of EDEM in the well-known EDEM-ERdj5-BiP ERAD complex [51], but this hypothesis requires further investigation.

5.3 Early Lectin Chaperone Interaction Networks

As nascent polypeptide chains enter the ER, a large proportion of them are glycosylated on Asn-X-S/T consensus sequences with Glc3Man9GlcNAc2 N-glycans by the translocon-associated oligosaccharyltransferase (OST). Aside from providing an additional mechanism for directional peptide sequestration in the ER, the glycan acts directly to increase hydrophilicity of the protein and indirectly as a ligand for recruitment of the nascent chain to the membrane anchored lectin chaperone calnexin and its soluble counterpart calreticulin. The terminal two glucose moieties must first be trimmed by glucosidase I (translocon associated), and glucosidase II, to generate the Glc1Man9GlcNAc2 N-glycan recognized by calnexin/calreticulin. The structural determinants of glycan recognition by calreticulin have been

determined, and are illustrated in Fig. 10.1a [63]. To efficiently capture nascent glycoproteins as they enter the ER, calnexin associates directly with the ribosome-translocon complex through its cytosolic tail [64]. The lectins recruit the protein disulfide isomerase ERp57 [65, 66] (Fig. 10.1b), or the peptidyl prolyl cis-trans isomerase cyclophilin B [13, 67] (Fig. 10.1c) to the nascent chain to aid the folding process (Fig. 10.1d). In yeast, Mpd1p (a yeast PDI family protein) interacts with Cne1p (a yeast homolog of calnexin) [68].

A significant body of research has been dedicated to understanding the specific roles of the ERp57-calnexin/calreticulin interaction in the folding of glycoproteins [69, 70] and also in the MHC Class I peptide loading complex with tapasin [71, 72]. Structural insights, NMR, mutagenesis, and isothermal titration calorimetry (ITC)-binding studies have defined the molecular details of the interaction between the extended proline-rich (P) domain of calnexin, and the noncatalytic bb' domains of ERp57 [66]. The molecular details have allowed theoretical modeling of the interaction, revealing a complex that positions the thiol active sites of ERp57 in proximity to a glycoprotein substrate bound to the lectin-binding site of calnexin [69] (Fig. 10.1d). Specific abrogation of the ERp57/calnexin interaction through the point mutation (R282A) of ERp57 has made it possible to ascertain the importance of this interaction for recruitment of ERp57 to specific glycoprotein substrates or complexes containing calnexin/calreticulin [73]. The ERp57-calnexin/calreticulin interaction is crucial for recruitment of ERp57 to the majority of its glycoprotein substrates, but is not required for interaction with tapasin or the primary oxidant Ero1 [73].

The ERp57/calnexin or calreticulin and cyclophilin B/calnexin or calreticulin complexes serve as examples of how ER complexes can be best defined through detailed molecular characterization that subsequently provides the means to define interaction importance *in vivo* using targeted genetic manipulations. In the case of the cyclophilin B/calnexin or calreticulin interactions, the specific functional consequences remain to be elucidated, but genetic diseases linked to mutations of cyclophilin B are providing clues [74] (discussed in a subsequent section below).

Interaction mapping in the ER has revealed much more interconnections between different functional categories of chaperones and foldases than previously anticipated, and the lectin chaperones are no exception (Fig. 10.2). Besides the surprising interactions of both calnexin and calreticulin with cyclophilin B, novel disulfide isomerase interactions have been identified for both lectin chaperones. Calnexin interacts with ERp29 [75] while calreticulin interacts with PDIr [13, 76, 77]. ERp29 does not contain a thiol-reactive active site, and is comprised of a helical D domain similar to Drosophila Wind protein [78], and a thioredoxin-like b domain. Its function appears to be in chaperoning a broad range of secreted and ER-resident proteins [79] including thyroglobulin, and PERK [75]. More recently, ERp29 has been implicated in trafficking of wild-type and F508del CFTR. ERp29 expression increases significantly in response to low concentrations of 4-phenylbutyrate (4PBA) that do not induce UPR, suggesting that 4PBA induces ERp29 expression through an unknown mechanism independent of UPR [80]. Increased levels of ERp29 enhance trafficking to the cell surface of both the mutant and wild-type CFTR channels in CF epithelial cells [80].

