THE ROLE OF THE Kar2p/BiP CHAPERONE IN ER QUALITY CONTROL

A Dissertation in
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by
Chia-Ling Hsu

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The dissertation of Chia-Ling Hsu was reviewed and approved* by the following:

Davis T. W. Ng  
Associate Professor of Biochemistry and Molecular Biology  
Dissertation Advisor  
Chair of Committee

Sarah Ades  
Assistant Professor of Biochemistry and Molecular Biology

David S. Gilmour  
Professor of Molecular and Cell Biology

Douglas Cavener  
Professor of Biology

Richard J. Frisque  
Professor of Molecular Viology  
Head of the Department of Biochemistry and Molecular Biology

*Signatures are on file in the Graduate School
ABSTRACT

The unfolded protein response (UPR) is an interorganellar signal transduction pathway that monitors endoplasmic reticulum (ER) homeostasis. Whole genome transcriptional analysis in budding yeast has revealed 381 genes regulated by the UPR. About half of those genes encode functions throughout the secretory pathway including protein folding and modification, protein quality control, lipid synthesis, protein trafficking, and vacuolar (lysosomal) proteases. Previous experiments have shown that the UPR is essential for ER stress tolerance but how the upregulation of individual genes contributes to stress tolerance is unknown. To answer this question, I devised an approach that uncouples the expression of individual target genes from UPR regulation.

The KAR2 gene was selected as the first target for analysis. KAR2 encodes Kar2p/BiP, a highly conserved protein belonging to the Hsp70 family of molecular chaperones. Kar2p is the first UPR target discovered and plays multiple functions in the ER including protein translocation, protein folding, ER associated degradation (ERAD) and karyogamy. These essential functions (protein translocation and folding) raise an interesting conundrum. Since half of UPR targets encode proteins that enter the secretory pathway, Kar2p is, in fact, an essential facilitator of the UPR at its last stage, the synthesis of target proteins. How then, is it possible to activate the UPR when a key facilitator is also a target gene that is immediately needed to deal with the misfolded products of stress (ERAD)?
To address this question and to study the contribution of individual target genes to the ER stress response, we engineered a strain that specifically uncouples Kar2p regulation from the UPR without compromising the activation of other target genes. Under stress, Kar2p upregulation was essential for cell viability. I showed that Kar2p upregulation was required to rid aberrant proteins through both the ERAD pathway and the ER-to-vacuolar overload pathway. Surprisingly, the essential functions of protein translocation and folding were unaffected when Kar2p levels became limiting under stress. This strategy allows Kar2p to temporarily dispense with its role in ridding misfolded proteins to facilitate the UPR until homeostasis is restored. The physiological importance of this ‘triage’ mechanism was apparent because a temporary disruption of Kar2p essential functions was lethal. In contrast, cells could fully survive a temporary disruption of Kar2p's quality control functions.

On the other hand, if Kar2p was limiting for prolonged periods, cells would die. I demonstrated that under stress, the clearance of misfolded proteins is essential. Accordingly, in the strain defective in upregulating KAR2 expression by the UPR, cells eventually died because they could not efficiently degrade misfolded proteins in the ER, this resulted in the accumulation of misfolded proteins in the ER, which caused cellular toxicity. In addition, I also found that a potential export signal in misfolded proteins was formed despite the misfolding of proteins and function in an ER export event.
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1.1 Protein folding in the endoplasmic reticulum (ER)

1.1.1 The endoplasmic reticulum (ER): A protein-folding compartment

The most outstanding characteristic of eukaryotic cells is compartmentalization by membrane-bound organelles. Each organelle is equipped with a specific set of protein and lipid compositions, generating a specialized environment with a distinctive morphology to carry out their cellular functions. The endoplasmic reticulum (ER) is one such vital organelle. The ER is an interconnected network of membrane-enclosed tubules, vesicles and sacs (cisternae) that extends from the nuclear membrane throughout the cytoplasm. It provides a unique oxidizing compartment for many functions including synthesis of lipids and sterols, storage of free calcium, protein folding, ER quality control and protein degradation.

In effect, the ER is the major site dedicated to biosynthesis of all cell surface proteins, secreted proteins and proteins that reside in any compartment along the secretory pathway. Together, these ER clients represent nearly one third of all eukaryotic proteins, as has been determined in S. cerevisiae (Ghaemmaghami et al., 2003). The nascent polypeptides start their journey in the secretory pathway (Figure 1-1) with translocation across the ER membrane via a translocon pore (Walter and Johnson, 1994),
either during translation (co-translational) or when translation is completed (post-translational). They enter the lumen in an unfolded form and their subsequent maturation is assisted by ER chaperones and folding enzymes. These folding factors function in catalyzing slow folding events and in preventing polypeptides from undergoing unproductive interactions with themselves and their environment, a process that leads to protein aggregation (Gething and Sambrook, 1992). The ER folding machinery is unique in the sense that most of its clients would leave the compartment once they are properly folded. Thus, in order to ensure only fully folded proteins are transported to their sites of function, a mechanism termed ‘ER quality control’ (ERQC) monitors the folding state of newly synthesized proteins (Ellgaard and Helenius, 2003). Through this mechanism, immature precursors are kept in the ER until they reach their native conformation. ERQC is also involved in degrading terminally misfolded protein via the ER-associated degradation pathway (ERAD) (Figure 1-1) (Finger et al., 1993). Recently, studies on the degradation of mutant Z variant of human serum protein α1-antitrypsin (known as α1-proteinase inhibitor or A1PiZ) even suggests a potential link between ERQC and an ER-associated autophagy pathway (Kruse et al., 2006a).
Figure 1-1: Protein folding in the endoplasmic reticulum: knowing right from wrong. Secretory and membrane proteins are synthesized and translocated into the ER via Sec61 translocon pore (entry). In the ER lumen, they obtain their native conformation by the assistance of ER chaperones and folding factors (Folding). Fully folded protein can pass strict quality control (QC) and transport to the Golgi, where they obtain further modification. After that, proteins are transported to their final destination which could be the extracellular space or lysosomes. If proteins are misfolded, they are recognized, retained and then degraded by ERAD or autophagy (which are likely mutually regulated, as indicated by blue arrows). Some soluble ERAD substrates are first transported to the ER-Golgi intermediate compartment (ERGIC) or cis-Golgi and subsequently re-enter the ER before degradation. The accumulation of misfolded proteins would activate resident ER stress sensors and the unfolded protein response (UPR) would be elicited. The UPR can activate multidimensional mechanisms to handle the increase of misfolded proteins (orange arrows) such as selective inhibition of protein entry into the ER and increasing the capacity of folding and degradation (via ERAD and autophagy). Brown arrows indicate the direction of vesicles moving among different compartments.
Specialized protein modifications occur within the ER lumen and these include, signal sequence cleavage to generate new N-terminus, the removal of the C-terminal glycosylphosphoinositol (GPI) anchor signal for attachment of GPI anchor (Takeda and Kinoshita, 1995), the formation of disulfide bonds in the unique oxidizing environment of the ER (Tu and Weissman, 2004), and co-translocational addition of N-linked oligosaccharides (van Anken and Braakman, 2005). Although the ER provides an environment optimized for the folding of newly synthesized polypeptides, folding of proteins can and does fail. The ER exerts some measures for responding to the presence of misfolded proteins. Apart from ERQC mechanism and the ERAD pathway, the accumulation of misfolded proteins in the ER lumen also elicits an ER-dedicated stress response, termed the unfolded protein response (UPR) to relieve such a stress (Figure 1-1). The UPR activates multidimensional mechanisms to handle the increase in misfolded proteins (Ron and Walter, 2007): (i) decreased protein translation, (ii) remodeling of the ER so as to augment its folding capacity and ERAD, (iii) decreased entry of proteins into the ER (Kang et al., 2006), and (iv) selective degradation of certain mRNAs that encode secretory proteins (Hollien and Weissman, 2006). Eventually, the UPR may induce pro-apoptotic program when folding problems in the ER can no longer be overcome (Schroder and Kaufman, 2005; Travers et al., 2000).

1.1.2 Targeting of ER client proteins into the ER lumen

In eukaryotes, the initial step of biogenesis of proteins destined for the secretory pathway is their targeting to and subsequent translocation across the ER membrane.
Targeting and translocation can occur during translation (co-translational) or when translation is complete (post-translational) and can either be dependent or independent of the signal recognition particle (SRP). While it has been studied in detail in yeast, post-translational translocation into mammalian ER remains poorly understood.

The selection of the SRP-dependent or SRP-independent pathway is determined mostly by the characteristics of the signal sequence. Generally, an ER-targeting signal sequence comprises a stretch of ~17 to 35 hydrophobic residues at the N-terminus of the ER clients, which targets them to the ER membrane (Walter and Johnson, 1994). These target sequences frequently contain a central hydrophobic core (h-region) and hydrophilic N-terminal and polar C-terminal regions (Martoglio and Dobberstein, 1998). Studies in yeast *S. cerevisiae* have revealed that the h-region of a signal sequence is the parameter distinguishing SRP-dependent from SRP-independent pathways. Signal sequences directing ER clients into the SRP-dependent pathway have a more hydrophobic h-region than those mediating SRP-independent targeting (Ng *et al*., 1996; Zheng and Gierasch, 1996).

In the case of SRP-dependent pathway (Figure 1-2), translocation is co-translational: SRP binds to the signal sequence as it emerges from the surface of the large subunit of the ribosome, stalls translation, and docks the nascent chain/ribosome-SRP complex onto the translocon in the ER membrane via binding to its membrane-localized receptor, SRP receptor (SR) (Walter and Johnson, 1994). Both SRP and SR are GTPases. Structural analyses using bacterial homologue of SRP (called Ffh) and SR (called FtsY) have revealed that the two partners together form a symmetry composite active site at the dimer interface, in which both enzymes reciprocally stimulate each other’s GTPase
activity by interactions between two GTP molecules across the dimer interface, and by extensive conformational changes that position catalytic residues in each active site to the vicinity of the substrates (Egea et al., 2004; Focia et al., 2004). Subsequently, the reciprocal activation of GTP hydrolysis would drive disassembly of the SRP-SR complex (Miller et al., 1993). As a result, the nascent polypeptides directly enter the translocon pore.

In addition to SRP-dependent co-translational pathway, a post-translational mode is also found in all eukaryotes (Figure 1-2). However, in higher eukaryotes, neither the mechanism nor components involved have been identified (Schlenstedt et al., 1990). In contrast, the post-translational translocation apparatus is best studied in S. cerevisiae. The pathway requires a heptameric complex (Sec complex) comprising the trimeric Sec61 complex (Sec61p, Sss1p, and Sbh1p) and the tetrameric Sec63 complex (Sec62, Sec63p, Sec71p, and Sec72p) (Panzaer et al., 1995), cytosolic chaperones (Wilkinson et al., 1997) and two ER luminal chaperones, Kar2p (yeast BiP: a member of the Hsp70 family of ATPase) (Vogel et al., 1990) and Lhs1p (Tyson and Stirling, 2000). The Sec complex is sufficient to carry out post-translational translocation of pre-pro-α-factor into reconstituted proteoliposomes in vitro in the presence of Kar2p (Panzaer et al., 1995). Upon completion of translation, these polypeptide chains bind to the cytoplasmic side of the ER membrane in an ATP-dependent manner. Although this binding involves interactions with Sec61p, Sec62p and Sec72p (Lyman and Schekman, 1997; Matlack et al., 1997), translocation only takes place in the context of the intact heptameric complex (Pilon et al., 1998).
Figure 1-2: The SRP-dependent translocation and the SRP-independent translocation pathways.
The scheme shows the first steps in SRP-dependent and SRP-independent protein transport across the ER membrane. Both pathways converge at the ER translocon formed by the Sec61 complex in eukaryotes (for details, see text). The selection of the SRP-dependent or SRP-independent pathway is determined mostly by characteristics of the hydrophobic core of the signal sequence (yellow pipe shaped object) (For details, see text).
1.1.3 The ER translocon

At the membrane, two translocation pathways converge at the ER translocon. The core of the translocon is composed of a hetero-trimeric complex of membrane proteins which is evolutionarily conserved in all classes of organisms, called Sec61 complex in eukaryotes and SecY complex in bacteria and archaea (Rapoport et al., 1996; Tyedmers et al., 2000). The α-subunits (Sec61α in mammals, Sec61p in *S. cerevisiae*, SecY in bacteria and archaea) and γ-subunits (Sec61γ in mammals, Sss1p in *S. cerevisiae*, SecE in bacteria and archaea) are essential for cell viability. Both proteins show high sequence similarity among these organisms and have similar topologies: the α-subunit contains ten membrane-spanning segments; the γ-subunit, in most organisms, spans the membrane once via a transmembrane segment close to the C terminus. The β-subunits (Sec61β in mammals, Sbh1p in *S. cerevisiae*, Secβ in archaea), however, are non-essential for cell viability. They are conserved in eukaryotes and in archaea but share no sequence homology to the corresponding SecG subunits in bacteria (Rapoport et al., 1996; Tyedmers et al., 2000).

The α-subunit forms the channel pore, which is a passive conduit and must therefore associate with partners that provide the driving force for polypeptides to be translocated (Matlack *et al.*, 1998). In co-translational translocation, the elongating nascent chains are transferred directly from the tunnel of the ribosome into the channel conduit and the energy is driven by GTPase hydrolysis during translocation (Figure 1-2) (Miller *et al.*, 1993). In post-translational translocation in yeast (and probably in other eukaryotes), the partners are the tetrameric Sec63 complex and lumenal ATPase BiP.
BiP serves as a molecular ratchet, preventing back-sliding of a polypeptide chain through the channel back into the cytosol (Matlack et al., 1999). Interaction of BiP with the polypeptide is mediated by the J-domain of Sec63p (Misselwitz et al., 1999). Hydrolysis of ATP bound to BiP is required for the association-dissociation cycle. In the eubacterial post-translational translocation, the cytoplasmic ATPase, SecA, is the interacting component. ATP hydrolysis by SecA is required to push polypeptides through the channel (Economou and Wickner, 1994).

Significant insight into the function of the Sec61p/SecY was obtained from structural studies. The early studies in cryo-EM reconstitution, including using purified yeast Sec61 complex bound to the ribosome in detergent (at ~26 Å resolution) (Beckmann et al., 1997) and using mammalian and yeast ribosome-channel complexes derived from solubilized native membranes or reconstituted proteoliposomes (at ~27 Å resolution) (Menetret et al., 2000), suggest that the translocon is composed of three or four copies of the Sec61 complex with a large central pore formed at the interface of these complexes. However, recently, a high-resolution X-ray structure (at ~3.2 Å resolution) obtained for the SecY complex from the archaea Methanococcus jannaschii revealed that a single copy of SecY complex is sufficient to form the translocon pore (Van den Berg et al., 2004). In this map (Figure 1-3), the α-subunit has two linked halves, comprising of transmembrane segments (TM) 1 to 5 and 6 to 10, with funnel-shaped openings to either side of the ER membrane. These two halves are clamped together by the γ-subunit (Figure 1-3, step1). A cytoplasmic funnel leading into the channel is plugged by a short helix, dubbed the ‘plug’ (Figure 1-3, step1) and the plug probably moves from the extracellular side halfway through the membrane into a cavity on the
extracellular side of the channel, bringing it close to the TM helix of the γ-subunit when a nascent polypeptide is translocated (Figure 1-3, step 3). The channel opening may be triggered by the binding of a channel partner (the ribosome, Sec62/63 complex or SecA) (Figure 1-3, step 2 indicates the situation with a ribosome) and intercalation of a signal sequence (or a TM segment in the case of a membrane protein) between TM2b and TM7 at the front of the molecule (Figure 1-3, step 3). This would create an ‘hourglass’ shaped channel with a narrow constriction in the middle of the membrane (Figure 1-3, step 3). This constriction is formed by a pore ring of six hydrophobic residues with branched side chains, which are often isoleucines. This pore ring may form a seal around the translocating polypeptide, preventing other molecules from permeating the membrane (Figure 1-3, step 4). Finally, when the polypeptide has passed through, the plug returns to its close-state position (Figure 1-3, step 5).
Figure 1-3: A model for translocation of a secretory protein. Based on the X-ray structure obtained for the SecY complex from the archaea *Methanococcus jannaschii*, a model for the translocation of secretory proteins is proposed by Van den Berg *et al.* The scheme shows different stages during translocation. The figure is extracted from Van den Berg *et al.* (Van den Berg *et al.*, 2004).
1.1.4 Protein folding in the ER: the role of ER chaperones

In order to be biologically active, proteins must fold into their native conformation. For proteins that enter the secretory pathway, folding process commences co-translational/translocationally and continues post-translationally in the ER until the native protein structure is reached. This conformational maturation of proteins is aided and monitored by a number of ER chaperones folding enzymes in a complex process. Molecular chaperones, in general, function in preventing aggregation of immature polypeptides, facilitating protein maturation and retaining folding proteins in appropriate micro- or macro-environments enriched with folding enzymes. Classical chaperones are divided into five classes including Hsp40 (ERdj1 to 5, Scj1p and Jem1), Hsp70 [glucose-regulated protein (GRP)78/BiP and GRP170/ORP150], Hsp90 (GRP94/gp96), GrpE-like (BAP/SIL1 and GRP170/ORP150), lectins (Calnexin, Calreticulin, EDEM1, EDEM2, and EDEM3) (Table 1-1) (Hebert and Molinari, 2007; van Anken and Braakman, 2005). To date, the components and the mechanisms of two chaperone systems have been best characterized, which are the molecular chaperone BiP/GRP78 and the lectin chaperones.
Table 1-1: Molecular chaperones in the ER.

<table>
<thead>
<tr>
<th>Family</th>
<th>Mammals</th>
<th>Synonym(s)</th>
<th>Yeast</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp40</td>
<td>ERdj1</td>
<td>Mtj1</td>
<td></td>
<td>Cofactors for Hsp70</td>
</tr>
<tr>
<td></td>
<td>ERdj2</td>
<td>Sec63</td>
<td>Sec63p</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERdj3</td>
<td>HEDJ, ERj3, ABBP-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERdj4</td>
<td>Mdj1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERdj5</td>
<td>JPD1</td>
<td>Scj1p</td>
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</tr>
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<td>Jem1p</td>
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<td>Hsp70</td>
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<td>BiP</td>
<td>Kar2p</td>
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<tr>
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<td>GRP170</td>
<td>ORP150, CBP-140</td>
<td>Lhs1p</td>
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<td>SIL1</td>
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<td>Cofactors for Hsp70</td>
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<td>ORP150, CBP-140</td>
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<td>Cne1p</td>
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<td>Glycoprotein-dedicated chaperones</td>
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<tr>
<td></td>
<td>EDEM1, EDEM2, EDEM3</td>
<td></td>
<td></td>
<td>Htm1p</td>
</tr>
</tbody>
</table>
Folding in the molecular chaperone BiP/GRP78 system

BiP/GRP78 (BiP, immunoglobulin heavy chain–binding protein; Grp78, 78-kDa glucose-regulated protein), the Hsp70 member, is the first eukaryotic ER chaperone to be identified as it associates with incompletely assembled antibody Ig heavy chains. BiP has been referred to be a master regulator of the ER (Hendershot, 2004) because it plays a critical role in controlling or contributing to several vital ER functions: (1) it maintains the permeability barrier of ER translocon by sealing the lumenal side during the early stages of protein translocation (Alder et al., 2005); (2) it facilitates translocation of growing nascent polypeptides across the ER membrane by acting as a molecular ratchet (Matlack et al., 1999); (3) it prevents immature proteins from aggregating by promiscuously binding to exposed hydrophobic domains (Blond-Elguindi et al., 1993; Flynn et al., 1991; Sanders et al., 1992; Vogel et al., 1990); (4) it participates in multiple steps during protein folding and oligomerization (Gething and Sambrook, 1992; Haas and Wabl, 1983); (5) it controls and maintains calcium homeostasis in the ER (Lievremont et al., 1997); (6) it maintains ultimately unfolded proteins in a dislocation-competent state so for retro-translocation to the cytosol for their degradation (Cabral et al., 2002); (7) it contributes to the regulation of the unfolded protein response (UPR) (Kimata et al., 2003; Oikawa et al., 2007; Todd-Corlett et al., 2007).