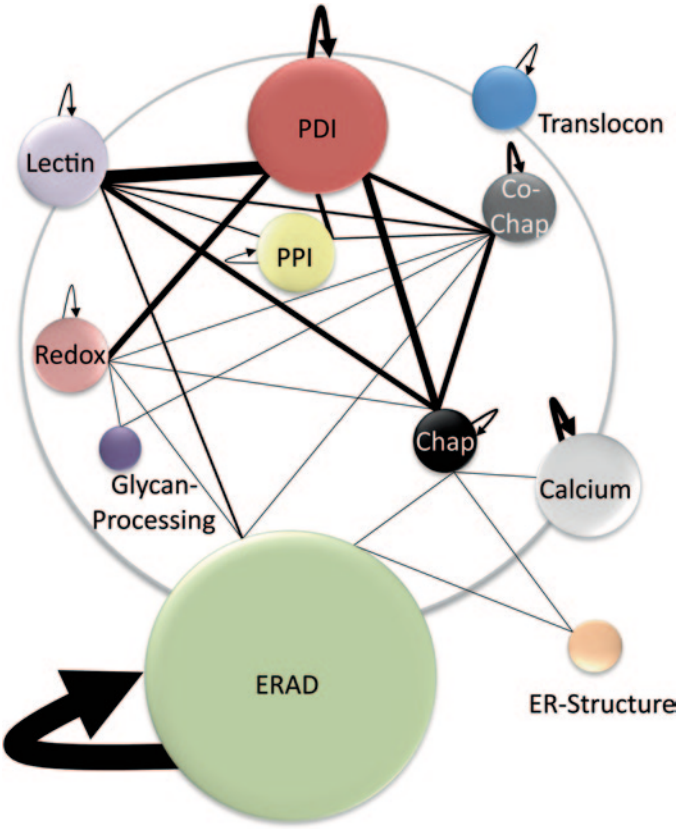

Fig. 10.2 Physical interactions of functional complexes in the ER. Represented are functional complexes that have been shown to associate directly. The size of the spheres correlates to the number of members for each functional complex found to be involved in direct interactions (as provided in Table 10.2). The weight of the edges indicates the number of literature-documented interactions between different pairs of functional complexes, or interactions of a functional complex with itself.

This pivotal role for ERp29 as a chaperone may also be related to its emerging function as a tumor suppressor [81]. PDIr is less well-studied, but its domain architecture is unique among ~20 ER-resident PDI family proteins [57], containing three slightly different thiol-reactive active sites. The active sites are found in three adjacent C-terminal thioredoxin domains with a single N-terminal non-catalytic thioredoxin-like domain mediating interactions with ERp72 and Calreticulin [77]. A PDIr-ERp72 complex would provide a large number of thiol reactive active sites in a single unit (six), and it is unclear what advantage this might provide. PDIr contains three distinct active sites CSMC, CGHC, and CPHC, and its thiol oxidase activity is minimal compared to other human PDIs [82] suggesting that it may play a more specialized role in isomerization. Perhaps calreticulin associates with both a potent oxidase (ERp57), and a more specialized isomerase (PDIr) to recruit slightly different activities to N-glycoproteins during different stages of protein folding.

Table 10.2 Functional connections between different classes of ER proteins, indicating the number of individual connections, referenced by PubMed-IDs

Class I	Class II	Count	PMID
Lectin	PDI	17	18653895, 22665516, 15865205, 10436013, 14988724, 23614004
Chaperone	PDI	10	19887585, 18653895, 15865205, 22665516, 10436013, 14988724, 23614004
PDI	Redox	8	19887585, 22119785, 22665516, 23979138, 22451649
Chaperone	Co-chaperone	7	12356756, 17567950, 18923428, 22267725, 22665516, 18653895
Chaperone	Lectin	7	22119785, 22665516, 12610305
Co-chaperone	PDI	6	22665516
PDI	Proline isomerases	6	12204109, 22665516
Chaperone	Proline isomerases	5	14960307, 18946027, 20801878
Co-chaperone	Lectin	3	22665516
ERAD	Lectin	3	22119785
Co-chaperone	Proline isomerases	2	22665516
Lectin	Proline isomerases	2	20801878
Redox	Glycan processing	1	16129668
Chaperone	Calcium	1	17981125, 23760505
Chaperone	ER structure	1	22689054
Chaperone	ERAD	1	23859598
Chaperone	Redox	1	22665516
Co-chaperone	ERAD	1	22267725
Co-chaperone	Redox	1	22119785
ERAD	ER structure	1	23790629
ERAD	Redox	1	22119785
Lectin	Redox	1	19887585
Co-chaperone	Glycan processing	1	22665516

ER Endoplasmic reticulum, *PDI* Protein disulfide isomerase, *ERAD* ER-associated degradation

An unexpected role for calnexin in regulating the transcriptional response to epidermal growth factor (EGF) receptor stimulation and apoptosis has been recently uncovered. Calnexin is highly abundant and has been found to associate with areas of the ER involved in mitochondrial contacts. The cytosolic domain of calnexin is cleaved by caspase-8 in response to EGF signaling, and this cytosolic peptide of calnexin translocates to the nucleus where it binds a protein inhibitor of activated STAT3 (PIAS3) [83]. Competitive binding of the STAT3 inhibitor enhances the STAT3-mediated response to EGF, linking calnexin to this critical apoptotic signal [83]. However, more than this, calnexin responds to ER stress by rendering its cytosolic peptide uncleavable by an unknown mechanism dependent on the glycan-binding luminal domain of calnexin. Previous studies have shown that calnexin can promote apoptosis through caspase-8 mediated cleavage of Bap31 in response to prolonged ER stress [84]. Inhibition of apoptosis through caspase-8 mediated cleavage of the calnexin cytosolic domain [83, 85] seems to indicate that calnexin can act as a switch to either promote or inhibit apoptosis. The interactions and regulation

of calnexin at ER-mitochondrial membrane interaction sites will be a subject of intense interest, relating ER stress, oncology, and potentially providing additional insight into the established role of ERp57 in cancer [86–88].

6 Protein Disulfide Isomerase Interactions

A critical role in protein folding in the ER is performed by protein disulfide isomerases (PDIs) that introduce disulfide bonds between cysteine residues (oxidation), re-arrange already formed disulfide bonds (isomerization), and break incorrectly formed disulfide bonds (reduction). These functions are performed by thiol reactive di-cysteine motifs (CXXC, commonly CGHC) that are found in the active domains of PDIs. There are 20 different PDI-like proteins in the ER, and the majority of them contain thiol-reactive active sites. The versatility of PDIs to perform reactions in opposite directions has led to much interest into their mechanisms of action, interactions, and regulation, particularly focused on the most abundant and prolific family member (PDI). Several key aspects of PDI activity have been worked out. For example, in reduction and isomerization reactions, the more C-terminal cysteine within the active site of PDI provides an "escape-pathway" [89]. This property has been used to trap substrates with second cysteine mutants of PDI family proteins [90], and has provided some insight into their substrate preferences.