BiP contains two main functional domains, an N-terminal ATPase domain and a C-terminal substrate-binding domain (SBD). BiP, like all Hsp70 family members, binds both adenosine triphosphate (ATP) and adenosine diphosphate (ADP), which serve to regulate its binding and release from nascent chains (Bukau and Horwich, 1998). The
binding of BiP to unfolded protein substrates is modulated by nucleotide (ATP or ADP) binding to its N-terminal ATPase domain. When BiP is in an ATP-bound state, which represents an ‘open’ configuration, it can associate with unfolded protein substrates through a SBD. Subsequently, Hsp40-like co-chaperones bind to the ATPase domain of BiP and catalyze the rapid hydrolysis of ATP to ADP, ‘locking’ BiP onto the substrate. Finally, the exchange of ATP back into the nucleotide binding cleft can induce a conformation change, which ‘reopens’ BiP and releases the substrate (Bukau and Horwich, 1998). These binding and release cycles are thought to assist the nascent polypeptides in folding.

Hsp70s are regulated by Hsp40-like cofactors (homologous to E. coli DnaJ proteins) and by the nucleotide-exchange or -releasing factors. DnaJ family proteins preferentially bind to the ATP form of their partner Hsp70s and stimulate their ATPase activity (Walsh et al., 2004). DnaJ is first identified as a cofactor of DnaK, the bacterial Hsp70 homologue, which stimulates the ATPase activity of DnaK and helps replicase λ phase DNA in host cells (Yochem et al., 1978). To date, many ER-localized DnaJ-domain containing proteins have been cloned in the different species: the yeast ER contains five DnaJ proteins (Sec63p, Scj1p, Jem1p, Hlj1p and Erj5p) (Cheetham and Caplan, 1998; Walsh et al., 2004), while the mammalian possesses at least five DnaJ orthologues (ERdj1 to 5) (Shen et al., 2002b) and one of these, ERdj3 has been shown to interact with misfolded protein in the ER (Meunier et al., 2002). In addition, nucleotide exchange factors assist in the swapping of ADP bound to Hsp70s for ATP. These factors help create an open configuration, leading to the release of peptides from Hsp70. The bacterial exchange factor GrpE is the most thoroughly studied (Liberek et al., 1991).
Recently, two mammalian ER nucleotide exchange factors, BAP/SIL1 (Chung et al., 2002) and GRP170/ORP150 (Weitzmann et al., 2006) are cloned. Both proteins have their homologues in the ER of yeast, which are Sil1p and Lhs1p (a member of heat shock family), respectively. Sil1p and Lhs1p are also shown to be nucleotide exchange factors for the yeast homologue of BiP, Kar2p (Steel et al., 2004).

A biochemical study, in which a large ER-localized multiple protein complex formed by a subset of ER chaperones which include BiP, GRP94, calcium-binding disulfide isomerase protein 1 (CaBP1), protein disulfide isomerase (PDI), cyclophilin B (an ER peptidyl-prolyl-isomerase), ERdj3, ERp72, GRP170, UDP-glucosyltransferase (UGGT) and stromal cell derived factor 2-like 1 (SDF2-L1, a member of the protein O-mannosyltransferase family) was observed in the assays using a chemical cross-linker to stabilize chaperone interactions (Meunier et al., 2002). The data suggest that this complex forms in the absence of nascent polypeptides synthesis. The whole complex may represent an ER network that can bind to unfolded protein substrates instead of existing as free pools that assemble onto substrate proteins. Moreover, it also showed that the entire complex can bind to unassembled Ig heavy chains, indicating that it is a functional chaperones complex whose organization may augment the local concentration of chaperones and folding enzymes on unfolded protein substrates (Meunier et al., 2002).

GRP94 is a major component in this complex. GRP94, only found in vertebrates, is the ER-localized Hsp90 member (Lee, 2001). GRP94 contains three functional domains: an N-terminal regulatory domain, a central substrate-binding domain and a C-terminal dimerization domain. It remains elusive as to whether the chaperoning mechanism of GRP94 is similar to Hsp90. Similar to its cytoplasmic paralog Hsp90, ATP
binding to the N-terminal domain of GRP94 seems to control substrate binding, although the precise mechanism of regulation remains unclear (Dollins et al., 2005; Soldano et al., 2003). However, in contrast to its cytoplasmic counterpart, it does not appear to act as an ATPase or have any cofactors. Therefore, how ATP hydrolysis is coupled to chaperone activity of GRP94 is still unknown.

Several lines of evidence imply that GRP94 plays a role in the protein folding process. It helps with the maturation of many ER clients including immunoglobulin chains, MHC class II, thyroglobulin and procollagen (Ferreira et al., 1994; Kuznetsov et al., 1994; Melnick et al., 1992; Schaiff et al., 1992). In addition, it has also been proposed that, in the ER, BiP and GRP94 act in tandem on overlapping population of folding intermediates and that GRP94 seems to assist in a more advanced step in folding, perhaps aiding the assembly of immunoglobulin substrates since immunoglobulins are transferred from BiP to GRP94 (Melnick et al., 1994).

Folding in the lectin chaperone system

The majority of the proteins that travel along the secretory pathway receive multiple N-linked glycans. This hydrophilic modification can influence the conformation of the polypeptide backbone, thus, counteracting the overall hydrophobicity of the polypeptide chain. They also provide binding sites for lectin chaperones. In contrast to BiP, which binds directly to hydrophobic patches in the polypeptide, the lectin chaperones bind the hydrophilic N-linked modification. However, both chaperone systems appear to play similar roles in assisting the overall fidelity of the folding process.
Preassembled core glycans comprising three glucose, nine mannoses, and two N-acetyl glucosamine residues (Glc$_3$Man$_9$GlcNAc$_2$, figure 1-4A) are synthesized in a cascade of enzymatic reactions coupling monosaccharide units to lipid dolichol moieties. First, a Man$_5$GlcNAc$_2$ precursor linked to dolichol by two phosphates is made at the cytoplasmic leaflet of the ER membrane. Next, this precursor flips to the lumenal leaflet, presumably by an unidentified flipase, and is then elongated to Glc$_3$Man$_9$GlcNAc$_2$-PP-dolichol (Helenius and Aebi, 2002). From the dolichol precursors, preassembled glycans (Glc$_3$Man$_9$GlcNAc$_2$, figure 1-4A) are transferred by the oligosaccharyl transferase (OST) complex onto nascent polypeptide chains at the side chains of asparagines within Asn-X-Ser/Thr consensus sites (where X stands for any residue except proline) (Kornfeld and Kornfeld, 1985; Marshall, 1972). OST is part of the ER translocon (Johnson and van Waes, 1999). Accordingly, the transfer generally occurs co-translationally once the nascent chain emerges from the translocon pore, at a distance of 12-14 amino acids from the ER membrane (Nilsson and von Heijne, 1993), which corresponds to ~65 amino acids from the peptidyltransferase center inside the ribosome (Mingarro et al., 2000). As soon as an N-linked glycan is added onto a polypeptide, it is then rapidly trimmed by the sequential actions of glucosidase I and II to generate a monoglycosylated side chain (Glc$_1$Man$_9$GlcNAc$_2$), which is a binding site for the lectin chaperones calnexin and calreticulin (Figure 1-4B, step 1).
Figure 1-4: The fate of newly synthesized glycoproteins in the ER lumen.

(A) Structure of protein-bound oligosaccharides. The high mannose core glycan is covalently attached to Asn-X-Ser/Thr consensus sequence of nascent polypeptide chains once nascent chains emerge from the translocon pore. Red triangles, glucose; orange circles, (1-2)-linked mannoses; green circles, other mannoses; black squares, N-acetylglucosamine residues. (B) Nascent chains enter the ER lumen through the Sec61 translocon (upper left). Glycoproteins may fold properly after a single run of binding to the Cnx/Crt, thus leaving the ER for delivery at the site of activity through the secretory pathway (export, green arrows). Ultimately misfolded proteins are retro-translocated into cytosol and degradation (retro-translocation, red arrows). Individual steps are thoroughly discussed in the text. The figure is extracted from Hebert and Molinari, 2007 (Hebert and Molinari, 2007).
Both calnexin (Cnx) and calreticulin (Crt) were initially named for their calcium-binding ability (Fliegel et al., 1989; Wada et al., 1991). Cnx is a 90 kDa type I ER membrane protein that contains a single lumenal carbohydrate binding domain. The X-ray crystal structure of this carbohydrate binding domain reveals that it is formed by a β-sandwich structure, which is commonly found in leguminous lectins (Schrag et al., 2001). A long hairpin motif stretches out from the carbohydrate binding domain, forming a second domain termed the P-domain. The P-domain, named for its richness in proline residues, creates an arm-like structure that recruits a glycoprotein-dedicated oxidoreductase involved in disulfide bond formation and isomerization, called ERp57 (Frickel et al., 2002; Kozlov et al., 2006). The tip of the P-domain associates with the b’-domain of ERp57 (Frickel et al., 2002). Crt is a 60 kDa soluble paralog of Cnx (Fliegel et al., 1989). While its crystal structure has not been solved, its strong sequence homology to Cnx implies that it will have a similar conformation composed of a single carbohydrate-binding domain with a slightly shorter P-domain.

Either protein forms a tandem with ERp57. The architecture of this ERp57-Cnx/Crt complex is unique; it provides a cage for its clients (Frickel et al., 2002). One can envision that the Cnx/Crt transiently fix folding intermediates to form a specific conformation necessary for the formation of certain disulfide bonds. ERp57, as a close partner of the Cnx/Crt, could then fulfill the role of catalyst. Indeed, ERp57 is shown to form transient disulfide bonds with Cnx and Crt-bound glycoproteins (Molinari and Helenius, 1999). Apparently, the Cnx/Crt lectins recruit ER clients for ERp57. In addition, association of ERp57 with ER clients may continue even after their dissociation from
Cnx or Crt. It, therefore, is possible that ERp57 has an additional role in client binding after the lectins have dissociated (Frenkel et al., 2004).

The Cnx/Crt cycle in this chaperone system is controlled by the glucosidase and transferase (Figure 1-4B). Soon after synthesis of N-linked core glycans, glucosidase I and II sequentially trim the first and the second glucose residues from the A branch of the oligosaccharide moieties (Figure 1-4B, step 1). This generates the monoglucosylated glycans which provide the initial binding sites for the lectin chaperones. At this point, Cnx/Crt association may deliver newly synthesized polypeptides to the ERp57, which catalyzes the rate-limiting step of protein folding in the ER lumen, namely formation and isomerization of disulfide bonds (Jessop et al., 2007). Folding substrates are eventually released from ERp57-Cnx or ERp57-Crt complexes (Figure 1-4B, steps 2 and 2a) and the third glucose is then rapidly removed by glucosidase II creating the unglucosylated substrate, thus, preventing immediate reassociation with the lectin chaperones (Figure 1-4B, step 3 for native proteins, step 3a for folding intermediates, and step 3b for folding-incompetent polypeptides).

Native and fully assembled proteins can leave the ER for delivery at the site of activity through the secretory pathway. Proteins that have not reached their native conformation are normally not released into secretory pathway, although some exceptions are known (Gurkan et al., 2006). Their forward transport is inhibited due to the recruitment of ER-retained molecular chaperones by exposed ‘misfolded determinates’ (for example, hydrophobic patches exposed on protein surface or unpaired cysteines). Non-native folding intermediates are recognized by the ER folding sensor UDP-glucose:glycoprotein glucosyltransferase (GT). GT regenerates the monoglucosylated
state of non-native polypeptides, allowing them to rebind to the lectin chaperones to complete the folding program in the Cnx/Crt chaperone cycle (a process belonging to ER quality control, as described below in the chapter 1.2.2) (Figure 1-4B, 4a). *S. cerevisiae* lacks this cycle; it has no GT or Crt, and the function of its Cnx homolog is still unclear. Correct folding in mammalian cells results in GT no longer recognizing the glycoprotein because re-glucosylation by GT happens only if the glycoprotein is incompletely folded (Parodi, 2000); it ignores native proteins that are released into the secretory pathway (Figure 1-4B, 4) and ultimately misfolded proteins that are prepared for retro-translocation into the cytosol for degradation (via ERAD pathway: the mechanisms for recognizing and disposing of glycoproteins will be discuss later) (Figure 1-4B, 4b). Therefore, the glucose here serves as a selective tag for immature glycoproteins –it is, in a sense, a ‘stamp of disapproval’ that informs the system that this protein is not yet ready to be exported. The fate of newly synthesized glycoproteins in the ER is depicted in figure 1-4B: glycoproteins may fold properly after a single run of binding to the Cnx/Crt (green arrows); however, cycling by the Cnx/Crt controlled by glucosidase II and GT (yellow arrows) can lead to proper maturation and transport (green arrows) or ERAD (red arrows).

The link between BiP and the lectin chaperone system

As for the folding of glycoproteins, the molecular chaperone BiP often binds first to exposed hydrophobic patches, and the glycoprotein may subsequently be handed over to the Cnx/Crt chaperone system to complete maturation (Molinari and Helenius, 2000).
Exceptions are glycoproteins displaying N-glycans in the very N-terminal portion of the molecule (Molinari and Helenius, 2000). As N-glycosylation and binding with Cnx/Crt can start if the first monoglucosylated oligosaccharides is located within approximately the first 50 residues of the polypeptide, this can bypass BiP assistance to the nascent chain. However, BiP can also intervene after substrate release from the Cnx/Crt system to bind extensively misfolded polypeptides.

1.2 Protein quality control in the ER

Besides providing an optimized environment for protein folding, the ER has a crucial quality-control (QC) role for ‘proof-reading’ newly synthesized proteins, so that only correctly folded proteins are sent to their final destination. This monitoring mechanism must be able to distinguish native from non-native protein conformations (including molecules that are actively folding and irreversibly misfolded) based on common structural and biophysical features and then handle each form differently. Native and fully assembled proteins destined for ER export are packaged into trafficking vesicles for delivery at the site of activity through the secretory pathway, while misfolded proteins are actively extracted from the folding machinery before being sorted for degradation (via ER-associated pathway, ERAD pathway). Folding polypeptides are retained in the ER and protected from degradation pathways.
1.2.1 ER quality control (ERQC): native proteins versus nonnative conundrum

The export of native proteins is the easiest to envision. Native proteins do not expose hydrophobic regions and unpaired cysteine residues and do not form aggregates that elicit recognition by ER-localized chaperones and modifying enzymes. This is a hotspot crucial for displaying differences from nonnative polypeptides. For these nonnative folding intermediates, one mechanism for ER retention is dependent on interactions with ER-localized chaperones that carry specific retrieval or retention sequences at their extreme C-termini, for example, luminal H/KDEL-related sequences for soluble proteins or cytosolic KKXX-related extensions for transmembrane proteins (Pelham, 1989).

*Export from the ER*

Unless retained by interactions with ER resident proteins, ER clients may leave the ER and travel to downstream compartments of the secretory pathway. Thus, when ER clients have fully matured and no longer interact with ER-localized chaperones, they can exit the ER by being captured into ER-derived transport vesicles, know as coat protein complex II (COPII) vesicles. Export from the ER occurs at ER exit sites (specialized regions that are enriched in the COPII machinery) (Mezzacasa and Helenius, 2002). While many ER clients may be packaged into COPII vesicles aspecifically, representing bulk flow from ER to Golgi (Wieland *et al.*, 1987), several other ER clients are actively transported out of the ER by interactions with specialized export machineries (Figure 1-4 B, 4). For example, in mammalian cells, a subset of soluble glycoproteins, including
blood coagulation factor and cathepsin-Z, is recruited by the mannose specific lectin ERGIC53, a hexameric membrane lectin which cycles between the ER and Golgi compartments (Appenzeller et al., 1999; Nichols et al., 1998). In yeast, the integral ER membrane protein, Erv29p, determines that the soluble cargo proteins glyco-pro-α-factor, carboxypeptidase Y and proteinase A are packaged and sent to the Golgi (Belden and Barlowe, 2001; Caldwell et al., 2001; Otte and Barlowe, 2004), whereas the p24 protein, Emp24, serves as an export receptor for specific secretory cargo, GPI-anchored ER proteins, like Gas1p (Muniz et al., 2000). ERGIC53, p24 proteins and Erv29p are proposed to recognize and bind to specific export signals contained within distinct soluble cargo molecules.

Export of ER membrane clients can also be mediated by signals located at the cytoplasmic side of the ER membrane. The so-called di-acidic sequence, DXE (where X represents any residue) within their cytoplasmic domains can serve as export signals (Nishimura and Balch, 1997) because it is recognized by the COPII subunit Sec24, which is responsible for efficiently recruiting cargo into nascent vesicles as they are formed from the ER membrane (Miller et al., 2002; Miller et al., 2003; Votsmeier and Gallwitz, 2001). Other cytoplasmic ER export motifs such as LXX\(^{\pm.5}\)M and YNNSNPF in SNARE proteins confer association with Sec24 and subsequent vesicular trafficking in a similar manner as the di-acidic sequence (Mossessova et al., 2003).

Why are export signals only recognized as such when ER clients have completely matured? It has been proposed that ‘unmasking’ of export motifs may provide a positive determinant for QC. For example, di-acidic export signals contained in the cytoplasmic domains of CFTR (cystic fibrosis conductance regulator) may be buried while the protein
is still actively folding, but become accessible to Sec24 once it has correctly folded (Wang et al., 2004). The opposite seems to be true as well for ATP-sensitive potassium channel, which is comprised of four α- and four β- subunits in its mature form. The monomeric subunits contain RXR ER retention signals that only are ‘masked’ when the subunits correctly assemble into the mature hetero-octamer (Zerangue et al., 1999).

Still, it is hard to imagine that ‘masking’ of retention signals and/or ‘unmasking’ of export signals determines the export of every ER client. Surprisingly, recent studies found that misfolded proteins can be packaged into COPII vesicles at ER exit sites. Analysis of the interplay between protein stability, folding and the capacity of ER to sort cargo to the COPII machinery showed that protein export from the mammalian ER is energetically permissive (Sekijima et al., 2005) and that ER export signals mediate the export of misfolded proteins, at least in yeast (Kincaid and Cooper, 2007). For example, in the case of transthyretin (TTR) disease-associated variants, the ERQC only prevents forward transport of the most highly destabilized structures but ignores other destabilized proteins such as those that can lead to disease states in spite of their compromised folding. The data indicates that misfolded proteins can be packaged into COPII vesicles at ER exit sites and be transported to the cell surface, which may lead to a new view of the ER as a QC device (Molinari et al., 2004) that takes into account the energetics of protein fold, independent of the acquisition or not of a native state, for ER export under normal physical conditions (Sekijima et al., 2005).
1.2.2 ERQC: the unfolded versus misfolded conundrum

Less evident is how QC machinery distinguishes unfolded (defined as an actively folding intermediate on its way to become a native molecule) from misfolded (defined as a terminally unstructured protein to be extracted from the folding machinery) because both conformers expose regions that attract ER-localized molecular chaperones. The regulation of these processes is better understood for a subset of misfolded N-glycosylated proteins.

For the QC of glycoprotein folding, as described in chapter 1.1.4 (folding in the lectin chaperone system), two enzymes, glucosidase II and GT, in connection with the Cnx/Crt cycle are of importance. As depicted in figure 1-5A, after the removal of the first glucose residue in the A branch by glucosidase I, the inner α1,3-linked glucose residues can be trimmed by glucosidase II (Brada and Dubach, 1984; Burns and Touster, 1982). The presence of three or two glucose residues can be considered to represent a ‘trimming glyco-code’ whereas one glucose residue represents a ‘trimming and folding glyco-code’ (Figure 1-5B). The resultant monoglucosylated glycans depicted in figure 1-5B (left side) may be bound by Cnx or Crt. Thus, if folding intermediates are considered, binding to Cnx or Crt can exclude them from degradation. While bound, substrates undergo conformational maturation assisted by a variety of folding enzymes (Figure 1-4B, the Cnx/Crt cycle). However, the complete deglucosylation (Figure 1-5B, right side) by glucosidase II can result in their release from the Cnx/Crt cycle. After this, the further trimming of mannose residue(s) in the B branch by ER α1,2-mannosidase(s) would cause glycans to switch from a trimming and folding glyco-code to an ‘ERAD glyco-code’. 
which opens the gate to retro-translocation and degradation of misfolded proteins (Figure 1-5C and figure 1-4B, red arrows).

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Figure 1-5: A schematic diagram representing the oligosaccharide trimming pathway by glucosidase I (Gls I), glucosidase II (Gls II), UDP-glucose:glycoprotein glucosyltransferase (GT) and ER-mannosidase I (ER-Man I).