The ER is, on average, more oxidizing than the cytosol, but recent evidence has called into question the long-held redox status of glutathione in the ER, and its capacity to oxidize PDIs based on their specific standard redox equilibria [91]. The biochemical standard reduction potential in millivolts ($E^{0\prime}$) is a measure of the tendency to gain or lose electrons at pH 7.0 in the presence of another electron acceptor or donor. $E^{0\prime}$ is measured in relation to glutathione. A lower $E^{0\prime}$ indicates that a particular species is more likely to be oxidized (or donate electrons) than a species with a higher $E^{0\prime}$. For example, human PDI has an $E^{0\prime}$ of approximately −180 mV [92] while *E.coli* thioredoxin has an $E^{0\prime}$ of −270 mV [93]. Thus thioredoxin is an efficient reductase of denatured proteins, ($E^{0\prime}$ of approximately −220 mV) [93] while PDI is an efficient oxidase of denatured proteins. A redox sensitive GFP revealed that the ER is not as oxidizing as suggested, having an $E^{0\prime}$of −208 +/−4 mV at pH 7.0 [91]. This less oxidizing environment introduces a challenge for PDI family members because their reducing potentials vary between −219 mV (for the very reducing ERdj5) [51] to −157 mV (for the more oxidizing a' domain of ERp57) [94]. Thus, for the more oxidizing PDIs, specific sources of oxidative equivalents that can accept electrons from these PDIs are required to facilitate their functions in oxidative protein folding. Such systems of electron transport (described below) are stabilized by local interactions and structural properties of the proteins involved [95]. One potential benefit of a less oxidizing ER is that PDIs should have more targeted control over the disulfide bonding process, with less rapid formation of potentially incorrect disulfides spontaneously upon ER entry.

6.1 PDI Interactions with Electron Acceptors and Donors

Acting upstream of PDI-mediated oxidation of cysteine thiols is ER oxidoreductin-1 (Ero1) [96] that is a flavin adenine dinucleotide (FAD)-binding protein that oxidizes PDI [89, 97], allowing it in turn to oxidize protein substrates. Studies in yeast have uncovered that the oxidative function of Ero1p is modulated tightly by Pdi1p [98], and similar regulation occurs with the mammalian proteins [99]. In mammals there are two isoforms of Ero1 (α and β), and Ero1α is the most abundant and ubiquitous isoform, and appears to function most prominently as a source of oxidative equivalents [100], while Ero1β has a more specialized role in the pancreas [101]. In S. *cerevisiae* and C. *elegans*, Ero1p is essential for viability, but neither Ero1α or β are essential for viability of mice [101], indicating that in mammals additional sources of disulfide bond equivalents are also important. These alternative sources include but may not be limited to glutathione and glutathione peroxidases, peroxiredoxin IV, vitamin K epoxide reductase, sulfhydryl oxidases, and the selenoprotein Sep15. Particularly in mammals, low molecular weight oxidants may contribute quite significantly to oxidative protein folding, and this area requires more research [102]. Ero1α is exquisitely redox sensitive [99, 100], and studies of the functional relationship between mammalian Ero1α and PDI have revealed that PDI regulates Ero1α oxidative function through rearrangement of a regulatory disulfide between C94 and C131 (inactive) or C94 and C99 (active) [103–105]. The functions and interactions of the alternative sources of oxidative equivalents are less well-characterized, but interactions with substrates and PDI family members [106] and other chaperones [13] are providing clues.

Recent work has determined a hierarchy of interaction of several of the most abundant PDIs with Ero1α [107] and other sources of oxidative equivalents [106]. While many of the soluble oxidoreductases in the ER (PDI, ERp57, ERp72, ERp46, and P5) are capable of complexing with Ero1α in isolation, PDI appears to be the most engaged with Ero1α, and can also act as a dispenser of oxidative equivalents to less oxidizing PDI family proteins [107]. Furthermore, the two separate redox-active sites of PDI have distinctive roles where the a domain accepts electrons from reduced glutathione while the a' domain accepts electrons from other PDIs [107]. These findings hint at an electron transport system where PDI and Ero1α act as a regulatory hub, and the biochemical redox potentials of the other PDI family members provide a variety of oxidative potentials to substrates with varying needs. This model is consistent with an earlier notion that the many different protein disulfide isomerases may provide slightly different redox capabilities to suit different substrates to which they are recruited [69], and that these are tightly regulated through the redox interactions of the PDIs with Ero1α [99].