The figure is extracted from Roth, J. et al., 2008 (Roth et al., 2008).
Mannose trimming allows discrimination between unfolded and terminally misfolded glycoproteins

In *S. cerevisiae*, kifunensine (a mannosidase inhibitor)-sensitive Mns1p is the glycanase involved in the removal of this specific mannose from the B chain of the glycan, leaving the structure Man₈GlcNAc₂ (Figure 1-5C) (Byrd *et al*., 1982). In mammalian cells, the corresponding enzyme, the ER α-mannosidase I, shows the same specificity for the mannose residue on the B chain and can sometimes remove an additional mannose residue (Helenius, 1994). It appears unlikely that the removal of the terminal mannose residue in the B branch contributes to the same strength of the signal in yeast as that in the mammalian cell. In mammalian cell, in fact, N-glycosylated substrate cycling in the lectin chaperone system provides good shielding from disposal. The removal of the single mannose residue at the terminus of the B branch is not sufficient to abolish it (Hebert *et al*., 2005). Thus, in mammals, unlike in yeast where the sole ER mannosidase is responsible for removal of mannose residues, there are several other α₁,₂-mannosidases that are sensitive to the same inhibitors (e.g., kifunensine) and that may recycle from the Golgi to the ER, namely mannosidases IA (also called Man9-mannosidase), IB and IC (Herscovics, 2001). These enzymes might act alone or in combination with ER mannosidase I.

Initially, ER mannosidase I was thought to only remove the terminal mannose residue on branch B and generate a precursor for ERAD pathway. However, it was observed that *in vitro*, at high concentrations, the enzyme can proceed with the removal of other mannose residues on branch A and C (Herscovics *et al*., 2002). This extensive trimming can generate the Man₇GlcNAc₂, Man₆GlcNAc₂, and Man₅GlcNAc₂ glycans,
which have been proposed as favored for ERAD since they lack the specific mannose receptor for glucose transfer to reenter the Cnx/Crt cycle (Helenius, 1994). Can these unphysiological conditions be achieved in vivo? Possibly, at least three possibilities exist:

(1) ER mannosidase I and the ERAD substrates are segregated to a specialized sub-compartment where the mannosidase concentration reaches high levels similar to those observed in vitro (Huyer et al., 2004), (2) intervention of other endomannosidase(s) that cleaves A branch mannoses in Golgi, or (3) intervention of a new set of recently characterized mannosidase-like proteins, EDEM1, EDEM2, and EDEM3 (Olivari and Molinari, 2007), which are thought to work downstream of the Cnx/Crt cycle as a post-retention step in targeting substrates to ERAD pathway.

1.2.3 The role of chaperones in ERQC

For those proteins that do not use the Cnx/Crt cycle, chaperones play a number of roles to prepare these substrates for the ERAD pathway when they fail to fold or assemble. Several examples are listed below:

(1) BiP (heavy chain binding protein):

It is well studied that BiP plays a role in retaining unassembled immunoglobulin heavy chains, for which its name was derived (Haas and Wabl, 1983). This function is prevalent; many misfolded ER lumenal proteins have been shown to specifically interact with BiP. BiP, with its J-domain containing co-chaperones, Scj1p and Jem1p, can also prevent substrates from aggregation so that it retains soluble ERAD substrates in a retro-translocation-competent conformation for degradation (Nishikawa et al., 2001).
(2) PDI (protein disulfide isomerase)

PDI is an abundant oxidoreductase enzyme in the ER that has been extensively studied in mammalian cells. It has been shown that PDI is needed for the retention and subsequent degradation of a wide range of substrates (Bottomley et al., 2001; Gillece et al., 1999). In mammals, PDI forms mixed disulfides with the pancreatic isoforms of β-secretase BACE457 that failed to fold following attempts in the Cnx/Crt system. And together with BiP, they direct these misfolded isoforms for retro-translocation (Molinari et al., 2002).

Recent evidence suggests that PDI plays a border role of actively unfolding and targeting substrates to the ERAD pathway. In the case of cholera toxin (CT) (an A/B5 subunit protein), PDI, functioning as a thiol-reductase, can relieve oxidized A subunit (an A1 chain covalently attached to the A2 chain through a disulfide bond) from its disulfide bond. CT enters the cells via endocytosis and travels retrograde through the secretory pathway until it reaches the ER. In the ER, a portion of the A subunit, the A1 chain (the active domain), must be reduced and unfolded for retro-translocating to the cytosol to cause disease. To search for this ‘unfoldase’, Tsai et al. developed and followed a toxin unfolding activity. The activity identified two members of PDI family – PDI and Ero1α – which are essential for this thiol-reduction (Tsai and Rapoport, 2002; Tsai et al., 2001). When PDI is oxidized, it releases the A1 chain and the A1 chain rapidly and spontaneously unfolds. Thus, PDI seems to act as a chaperone that is driven by a redox-cycle rather than an ATP-cycle (Tsai et al., 2001). The ER lumenal thioreductase, Ero1α, plays a role in oxidizing PDI to catalyze the release of the A1 chain (Tsai and Rapoport, 2002). In this case, the toxin adopts a pre-existing chaperone function of PDI to retro-
translocate to the cytosol where it obstructs ribosomal function. This implies that if
misfolded proteins are retro-translocated though the same route, reduction of disulfide
bonds are required to allow these proteins to unfold completely.

(3) ERp44:

ERp44 is also an oxidoreductase belonging to the PDI family. It has been shown
that ERp44 is a key element required for thiol-mediated retention of Ero1α and other
clients (Anelli et al., 2003). ERp44 forms reversible mixed disulfides with Ero1α (no
known ER localization motifs) through its free cysteine (C29), hence mediating Ero1α's
ER localization (Anelli et al., 2003). Through the same mechanism, ERp44 also prevents
the secretion of unassembled IgM with unpaired cysteines (Anelli et al., 2003).

(4) Eps1p:

Eps1, another PDI family member, is required for the retention of misfolded yeast
Pma1p (Pma1-D378N: an ERAD substrate). In cells deleted for the EPS1 gene, Pma1-
D378N leaves the ER and trafficks efficiently to the plasma membrane but the
localization of normal ER resident protein is unaffected. Together, these studies
[examples (2), (3) and (4)] show that one major function of ER lumenal oxidoreductases
is to retain misfolded and misassembled proteins in the ER.
1.2.4 ER-associated degradation (ERAD) pathway

Some ER clients never reach their native conformation because they misfold beyond rescue. This implies that ERQC must be coupled to efficient proteolytic systems which allow misfolded proteins to be degraded. Otherwise, they would fill the ER lumen, hence causing disequilibrium of ER homeostasis. In this process, folding failures need to be recognized as such in the ER lumen, for instance, by the lectin EDEM. Once recognized, misfolded proteins likely undergo partial unfolding and reduction before retro-translocation. As described above, both BiP and PDI family members are involved in this process (Molinari et al., 2002; Nishikawa et al., 2001; Tsai et al., 2001). Recently, ERdj5 was also found to play an important role in the retro-translocation for certain substrates that form interchain disulfide bonds (Cunnea et al., 2003; Hosoda et al., 2003). ERdj5 is an interesting protein that contains a both J-domain and an oxidoreductase moiety. Both activities are essential, suggesting that ERdj5 may couple reduction and BiP-mediated unfolding.

The retro-translocation

Retro-translocation is the least known step in ERAD. To date, the identity of a retro-translocation conduit remains controversial because of the topological diversity of ERAD substrates. The earliest candidate for the retro-translocation pore was the import pore complex (Sec61 complex). As described above, Sec61 protein itself is a passive conduit so the involvement of accessory factors is needed to provide the driving force and thus directionality for protein import. However, it is possible that the Sec61 protein is not
limited to unidirectional protein passage. Retro-translocation could be ER translocation in reverse that depends on Sec61, in combination with a different set of cofactors. Indeed, several lines of evidence support a role of the Sec61 complex in protein retro-translocation.

The major histocompatibility complex (MHC) class I mediate antigen presentation in immune cells. In order to escape this defense, the human cytomegalovirus expresses proteins US2 and US11 to target MHC class I heavy-chain protein to ERAD pathway for degradation. In cells expressing US2 and Sec61α, a subunit of the Sec61 channel was found in association with heavy chain (Wiertz et al., 1996). In addition, analyses in yeast showed that a variety of yeast sec61 mutants reduced the turnover rate of misfolded protein substrates (Pilon et al., 1997; Plemper et al., 1997). These studies raise the intriguing possibility that the ER translocon does double duty as the ‘retro-translocon’.

Recently, several studies have raised doubts regarding Sec61’s identity as the retro-translocon. Firstly, Tirosh and colleagues demonstrates that a tightly folded protein, methotrexate-bound dihydrofolate reductase (DHFR), can be fused to MHC class I heavy-chain without affecting their US2/11-mediated degradation (Tirosh et al., 2003). The smallest cross-section of DHFR is estimated to be 40 Å, suggesting that the putative channel must accommodate large proteins. Secondly, N-linked carbohydrates on misfolded glycoproteins were found to increase the size of the exported molecules even further because sugar moieties are not removed until glycoproteins reach the cytosol (Blom et al., 2004). However, the constriction of the ER translocon (10-12 Å) that has been proposed based on the crystal structure of archaea SecY translocon (Van den Berg
et al., 2004) seems too narrow to allow retro-translocation of fully folded proteins and glycosylated misfolded proteins. Although this proposed translocon is inconsistent with the observed function of the translocon in exporting large glycosylated and folded proteins, it should be noted that the crystal structure does not provide information on the flexibility of the channel. Indeed, other studies have estimated that the translocon has a diameter of 40-60 Å (Hamman et al., 1997; Wirth et al., 2003). Alternatively, Sec61 may forms oligomers or cooperate with other factors in the retro-translocation process.

Another protein suggested to form a retro-translocon in the ER is the mammalian protein Derlin-1 and its yeast homologue Der1p (Knop et al., 1996; Lilley and Ploegh, 2004; Ye et al., 2004). Both are multi-spanning transmembrane proteins that are needed for the degradation of certain ERAD substrates. Whereas Der1p-interacting proteins are unknown, Derlin-1 is found in a complex containing MHC class I heavy-chain that is targeted for degradation by US11. In Lilley and Ploegh’s study, the profile of proteins interacting with wild-type US11 was compared to that with a defective US11 mutant and Derlin-1 was found to bind to wild-type US11 but not to the mutant, suggesting a direct role of Derlin-1 in US11-mediated retro-translocation (Lilley and Ploegh, 2004). In the other study done by Ye et al., Derlin-1 and a novel transmembrane protein termed VIMP (VCP-interacting membrane protein; VCP is another name for p97 (see below)) were identified to be co-purified with a membrane receptor for cytosolic p97 (Cdc48 in yeast) (Ye et al., 2004). p97 is an cytosolic AAA-ATPase which provides the mechanical energy for nearly all substrates examined to date to be extracted from the ER membrane during the retro-translocation process. In addition, VIMP was suggested to play a role in anchoring cytosolic p97 to Derlin-1 at the ER membrane.
Taken together, these studies showed that US11 delivers MHC class I heavy-chains to Derlin-1 through direct interaction and the subsequent retro-translocation is driven by the p97/Ufd1/Npl4 complex. A missing point in the whole story is the identity of the retro-translocon. As subunits of the Sec61 translocon were not found associated with any component in this system, it led to the proposal that Derlin-1 may be part of a retro-translocon used by a subset of substrates (Ye et al., 2004). However, on the basis of current information, other solutions are equally attractive, particularly as Derlin-1 and Der1p are only needed for the turnover of a subset of ERAD substrates. Other candidates include the membrane-bound ubiquitin ligases Hrd1p and Doa10p. Both possess multi-spanning transmembrane domains that could form a pore and could provide a physical link between retro-translocation and ubiquitination. To identify the true retro-translocon, the establishment of a in vitro reconstitution system for ERAD may be required ultimately, much like those set up for translocation into the ER (Gorlich and Rapoport, 1993; Panzner et al., 1995).