6.2 Complexes for Safe Disposal of Electrons from PDIs

Importantly, as Ero1α oxidizes PDIs it accepts electrons and must dispose of these safely [108–110]. Electrons are transferred to molecular oxygen, forming hydrogen

peroxide that must be neutralized by ER-resident peroxiredoxin 4 (PrxIV) [111, 112] or glutaredoxins (GPx7, GPx8) [110], or properly directed for use in other functions such as ROS signaling [113]. Importantly, other proteins including nicotinamide adenine dinucleotide oxidase (NOX) family members that can reside in the ER (NOX2 and 4) also produce peroxides and are involved in signaling the UPR, calcium release regulation, and interactions with PDIs [114]. The protein complexes involved in these processes are not currently well characterized. However, in the case of PrxIV, which requires a disulfide in its active site to be reduced prior to reaction with H_2O_2, studies have shown it cooperates with PDI family members (PDI, ERp46, P5, ERp57, and ERp18) that reduce this disulfide bond, promoting PrxIV activity [115]. Other investigations have revealed a substrate-like specificity of ERp46 for PrxIV [90], and a physical interaction between ERp72 and PrxIV [13]. Redox active proteins in the ER exhibit close functional and physical interactions, and future studies will reveal more clearly how tightly knit these systems are.

6.3 Diverse Structures, Functions, and Interactions of PDIs

The PDI family of proteins is one of the largest families of ER-resident proteins, and not surprisingly, the members are varied in structure [57], function, and interactions. An important longstanding question has been: Why are there so many PDI family proteins in the ER? There appears to be at least two main categories of PDIs—those that interact directly with substrates (e.g., PDI can bind substrates directly), and those that interact with substrates through partner proteins (e.g., ERp57 interacts with calnexin or calreticulin to recruit glycoprotein substrates). This does not mean that a particular PDI that is capable of interacting with substrates on its own works independently of other ER resident proteins, but simply that it does not require those interactions for substrate binding. The specific roles of the many different PDIs is becoming more and more clear through studies looking at both how each one interacts with specific sources of oxidative equivalents [106], and by how each interacts with other ER resident chaperones and foldases [13].

While it is expected that redox active proteins involved in electron transfer reactions in the ER should be physically associated, additional insight into the varied functions of the different PDIs can be gained from understanding their unique interactions with proteins of different functional annotations. Guilt by association reveals a different but perhaps complementary perspective on the unique redox potential hypothesis for PDIs. This perspective suggests that the specific interactions of each PDI are critical for its unique substrate-specific functions.

The reason for so many different PDIs in the ER is a question that is gradually being answered. On the one hand, redundancy of PDIs allows the ER to adapt when overloaded with a particular substrate, and thus proteins with overlapping functions would be beneficial. Gene silencing studies of four abundant PDIs (PDI, ERp57, ERp72, and P5) and the resultant impact on the folding of a panel of five secreted proteins revealed that, at least for the specific substrates chosen, PDI is most important [17]. However, the other PDIs were able to compensate for PDI loss

quite effectively although a loss of both PDI and ERp57 impeded oxidative protein folding efficiency most significantly [17]. Given the many different client proteins passing through the ER, it is difficult to generalize these results more broadly, but the relative abundance of PDI and ERp57 supports their dominant roles in disulfide bond formation for non-glycosylated and glycosylated substrates, respectively. The specialized functions of other PDI family proteins continue to be uncovered. For example, the PDI family member anterior gradient-2 (AGR2) functions as a shuttling factor for mucins [116], and is implicated as an oncogene in a variety of cancers [117]. The role of AGR2 in cancer has prompted research into its physical and functional interactions, and the AAA+ tumor suppressor Reptin was found to bind its substrate-binding loop region in an ATP-regulated fashion [118]. It is alarming how many PDI family members have been implicated in cancer using unbiased genomic screens [119], hinting at additional unexpected and specialized interactions for this family to be uncovered in the future.

6.4 PDI and PPI Partnerships

While it is difficult to surmise the implications of all of the known PDI interactions, a trend of partnering with peptidyl-prolyl cis-trans isomerases is clear (Table 10.2). While disulfide and prolyl isomerases carry out very different biochemical functions, the utility of joining them together in a functional unit can be specifically appreciated for substrates such as Immunoglobulin G (IgG) where rate-determining steps for disulfide bond formation require prolyl-cis-trans isomerization [120, 121]. ERp72, PDI, and P5 can partner with cyclophilin B, and the assembly of C_H1 and C_L fragments of IgG in vitro is enhanced in the presence of both ERp72 and cyclophilin B [13]. This physical and functional cooperation appears to be replicated in several other PDI-PPI pairs including ERp29-FKBP23, ERp19-FKBP65, and ERp57-FKBP13. The functional implications of these other partnerships remain unexplored but results with cyclophilin B suggest that efficiency of protein folding could be enhanced by concerted action of both PDIs and PPIs on the same substrate.

6.5 Other PDI Interactions

The PDI family contains members involved in diverse ER functions. As the chaperone systems devoted to protein retention, secretion, quality control, and ERAD are being identified, more PDIs are finding their places involved in one or more of these processes. In addition, unexpected connections between PDI family proteins are being revealed, such as interaction between ERp57 and ERp27 (a non-redox active 2-domain PDI) [122]. This interaction may serve as an alternative means to recruit substrates to ERp57 via the recently defined chaperone-like binding activity of ERp27 [123]. ERp44 has been found to cycle between the ER and Golgi, engaging in a pH-dependent retrieval of incompletely disulfide bonded substrates like IgM

[124]. PDIs are also clearly involved in ERAD, with diverse substrate-binding capabilities likely contributing to ERAD of diverse substrates [125], and documented reduction of disulfides in terminally misfolded proteins by highly reductive ERdj5 [51]. The targeting of PDIs to ERAD is in at least some cases clearly determined through interactions with ERAD components, and it is likely that there are additional interactions between the different PDIs and ERAD machineries.

7 Interactions between Functionally Distinct ER Proteins

While it is to be expected that proteins of different functions will interact, the identification of these interactions provides a wealth of insight into how machineries assemble and are organized within the cell. Many such interactions have been recently identified in the ER, and here we attempt to highlight some of these novel complexes encountered by nascent chains, with a view towards their functional consequences.

Aside from its interactions with protein disulfide isomerases, the prolyl isomerase cyclophilin B interacts with GRp94, BiP, and calnexin/calreticulin [13]. These interactions suggest that cyclophilin B is a very versatile foldase, capable of being recruited to multiple chaperone complexes to aid the folding of proteins that may benefit from prolyl isomerization. The importance of cyclophilin B recruitment to specific chaperone complexes is supported by recent genetic evidence where a mutation within the polybasic region of cyclophilin B (G6R), known to be important for chaperone binding from NMR and crystallography studies [13, 67] leads to hyperelastosis cutis in inbred quarter horses [74]. Collagen assembly is impaired in fibroblasts from the horses, implying that in order for cyclophilin B to carry out its critical function for assembly of the collagen triple-helix, it must be properly targeted to chaperone complexes [74].

BiP and cyclophilin B are perhaps two of the best examples of proteins that function in very diverse capacities in the ER, depending on the complexes that they associate with. For example, BiP not only functions to bind and aid in the directional entry of nascent chains at the translocon when recruited by specific J-domain co-chaperones like ERdj1, 2, and 3 (described earlier) but also plays a crucial role in unfolding and disposal of terminally misfolded proteins as part of a complex with ERdj5 and EDEMs [51]. Similarly, cyclophilin B not only has roles in anterograde folding in the aforementioned chaperone complexes [74, 126] but also plays critical roles in ERAD of soluble proteins [127]. While the different chaperones and foldases in the ER may appear to have prominent roles in specific complexes, it is important to keep in mind that entirely new and diverse functions for these same chaperones may be uncovered through their associations with other complexes, particularly if they are relatively abundant in the ER.

This emerging trend of chaperone sharing between functional complexes can be seen in Fig. 10.2. The number of interactions between proteins in the ER that carry out diverse functions are illustrated by the thickness of the edges linking

different functional annotations (nodes). While we do not specifically describe each chaperone protein that finds itself playing multiple parts in the ER, it is clear that multi-function chaperones are common. Table 10.2 summarizes the number of interactions between different functional classes. Generally, as might be expected, the most abundant ER-resident proteins are found to interact with the largest variety of functional categories. The most connected functional classes are the lectins and PDIs followed by chaperones and PDIs. Less abundant ER proteins likely fill specialized roles in specific complexes that are spatially arranged in the ER in defined microdomains or subcompartments. These complexes help polarize the ER into regions specialized for entry, oxidative folding functions (like the Ero1 electron transport system previously described), quality control, retention, and ERAD. It appears that the functions of many proteins within the ER are more precisely defined by the complexes that they belong to than by their individual annotated functions. With further investigation, involvement of different chaperone proteins in diverse complexes and functions will be uncovered. These trends speak to the complex interplay between ER resident proteins and their versatility, and further agree with data from real time imaging of abundant chaperones under different conditions in living cells [19]. The most abundant chaperones diffuse freely throughout the ER, and upon increases in protein load, their localization to specific regions (likely complexes engaged with substrates involved in folding or ERAD) is very rapid. This intrinsic buffering capacity also provides feedback through, for example, lack of BiP availability, as a mechanism for initiating the unfolded protein response [128].

8 Cargo Sorting and Protein Retention Complexes

As proteins fold in the ER, their subsequent fate must be determined based on their fidelity. Proteins that are incompletely or improperly folded or assembled must be retained until defects are corrected, or the terminally defective protein is earmarked for ERAD. This is not a trivial process, and there appear to be glycan-dependent and independent sorting and retention mechanisms. Glycan independent mechanisms include interactions with BiP. There are also retrieval mechanisms that recapture proteins that have erroneously exited the ER in vesicles headed for the Golgi.

8.1 The UGGT Cycling Complex

UDP-glucose:glycoprotein glucosyltransferase (UGGT) functions to recognize and reglucosylate incompletely folded proteins on their high mannose N-linked glycans so that they can be recycled for productive folding attempts in association with calnexin/calreticulin. How exactly UGGT recognizes misfolding remains a mystery, but the mechanism appears to involve conformational changes in UGGT and binding through hydrophobic surfaces [129]. The recognition mechanism is distinct from active binding and release cycles typical of most chaperones. There are two

isoforms of UGGT in mammals (UGGT1 and UGGT2), and in *C.elegans* UGGT2 appears to have a distinct but critical function with weak or non-existent glucosylation activity (although the lack of observed activity may be due to low expression levels of UGGT2 compared to UGGT1) [130]. In mammalian cells, both isoforms have glucosylating activity [131, 132]. The selenoprotein Sep15 binds UGGT, forming a tight 1:1 complex [133, 134] that remains intact during non-reducing native gel electrophoresis for long time periods (our unpublished observations). This interaction is mediated by an N-terminal cysteine-rich domain of Sep15 that is necessary and sufficient to form a complex with UGGT [134]. Sep15 has been shown to significantly enhance the glucosylation activity of UGGT towards a misfolded substrate in vitro [131]. Studies in murine fibroblasts indicate that Sep15 expression is upregulated in response to sub-acute UPR (induced by the anterograde transport inhibitor brefeldin A or N-glycosylation inhibitor tunicamycin), but Sep15 is rapidly degraded in reponse to acute stress induced by the reducing agent dithiothreitol (DTT) or thapsigargin, a non-competitive inhibitor of the ER calcium pump (SERCA) [135]. This suggests that Sep15 may have a role in modulating UGGT activity to increase quality control stringency under moderate stress, but upon acute stress, degradation of substrates may be favored by removal of its UGGT-enhancing activity. However, Sep15 deficiency does not itself lead to UPR, suggesting that Sep15 has a somewhat limited substrate specificity, or that its functions can be compensated for by other yet undiscovered modulators of UGGT [135].