**Driving retro-translocation: the road to degradation**

By analogy to both co- and post translational translocation systems (see Chapter 1.1.3), protein export from the ER is supposed to require a driving force that determines the direction of transport. For retro-translocation of ERAD substrates, the cytosolic protein complex containing the AAA ATPase Cdc48p (AAA: ATPases associated with diverse cellular activities) is considered to be a key component providing the mechanical energy (Braun et al., 2002; Jarosch et al., 2002; Rabinovich et al., 2002; Ye et al., 2001).
Cdc48p is also termed p97 or VCP in mammals. Cdc48/p97 associates with two cofactors – Npl4 and Ufd1 – that bind ubiquitinated proteins (Meyer et al., 2002). This complex is involved in the turnover of a variety of cytosolic, nuclear, and ER-associated substrates, probably by promoting the disassembly of protein complexes prior their degradation (Figure 1-6).
Figure 1-6: Proteasomal degradation of ERAD substrates. ER misfolded proteins are recognized by different mechanisms of quality control. Once being recognized, terminally misfolded proteins are delivered to a putative channel that mediates their export from the ER. Retro-translocation is completed with the assistance of the Cdc48/p97 complex and membrane-extracted substrates are handed over to the 26 S proteasome by other factors such as Rad23p and Dsk2p. The figure is extracted from Meusser, B. et al., 2005 (Meusser et al., 2005).
How may Cdc48p-Npl4p-Ufd1p contribute to the retro-translocation process? Mutants in the ATPase domains of Cdc48p lose its ability to export substrates, indicating that Cdc48p-Npl4p-Ufd1p extracts proteins from the ER membrane in an ATP-dependent manner (Ye et al., 2003). This mode of action, similar to ‘motor’-like functions of Hsp70-type chaperones in the process of post-translational translocation (Matlack et al., 1999), would depend on binding of Cdc48p-Npl4p-Ufd1p to the substrates at a very early stage in the retro-translocation process and it would also require a docking site for Cdc48p-Npl4p-Ufd1p at the putative export pore (Ye et al., 2004; Zhong et al., 2004). Indeed, Cdc48p-Npl4p-Ufd1p is shown to interact with Derlin-1 and the ER-located E3, gp78. In addition, mammalian VIMP, an ER integral membrane protein, may play a role in recruiting cytosolic p97 to the membrane (Ye et al., 2004).

Alternatively, Cdc48p-Npl4p-Ufd1p may act downstream in the retro-translocation process to actively liberate the ubiquitinated substrates that have crossed the ER membrane. During the maturation of yeast Spt23p, the Cdc48p-Npl4p-Ufd1p complex performs such an activity (Rape et al., 2001). The Spt23p precursor is an ER integral membrane protein (p120), which forms homodimers. Upon activation, a soluble fragment (p90: a transcription factor for expression of the fatty acid desaturase) is generated by proteolysis and is detached from its membrane bound partner (p120). The proteolysis is carried out by the proteasome and the process is dependent on ubiquitination of the protein. Specifically, the Cdc48p-Npl4p-Ufd1p complex binds to ubiquitinated p90 and functions to actively release it from the tightly associated unprocessed p120. Consistent with this view, polyubiquitinated ERAD substrates accumulate at the cytoplasmic side of the ER membrane in mutants deleted for NPL4 and

NPL4
*UFD1* genes, indicating that substrate molecules are able to cross the membrane independently of the Cdc48p-Npl4p-Ufd1p complex and this complex should function after export of molecules has been completed (Jarosch *et al.*, 2002). In this case, the directionality of passage may be assured by ubiquitinated-binding factors, which are probably located at ER membrane near the site of ubiquitination and prevent back-sliding of ubiquitinated molecules through the channel into the ER. The E3 ubiquitin ligase, gp78, contains a Cue domain at the cytoplasmic side, which may act in such a role (Fang *et al.*, 2001).

The Cdc48p-Npl4p-Ufd1p complex could also bind and help deliver ubiquitinated substrates from the site of ubiquitination to the 26S proteasome (Richly *et al.*, 2005). In this process, Cdc48p-Npl4p-Ufd1p interacts with the ubiquitin-chain elongating factor Ufd2p. Cdc48p-Npl4p-Ufd1p is also found to associate with Rad23p and Dsk2p, which subsequently hand over ubiquitinated substrates from the Cdc48p-Npl4p-Ufd1p complex to the 26S proteasome (Figure 1-6) (Medicherla *et al.*, 2004). On the one hand, Rad23p and Dsk2p can bind polyubiquitinated substrates through a ubiquitin association domain (Rao and Sastry, 2002), while on the other hand, they can recruit the 26S proteasome through a ubiquitin-like domain (Hartmann-Petersen *et al.*, 2003). Recent work suggests that Cdc48p-Npl4p-Ufd1p binds to mono-ubiquitinated or oligo-ubiquitinated (two or three ubiquitin molecules) Spt23-p90 (Richly *et al.*, 2005). After Ufd2p further elongates these ubiquitin chains, Spt23-p90 is delivered to Rad23p and Dsk2p, which preferentially bind longer ubiquitin chains. Finally, Rad23p and Dsk2p hand over their cargo to the 26S proteasome for degradation. This ‘escort pathway’ conveys Spt23-p90 from the site of
ubiquitination to the 26S proteasome through a cascade of factors, which have high
affinity for the attached ubiquitin moieties (Richly et al., 2005).

It seems tempting to conceive that a similar transport cascade of ubiquitin-binding
factors at the ER escorts the export of QC substrates. Rad23p, Dsk2p and Ufd2p facilitate
protein degradation in general, including ERAD substrates (Medicherla et al., 2004;
Richly et al., 2005). Rad23p and its mammalian homolog HR23B have been shown to
interact with Png1p, a cytoplasmic N-glycanase that is thought to remove glycans prior to
their proteolysis (Suzuki et al., 2001). In a proposed ‘guidance model’, after substrates
partially emerge from a retro-translocon, exposed regions may be immediately
ubiquitinated and are then trapped by ubiquitin-binding factors at the cytoplasmic side of
the membrane. Ubiquitinated substrates are subsequently handed over to downstream
factors which have increasing affinity for ubiquitin chains. The serial association of these
acting factors may mediate retro-translocation by a mechanism similar to a molecular
ratchet. However, whether the binding of these factors to ubiquitin moieties is sufficient
to drive the export process remains to be determined. It is plausible that Cdc48p-Npl4p-
Ufd1p not only functions as a ubiquitin-binding factor but also could actively liberate
exported proteins from the ER membrane. Furthermore, ‘motor’, ‘mobilizing’, or
‘guidance’ functions of Cdc48p-Npl4p-Ufd1p may not be mutually exclusive and, in fact,
translocation of different types of substrates may depend on replaceable modes of
Cdc48p-Npl4p-Ufd1p actions.
The earliest clue that linked the turnover of non-native ER-membrane bound proteins to the cytoplasmic ubiquitin proteasome system (See figure 1-7 for a description of the ubiquitin proteasome system (UPS)) was provided by two interesting findings. First, Ubc6p was found to participate in the turnover of mutant Sec61-2p (the product of an unstable yeast SEC61 allele) (Sommer and Jentsch, 1993). Shortly after, it was shown that the degradation of mammalian ΔF508 variant of cystic fibrosis conductance regulator (ΔF508CFTR) was ubiquitin/proteasome-dependent (Jensen et al., 1995; Ward et al., 1995). Furthermore, it became clear that the misfolded ER-lumenal protein carboxypeptidase (CPY*) was also degraded in a Ubc7p-dependant manner (Hiller et al., 1996). Both Ubc6p and Ubc7p reside at the cytoplasmic side of the ER membrane. While Ubc6p is an integral membrane protein with its catalytic domain cytosolically oriented (Sommer and Jentsch, 1993), Ubc7p is anchored to the membrane by another membrane protein, Cue1p (Biederer et al., 1997). The restriction of these Ubc enzymes to the ER membrane and the lack of UPS components in secretory compartments implied that ERAD substrates including lumenal and membrane proteins must be retro-translocated back to the cytoplasm for degradation. This process is summarized by a schematic diagram in figure 1-6.
Ubiquitin conjugation to a substrate involves a cascade of enzymatic reactions. First, ubiquitin (Ub) is activated through formation of an ATP-dependant high-energy thioester linkage between active site cysteine of the Ub-activating enzyme (E1) and the C-terminus of Ub. Next, activated Ub is then transferred to a cysteine side chain of an E2 Ub-conjugating enzyme (Ubc). E2s work together with Ub protein ligases (E3) to transfer Ubs to lysine residues of substrate proteins either directly from the E2 (as depicted in this figure) or through an E3-Ub intermediate. A polyubiquitin chain is formed on the substrate by the serial addition of Ubs to lysine residues of the previously attached Ub. For substrates destined for degradation, Ub itself is ubiquitinated usually at the K48 to form polyubiquitin chains. Polyubiquitin chains provide a signal recognized by specific subunits in the 19S capping complexes of the 26S proteasome. The AAA type-ATPases in the 19S cap are needed to transfer the polyubiquitin chains in the central chamber of the 20S core particle that possess proteolysis activity. In addition, deubiquitinating enzymes associated with the 19S cap remove Ubs from the substrates prior to terminal digestion. The figure is extracted from Meusser, B. et al., 2005 (Meusser et al., 2005).
ERAD substrates are not only ER misfolded proteins but also folded proteins such as HMGR, 3-hydroxy 3-methylglutaryl coenzyme A (HMG CoA) reductase which is a key enzyme of the mevalonate pathway. The regulated breakdown of HMGR is controlled by the ERAD system (Hampton, 2002). The mevalonate pathway produces sterols and a variety of isoprenoids in the end. When flux through the mevalonate pathway is high, the rate of HMGR degradation is high and enzyme levels tend to be low, whereas the degradation rate is low and enzyme levels tend to increase when flux is low. In *S. cerevisiae*, there are two isomers of ER transmembrane HMGR, Hmg1p and Hmg2p. Hmg2p is physiologically degraded by the ERAD pathway but Hmg1p is stable. The analysis of Hmg2p turnover led to the identification of several core components of ERAD (Hampton *et al.*, 1996) including Hrd1p/Der3p, an E3 ubiquitin ligase localized at the ER membrane. Hrd1p/Der3p consists of an N-terminal multi-spanning membrane anchor and a cytoplasmic C-terminal region bearing a RING-H2 motif (Bays *et al.*, 2001). Hrd1p/Der3p is also required for degradation of misfolded CPY* in vivo* (Bordallo *et al.*, 1998) and functions autonomously as an E3 ubiquitin ligase *in vitro* as demonstrated by its ability in ubiquitinating denatured proteins, suggesting that it functions as an E3 ubiquitin ligase in quality control (Bays *et al.*, 2001). Although the C-terminal RING-H2 domain of Hrd1p/Der3p is exposed to numerous ubiquitin E2s, Hrd1p/Der3p only employs Ubc7p and another E2 enzyme, Ubc1p (to a much lesser degree), in the execution of its function (Bays *et al.*, 2001; Friedlander *et al.*, 2000). Another ER integral membrane protein, Hrd3p, is associated with Hrd1p/Der3p that may target substrates to the site of retro-translocation via its large lumenal domain (Gardner *et al.*, 2000).
The surprising observation that the turnover of Hmg2p involves a quality control ligase led to the provocative hypothesis that a conformational change may turn Hmg2p into a quality control substrate, thereby triggering its degradation (Shearer and Hampton, 2004; Shearer and Hampton, 2005). In line with this assumption, Hmg2p turnover is strongly decreased by the chemical chaperone glycerol which has been shown to help loosely folded proteins maintain their native structure. In addition, in vitro conditions that destroy Hmg2p stability increase its protease sensitivity. These findings imply that conditional conformational changes could convert Hmg2p into a short-lived protein.