In *trypanosoma cruzi*, deletion of UGGT impedes interaction of *T. cruzi* cathepsin L (TcrCATL), a lysosomal protease, with calreticulin as expected. The efficiency of TcrCATL folding is drastically reduced in UGGT-null cells where the protein forms intermolecular disulfide bonded aggregates. Perhaps the most probable explanation for this result is that calreticulin-mediated recruitment of a *T. cruzi* homolog of ERp57 to the protein is not achieved (glucosylation of TcrCATL is performed exclusively by UGGT in *T. cruzi*), and incorrectly formed disulfide bonds cannot be resolved. However, ERp57 is one of the more oxidizing PDI family members while Sep15 is much more reducing, with a biochemical standard reduction potential of −225 mV (−157 mV for the a' domain of ERp57). This reduction potential is similar to that of ERdj5 which appears to function primarily to reduce disulfides of terminally misfolded proteins prior to retrotranslocation and proteasomal degradation. It is interesting to note that the *T. cruzi* genome does not appear to contain a clearly recognizeable homolog of Sep15, although a related trypanosome does (hypothetical protein STCU_06695 of *Strigomonas culicis*). There is at least one *T. cruzi* protein that shares 30% identity with the *S. culicis* protein, and may function like Sep15, but this remains to be determined.

A function for the Sep15-UGGT quality control complex in mammals may be not only to reglucosylate proteins with recognized flaws in folding but also to reduce or isomerise incorrect disulfide bonds [136]. Reduced disulfide bonds that require more oxidative "power" for fresh attempts to form correct bonds are then recruited back to more oxidizing ERp57 through Glc1-N-glycan interaction with calnexin/calreticulin. This may allow a superficial partitioning of the oxidative and reductive functions during protein folding, as appears to be the trend with the differing redox potentials of the PDIs described above, and their respectively distinct complexes.

The very tight 1:1 UGGT-Sep15 complex (K_d~40 nM) may serve to sequester Sep15 and ensure that Sep15 reductive activity is targeted to substrate proteins that interact with UGGT. Knockdown or overexpression of Sep15 in NIH-3T3 cells imbalances redox homeostasis, suggesting a pivotal role for this protein in ER redox [136]. Other Selenocysteine-containing proteins in the ER include SelM [137] and Fep15 [138], but their functions are not yet clear although they are unlikely to interact with UGGT because they lack the cysteine-rich N-terminus of Sep15 that mediates this interaction.

Other than membership in a multi-protein complex in the ER [6], there are few other known interactions of UGGT. How UGGT is recruited to this multi-protein complex is not clear. It appears that ERdj3 may play a role as it was recently found to interact with UGGT using an ER-tailored yeast two-hybrid system [13]. The functional significance of the ERdj3-UGGT interaction is not known.

8.2 Complexes that Mediate Exit from the Calnexin Cycle

Ultimately, proteins must either achieve a correct fold and bypass recognition by UGGT, bypass recognition while still aberrant due to UGGT saturation or other unknown mechanisms, or be deemed terminally misfolded and targeted for ERAD.

8.3 Complexes of the Mannose Trimming Timer

The most well-established model for N-glycoprotein folding quality control involves the successive slow trimming of mannose moeities from the glycan [139], resulting in eventual extraction of the protein from the calnexin cycle. In yeast, initial trimming is mediated by mannosidase 1 (Mns1p) followed by additional trimming carried out by Htm1p that forms a specific complex with Pdi1p [140]. Pdi1p interaction with Htm1p is mediated by a specific intermolecular disulfide bond with the C-terminal domain of Htm1p, and enhances the mannosidase activity of Htm1p [140]. The resultant trimmed glycan is recognized by the mannose-6-phosphate homology (MRH) domain of Yos9p that targets the protein for ERAD. Mammalian cells have a similar but more complex system involving the same initial trimming by ER mannosidase 1 followed by putative further trimming of mannoses by ER-degradation enhancing mannosidase-like (EDEM) proteins EDEM1, EDEM2, or EDEM3 (in vitro demonstration of EDEM mannosidase activities is still lacking). Similarly to yeast, the mammalian EDEMs appear to cooperate with PDI family proteins through interactions where EDEM3 interacts with ERp46 [90], and EDEM1 interacts with PDI [13]. EDEM1 also interacts with the reductive PDI family member ERdj5 [51] and EDEM2 and EDEM3 appear to also [13], suggesting they may cooperate in processing substrates for eventual retrotranslocation. The precise functional consequences of each PDI-EDEM interaction remain uncharacterized, but in the case of ERdj5-EDEM1, it appears to

target PDI activity to terminally misfolded proteins to prepare them for retrotranslocation [51]. BiP is also found in the complex with EDEM1 and ERdj5, converging four major functions in a single unit (J-domain co-chaperone, PDI, chaperone, and lectin; Fig. 10.2). EDEM1, EDEM2, and EDEM3 have been found to interact with GRp94 [13] while GRp94 also interacts with the mannose-binding lectin XTP3-B [4] that is tightly linked to the ERAD dislocation machineries through SEL1 L (Fig. 10.2). Interestingly BiP also interacts with OS-9, another lectin that binds SEL1 L. Trimmed glycans are recognized by the MRH domains of OS-9 and XTP3-B that can recruit misfolded proteins to the retrotranslocation channel through interactions with SEL1 L [4, 141]. However, interactions of OS-9 and XTP3-B with glycan independent chaperones, and their functional involvement in retrotranslocation of non-glycosylated substrates along with the EDEMs suggest that glycan dependent and independent functions converge at the retrotranslocon and share components.

8.4 Anterograde Trafficking Complexes for Folded Proteins

It is clear that trafficking of correctly folded proteins from the ER to the Golgi for further processing occurs with the involvement of cargo receptors including vesicle integral membrane protein VIP36, VIPL, and ER Golgi intermediate compartment protein-53 (ERGIC-53) in association with coat protein complex II (COPII) and COPI [142–144]. However, there are many details that remain to be worked out, and a family of closely similar abundant membrane proteins termed p24 s has long been implicated in ER-Golgi trafficking. The sequence of the p24 s (EMP24/GP25 LErp) and the members of the family are highly conserved from yeast to humans and they are located in the ER, COPI, and COPII vesicles and the cis-Golgi. While this high degree of conservation implies that they are involved in an essential cellular function, a multiple knock out of the 8 family members in *S.cerevisiae* yielded viable cells that showed a decreased rate of secretion of GPI-anchored proteins and increased leakage of BiP [144]. In contrast, a knock out of a single member in mammalian cells is lethal [146]. Recent results have shown that they are important in secretion of some of the Wnt ligands [147]. Increasing, but not yet definitive evidence is that they function as originally speculated, as cargo receptors for secreted proteins [145, 148]. A greater knowledge of their functional and physical interactions with other proteins will aid in defining their functions.

8.5 ER Mannosidase-I/COPI Interactions and Protein Retrieval

An apparent paradox in ER protein folding quality control has been the localization of all quality control components in the same intracellular organelle as components involved in ERAD. However, there have been plenty of indications that ER quality control and productive folding is somehow partitioned from ERAD [149, 150].

Recently, careful study of the localization of ER mannosidase I (ERManI) revealed that it primarily localizes to the Golgi where it is O-glycosylated [151]. Furthermore, ERManI has been shown to physically interact with the γ-COP subunit of COPI, responsible for Golgi-to-ER retrograde protein transport [152]. Mutations that disrupted this interaction rendered ERManI incapable of mediating efficient disposal of the ERAD substrate Null Hong Kong (NHK) AAT. These functionally significant interactions underscore the importance of understanding partitioning of ER quality control and the PPIs involved.

9 Interactions and Functions in ERAD

The half-lives of the component proteins of the ER varies considerably, indicating that there are underlying mechanisms that maintain the homeostasis of the ER. While the mechanisms that signal the increased need for proteins have been recognized by their participation in the unfolded protein response, those involved in the removal of ER proteins have not been as well studied. The role of autophagy in ER homeostasis has yet to be fully explored but the process known as ERAD is known in detail. The proteins that participate in the ERAD of both membrane and soluble secretory proteins have been identified principally through functional studies mainly in yeast [34].

The behavior of mutant proteins that cause protein trafficking diseases has generated interest in the mechanism of ERAD. More precisely, how does this quality control system function and how might it be subverted? For example, in the archetypical protein trafficking disease cystic fibrosis the mutant protein F508del-CFTR is recognized as misfolded, retained in the ER and retrotranslocated from the ER to be degraded by the proteasome. However, if cells are incubated at low temperature the mutant protein traffics to the plasma membrane and is functional. The precise mechanism of recognition of the misfolded F508del-CFTR protein is unknown, and there are no mutations that decrease the fidelity of the cellular protein quality control mechanism in the same way that the trafficking of F508del-CFTR is corrected by compounds [153, 154].

The ER Calnexin cycle that recognizes secreted N-glycosylated proteins and assists in their folding is well characterized [70, 155] as is the system for non-glycosylated secreted proteins [156]. The main components of this quality control system for recognition of misfolded proteins UGGT and BiP are well characterized. Misfolded glycoproteins show prolonged association with calnexin but compounds, such as deoxynojirimycin, that divert glycoproteins from the Calnexin Cycle do not correct the ER retention of F508del-CFTR. In addition, the F508del mutation is in the nucleotide-binding domain 1 of the CFTR molecule and located in the cytosol. Although there has been a comprehensive catalogue of the proteins that bind to CFTR [157] only, a knockdown of Aha1, which is a cochaperone of Hsp90, had any functional effect on the trafficking of F508del CFTR. Thus, although the role of the Calnexin Cycle in the quality control of soluble secretory

proteins is clear, the recognition mechanism of misfolded membrane proteins is not well understood.

The ER luminal components and the cytosolic ATPase of the retrotranslocation machinery are known. The connection of the most reductive PDI ERdj5, BiP, and EDEMs is mentioned above. This complex functionally links with the misfolded glycoprotein recognition receptors Hrd3 and Yos9 by an as yet unknown mechanism.