A second Ring-finger E3 ubiquitin ligase at the ER membrane is Doa10p, which functions with Ubc6p and Cue1p/Ubc7p. Doa10p is involved in removing both ER proteins and cytoplasmic targets (Swanson et al., 2001). As Hrd1p/Der3p and Doa10p are required for degrading distinct ERAD substrates respectively, different ERAD pathways may exist in the ER (Vashist and Ng, 2004). In S. cerevisiae, different E3 ubiquitin ligases could define different ERAD pathways (Carvalho et al., 2006). Proteins with misfolded luminal domains use the ERAD-L pathway, in which the Hrd1p/Hrd3p ligase forms a near stoichiometric membrane core complex by binding to the putative channel protein Der1p via Usa1p, a novel component. This core complex associates through Hrd3p with Yos9p, a substrate recognition protein in the ER lumen. ERAD substrates with misfolded intra-membrane domains require only some of the core complex components and may define a novel pathway (ERAD-M). Membrane proteins with misfolded cytosolic domains use the ERAD-C pathway and are directly targeted to the Doa10p ubiquitin ligase. All three pathways converge at the Cdc48p ATPase complex.
whose membrane recruitment is facilitated by Ubx2p. These results lead to a unifying concept for ERAD that may also apply to mammalian cells.

1.3 Unfolded protein response (UPR)

In eukaryotic cells, secreted proteins and proteins that reside in any compartment along the secretory pathway first enter the secretory pathway by translocation into the ER, where they fold and, if appropriate, become covalently modified and assembled into their final oligomeric complexes. Only correctly folded and assembled proteins, i.e., proteins that have passed the QC standards, are allowed to leave the ER and transit to intracellular organelles or the cell surface. As a processing plant for protein folding and posttranslational modifications, the ER is highly sensitive to disequilibrium in homeostasis. A number of cellular stress conditions, such as perturbation in calcium or redox status, elevated secretory protein synthesis, expression of misfolded proteins, glucose deprivation, altered glycosylation, and overloading of cholesterol can interfere with protein folding and then lead to the accumulation of unfolded or misfolded proteins in the ER, collectively termed ER stress. This stress causes a fundamental threat to cells so the ER has evolved an exquisite mechanism to ensure that the folding capacity of the ER is sufficient and adjusted appropriately according to the requirements within the ER, i.e., that ER homeostasis is maintained. Such homeostatic control is carried out by the action of signaling transduction pathways that bear sensors facing the ER lumen and effectors that convey the message from the ER to other cellular compartments. The first clue concerning the presence of such signaling events in cells was provided by the
observation that the expression of genes encoding ER chaperones is selectively activated by genetic and pharmacological manipulations that increase the load of misfolded ER proteins (Kozutsumi et al., 1988). At the core of this regulation is an evolutionarily conserved ER-to-nucleus signaling pathway called the unfolded protein response (UPR) (Bernales et al., 2006).

The UPR is initiated by the accumulation of unfolded or misfolded proteins in the ER (ER stress), which result when protein folding demand exceeds the protein folding capacity of the ER. Physiologically, conditions that induce the UPR by ER stress include: (1) the making of various professional secretory cells, such as plasma or pancreatic β cells, (2) impeded metabolic conditions, such as glucose deprivation, hyperhomocysteinemia and ischemia, (3) mutations in the genes encoding secretory or transmembrane proteins, which normally fold in the ER, such as α-1 antitrypsin and (4) infection by certain pathogens, such as hepatitis C. The UPR can also be triggered experimentally. Calcium depletion from the ER lumen, inhibition of N-linked glycosylation, reduction of disulfide bonds, expression of mutant, even some wild-type, secretory or transmembrane proteins may saturate folding pathways and clog the ER lumen (Kaufman, 2002).

1.3.1 Three UPR transducers

The basic signaling components in the UPR pathway was first characterized in the yeast S. cerevisiae more than a decade ago. The type I ER transmembrane protein kinase/endoribonuclease Ire1/Ern1p was found to be essential for cell survival in
response to ER stress and was identified as a stress transducer to initiate UPR signaling (Cox et al., 1993; Mori et al., 1993). Afterward, researchers found that all eukaryotes have not only conserved the essential and unique features of Ire1p-mediated UPR signaling pathway found in yeast but also evolved two additional sensors to generate a diversity of responses. In mammalian cells, the counterpart to yeast Ire1p has two isoforms: Ire1α and Ire1β. Although Ire1α is expressed in most cells and tissues, Ire1β is primarily restricted to epithelial cells of the gastrointestinal tract (Tirasophon et al., 1998; Wang et al., 1998). In addition to Ire1, higher eukaryotes utilize two additional UPR transducers: PKR-like ER-associated kinase (PERK) and activating transcription factor 6 (ATF6).

IRE1 is composed of an N-terminal ER-stress sensing domain in the ER lumen and a serine/threonine kinase domain, followed by C-terminal endoribonuclease (RNase) domain in the cytoplasm (Mori et al., 1993; Shamu and Walter, 1996). PERK contains a large ER lumenal stress-sensing domain that is functionally interchangeable with the IRE1 lumenal domain, a cytoplasmic serine/threonine kinase domain and a cytoplasmic eukaryotic translation-initiation factor 2α (eIF2α) kinase domain (Harding et al., 1999; Liu et al., 2000; Shi et al., 1998). ATF6 is a transcription factor with an N-terminal cytoplasmic basic leucine zipper (b-ZIP) domain and a C-terminal ER lumenal domain to sense stress (Haze et al., 1999). Three stress transducers are all localized at the ER membrane and constitutively expressed in all cell lines.

Upon ER stress, the UPR is activated to reduce the protein load that enters the ER, which is a transient adaption that is achieved by lowering protein synthesis and translocation into the ER, and to increase the protein-folding capacity to handle
unfolded proteins, which is a longer-term adaptation that entails transcriptional activation of UPR targets genes, including those that function as part of the ER protein-folding machinery and those that are required for ERAD pathway. The UPR is orchestrated by transcriptional upregulation of multiple genes mediated by IRE1 and ATF6, a general attenuation in translation initiation and a selective translation of specific mRNAs mediated by PERK.

1.3.2 UPR signaling mediated by IRE1 (Figure 1-8 for yeast and figure 1-9 for metazoans)

In response to ER stress, IRE1 undergoes dimerization/oligomerization in the plane of the membrane, thus promoting trans-autophosphorylation of on positive regulation sites within juxtaposed kinase domains (Shamu and Walter, 1996; Welihinda and Kaufman, 1996).

What events occur in the IRE1 lumenal domain to activate IRE1 upon ER stress? The previous prevailing model posed that in nonstressed cells, BiP binds to the lumenal domain of IRE1 where it acts as a negative regulator, thus repressing the activation of IRE1 (Bertolotti et al., 2000; Okamura et al., 2000). In response to the accumulation of unfolded proteins in the ER, BiP might preferentially associate with unfolded protein instead of IRE1. Because the region of BiP that interacts with IRE1 is the same region of BiP that binds to exposed hydrophobic patches in unfolded proteins, IRE1 is tuned on by dimerization/oligomerization of its lumenal domain when BiP is titrated away from it (Kimata et al., 2004). Recently, the X-ray crystal structure of luminal domain of yeast and human IRE1 has been determined (Credle et al., 2005; Zhou et al., 2006), providing
an attractive alternative model. According to the structural information, dimerization/oligomerization may be induced directly by binding of unfolded proteins to the IRE1 lumenal domain, which has an architectural resemblance to the peptide-binding domain of major histocompatibility complexes (Credle et al., 2005; Zhou et al., 2006).

In addition to triggering its protein kinase catalytic function, dimerization/oligomerization and auto-phosphorylation of IRE1 lead to the activation of its unusual effector function (site-specific RNase activity), which causes the endonucleolytic cleavage of the sole known substrate: an mRNA that encodes a bZIP transcription factor named Hac1p in yeast (Figure 1-8) (Cox and Walter, 1996) or XBP1 in metazoans (Figure 1-9) (Yoshida et al., 2001).