There are several candidates for the protein channel that facilitates exit of proteins from the ER. One is the translocon Sec61, or perhaps a version thereof, and more recently Derlin1 (Der1) has been recognized in yeast to form a complex together with the luminal Hrd3 that recognizes substrates, and Hrd1 that ubiquitylates them [158].

The cast of characters has probably been all identified, and some of the complexes that they form identified, but establishing how they function in ERAD needs a global approach.

A comprehensive approach to defining the components and organization of ERAD networks used several experimental approaches to define high confidence interactions [4]. The authors identified potential interacting ER proteins involved in ERAD with tagged proteins expressed in cells, and used purification protocols with different detergents. The complexes were analyzed by mass spectrometry and validated by combining the data from several approaches. They also integrated the data by detecting interactions of the ERAD components with a set of misfolded secreted and membrane proteins while correlating with gene expression data. These studies identified and confirmed complexes that perform the steps in the recognition, retrotranslocation, dislocation, and ubiquitination of misfolded proteins and also showed links to the 26 S proteasome. The details of these complexes has been extensively reviewed elsewhere [141]. This type of integrated approach promises to unravel the mechanism of how misfolded membrane proteins are recognized and how the high fidelity of the cellular protein quality control system is achieved.

10 Regulation of Interactions in the ER

10.1 Chaperone–Substrate Interactions

Regulation of interactions between substrates and chaperone proteins in the ER remains poorly understood although there are several well-characterized mechanisms such as glycan trimming, and emerging novel ones such as ADP-ribosylation of BiP [49]. Glycan trimming functions as both a mechanism for targeting proteins to their respective fates, and as a means to bring together ERAD machineries in the ER have been recently reviewed [139]. ADP-ribosylation was discussed above as a rapid regulator of BiP-substrate interactions to prevent non-productive binding during normal or minimal substrate load. A recent study uncovered a mechanism in

yeast whereby substrate proteins are rapidly and irreversibly tagged for degradation by O-mannosylation [159]. O-mannosylation reduces interaction of substrates with Kar2p, the yeast homolog of BiP. This tagging mechanism provides an alternative to glycan trimming to regulate chaperone-substrate interactions, freeing up chaperones for useful engagement of substrates not past the point of no return. Whether this same mechanism operates in mammalian cells remains to be explored. There is also evidence to suggest that calcium can regulate interactions of smooth ER with mitochondria [160] probably having a broader impact on chaperone-substrate interactions although the mechanisms that dictate these apparent generalized effects remain poorly understood.

10.2 Chaperone–Chaperone Interactions

The regulation of interactions between ER chaperones is far less understood than the regulation of chaperone-substrate interactions. Glycans play a role that is becoming increasingly appreciated (XTP-3B, SEL1 L, and OS-9 are brought together through glycan specific interactions). Due to the electrostatic nature of many ER chaperone-chaperone interactions (that don't involve unfolded protein-binding sites), it is likely that these interactions can be regulated by cation or anion concentrations in the ER, but how exactly this regulation could occur in a specified manner remains unclear. While novel interactions between ER chaperones and foldases continue to be uncovered, defining their respective regulatory mechanisms will become increasingly important to understand. One fascinating area of study is the use of small molecules to correct trafficking defects of mutant proteins linked to human diseases, and some correctors may work by disrupting or modulating ER chaperone complexes. These studies will undoubtedly reveal novel regulations in the ER, as observed for F508del-CFTR correctors such as latonduine, and PARP inhibitors [161].

11 Conclusions and Future Outlook

Our understanding of ER protein folding machineries and their interactions in the ER has advanced greatly in recent years. While earlier studies appeared to indicate a certain degree of separation between functional classes of ER proteins, particularly between the glycan-dependent lectins and glycan-independent BiP chaperones, it is now apparent that chaperones and foldases of very different functional annotations interact promiscuously. Far from being nonspecific and random, many of these novel interactions have specific functional implications, illustrating how tightly interconnected the ER folding machineries are. Beyond the specific interactions, there are many fascinating questions regarding ER complexes to be addressed in future work. Recent high-resolution microscopy and image reconstruction has revealed a "parking garage"- like structure of the ER that can be modulated by beta

sheet-rich proteins such as p180, Kinectin, and Climp63 [1]. There have also been a significant number of studies on the reticulons and their impacts on membrane curvature in the ER [2] while mitochondria associated ER membranes (MAMs) are becoming hotbeds of function related to apoptosis [162]. One obvious question will be—how do ER complexes localize within the ER? Are there particular complexes that contribute to, or are most associated with, particular morphologies/membrane arrangements? It seems clear that many ER chaperones can freely diffuse throughout the ER, but this free diffusion is slowed with increases in substrate load. Are there subregions in the ER that constitute specific targeting areas for chaperones and foldases, arranged spatially to best accommodate the folding needs of the cell? While versatile, these basic complexes may provide a backbone for additional regulatory interactions and organization so that, as in the case of the PDIs, oxidative protein folding is orchestrated such that thiol oxidation, reduction, and isomerisation are most efficient to fold proteins without compromising speed, fidelity, or undue production of reactive oxygen species. Perhaps the ER will emerge, as has the mitochondria, with complexes dedicated to specific functions in electron transport that can be arranged into supercomplexes that determine electron flux under different metabolic circumstances [163]. Certainly, there are many questions to be asked, and with the advent of enhanced resolution, sensitivity, and interaction validation methods, many novel ER chaperone interactions and functions will be discovered in the coming years.

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