IRE1 cleaves the precursor HAC1 or XBP1 mRNA at two sites, thereby excising an intervening intron from the mRNA. The severed exons are subsequently ligated by an action of tRNA ligase. The enzyme required for this ligation is Rlg1p in yeast (Figure 1-8) (Sidrauski and Walter, 1997) but has not been identified in higher eukaryotes.
Figure 1-8: Signaling by Ire1p in *S. cerevisiae*.
Upon accumulation of misfolded proteins in the ER, Ire1p undergoes dimerization/oligomerization and trans-autophosphorylation, thus eliciting the activation of its site-specific RNase activity. Ire1p then cleaves an inhibitory intron from *HAC1* mRNA and the severed exons are subsequently ligated by the action of tRNA ligase, Rlg1p. The sliced *HAC1* mRNA is efficiently translated to produce a Hac1p with 18 new amino acids at the C-terminus (green). Hac1p then relocates to the nucleus where it binds to the UPRE of target genes to activate their expression. The red arrow represents process that occurs when the UPR is off; the green arrows indicate processes that occur when the UPR is on. The figure is extracted from Kaufman, RJ (Kaufman, 1999).
Figure 1-9: Signaling by IRE1 in metazoans. Individual steps are thoroughly discussed in the text. The figure is extracted from Ron and Walter, 2007 (Ron and Walter, 2007).
The consequences of this IRE1-mediated splicing are different in yeast and metazoans. In yeast, the HAC1 mRNA intron represses translation and removal of this repression increases its translation efficiency and alters the C-terminal sequence of Hac1p to generate a potent transcriptional activator, which is the key activating event of the yeast UPR (Ruegsegger et al., 2001). The resultant spliced Hac1p activates transcription via direct binding to the cis-acting UPR element (UPRE, minimal motif TGACGTG(C/A)) upstream of many UPR target genes (Figure 1-8) (Patil and Walter, 2001). In contrast, in the case of XBP1, both the unspliced and spliced forms of mRNA produce functional proteins and together the proteins form a feedback system that control the duration of the UPR response (Figure 1-9) (Calfon et al., 2002; Yoshida et al., 2006). The spliced form of protein, XBP1s, is more stable (Calfon et al., 2002) and works as a potent activator of UPR target genes (Yoshida et al., 2001), whereas the unspliced XBP1, XBP1u, is labile and binds to XBP1s, which appears to accelerate degradation of XBP1s (Yoshida et al., 2006).

In addition to post-transcriptional regulation by IRE1-mediated splicing, HAC1 and XBP1 mRNAs are also UPR transcriptional targets. In yeast, under conditions of severe ER stress, HAC1 mRNA expression is upregulated independently of Ire1p and extra increase in the Hac1p transcription factor drives a qualitatively different transcription response termed the super-UPR (Leber et al., 2004). In metazoans, the amount of XBP1u mRNA increases relative to XBP1s mRNA as ER stress decreases, so does IRE1 activity (Yoshida et al., 2006). Thus, with more XBP1u available to inhibit the activity of XBPs, the UPR signaling can be rapidly terminated.
Metazoan IRE1 may function even beyond its nucleotylic activity. It has been shown that phosphorylated mammalian IRE1 can interact with TRAF2 (tumor necrosis factor receptor (TNFR)-associated factor-2), which allows it to signal to Jun N-terminal kinase (JNK) (Urano et al., 2000) and change intracellular signaling such as that resulting in insulin resistance (Figure 1-9) (Ozcan et al., 2004). The IRE1-TRAF2 complex is also connected to programmed cell death by its interaction with components of the cell death machinery such as caspase-12 (Yoneda et al., 2001). However, the physiological significance of this regulation remains unclear although indirect evidence does show contribution to the death of ER-stress cells.

Recently, the analysis of mRNA expression in Drosophila melanogaster cells during ER stress revealed that activated IRE1 can mediate the cleavage of various ER-associated mRNA, leading to their degradation (Figure 1-9) (Hollien and Weissman, 2006). This mechanism appears to reduce the load on the stressed ER, which may facilitate the reprogramming of protein synthesis and translocation mechanism in the ER. It is unknown whether IRE1 degrades these mRNA directly or it does so by activating or recruiting other RNases.

1.3.3 UPR signaling mediated by ATF6 (Figure 1-10)

A search for additional proteins that bind mammalian UPR-responsive promoter elements (ER stress response element, ERSE) led to the identification of ATF6 (Yoshida et al., 1998). In unstressed cells, ATF6 is synthesized as inactive precursor, anchored to the ER membrane through a transmembrane domain with a stress-sensing domain
projecting into the ER lumen. Upon ER stress, ATF6 is transported from the ER to the Golgi where it is cleaved by Golgi-resident site-1 protease (S1P) and site-2 protease (S2P) to yield a cytoplasmic b-ZIP transcription factor, ATF6f ('f' for fragment). Subsequently, ATF6f relocates to the nucleus to activate UPR target genes (Figure 1-10) (Shen et al., 2002a).

Figure 1-10: Signaling by ATF6.
The scheme shows transcriptional regulation mediated by transcription factor-6 (ATF6) and cyclic AMP response element binding protein hepatocyte (CREBH) upon ER stress. The details are thoroughly discussed in the text. The figure is extracted from Ron and Walter, 2007 (Ron and Walter, 2007).
Notably, S1P and S2P also process other proteins in addition to ATF6, such as the ER-associated transmembrane sterol-response element binding protein (SREBP), a transcription factor that control cellular sterol levels (Ye et al., 2000). In both cases, the activating proteolytic steps take place after trafficking of the inactive precursor to the Golgi. However, the initial trigger for the trafficking event is different for the two classes of proteins: liberation from cholesterol deprivation in the case of SREBPs, but the accumulation of unfolded proteins in the ER in the case of ATF6. This accumulation may involve direct or indirect sensing of unfolded proteins by the luminal domain of ATF6, which causes ATF6 to be released from the ATF6-BiP complex (formed in the unstressed condition through stable binding of BiP to the ATF6 luminal domain) and then be delivered to the Golgi (Shen et al., 2002a).

On the activation of the UPR, ATF6 upregulates a group of genes that encode ER-localized molecular chaperones and folding enzymes, whereas XBP1 upregulates a set of ER-localized chaperone genes which are essential for protein folding, maturation, and degradation (Lee et al., 2003; Okada et al., 2002). It was previously proposed that IRE1-mediated splicing and S2P-mediated ATF6 merge to regulate XBP1 in response to ER stress: XBP1 mRNA levels are induced by the active form of ATF6 to produce more XBP1 mRNA substrates for IRE1-mediated slicing (Lee et al., 2002; Yoshida et al., 2001). However, the XBP1 mRNA and protein levels were not altered in cells defective in ATF6 cleavage during ER stress (Lee et al., 2003; Lee et al., 2002). Induction of mouse ATF6 mRNA was partially compromised in the absence of XBP1; therefore, it was proposed that ATF6 works downstream of XBP1 in some cases (Lee et al., 2003).
These data suggest that XBP1 and ATF6 function largely in parallel pathways and may
interact with each other during ER stress.

Thus far, a number of ATF6-related transmembrane transcription factors have
been identified, which include LZIP (also known as lumen or cyclic AMP-responsive
element binding protein-3 (CREB3)), OASIS (also known as CREB3-like-1), CREB4,
CREBH (CREB-hepatocyte), and Tisp40 (transcript induced in spermiogenesis-40)
according to their predicted structure (Bailey and O'Hare, 2007). Among these factors,
CREBH has recently been found to be activated in response to ER stress, cleaved by
Golgi proteases and transported to the nucleus to induce appropriate adaptive responses
(Figure 1-10). However, CREBH does not activate transcription of UPR targets but,
rather, links ER stress in the liver to the secretion of serum proteins that are related to
inflammation (so called acute-phase responsive proteins) (Zhang et al., 2006). These
observations are an intriguing example of the integration of the UPR into broad
coordinated responses.

1.3.4 UPR signaling mediated by PERK (Figure 1-11)

The last ER stress transducer, PERK, superficially resembles IRE1 because both
are ER type I transmembrane proteins and the stress-sensing domain of PERK is distantly
related to that of IRE1 (similar in structure and function, and experimentally
interchangeable) (Liu et al., 2000). PERK, similar to IRE1, also contains a cytoplasmic
protein kinase domain which can undergo activating trans-autophosphorylation by
oligomerization upon ER stress. However, unlike IRE1, for which the only substrate is
itself, activated PERK phosphorylates the α-subunit of eukaryotic initiation factor-2 (eIF2α) on Ser51. This phosphorylation prevents the formation of the ternary translation initiation complex eIF2α–GTP–tRNA\textsubscript{Met}, thereby attenuating protein translation in general to reduce the load of newly synthesized proteins, many of which are destined to enter the already stress ER lumen (Figure 1-11) (Harding \textit{et al.}, 1999).

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**Figure 1-11: UPR signaling mediated by PERK.**

Upon accumulation of unfolded proteins (U-Pr) in the ER, BiP is released from PERK, thus permitting its dimerization and activation to phosphorylate Ser51 on eIF2α. The phosphorylated PERK may lead to either attenuation of global mRNA translation or activation of selective mRNAs, such as \textit{GCN4} or \textit{ATF4} mRNA. The figure was extracted from Zhang and Kaufman, 2006 (Zhang and Kaufman, 2006).
In addition to globally decreasing protein synthesis, phosphorylated eIF2α selectively stimulates translation of a specific subset of mRNA in response to stress. In yeast, upon amino acid starvation, phosphorylation of eIF2α by Gcn2 (general control non-derepressible-2; a kinase activated by uncharged tRNAs) promotes translation of GCN4 mRNA that encodes a b-ZIP transcription factor required for activation of genes involved in amino acid biosynthetic functions (Figure 1-11) (Hinnebusch and Natarajan, 2002). GCN4 mRNA contains short, inhibitory upstream open reading frames (uORFs) in its 5’-untranslated region. These inhibitory uORFs, which originally prevent translation initiation of the downstream GCN4-encoding ORF in unstressed cells, are bypassed only when eIF2α is phosphorylated and its activity is limited, thereby leading to ribosomes skipping the uORFs so that the GCN4 ORF can be translated. This feature is also conserved in mammalian cells to regulate translation during ER stress and amino acid starvation. For example, upon ER stress, phosphorylated eIF2α selectively promotes translation of ATF4 mRNA, which encodes the metazoan homologue of Gcn4p (Harding et al., 2000). ATF4 subsequently activates transcription of genes involved in amino acid transport and metabolism, oxidation-reduction reactions and ER-induced apoptosis (Harding et al., 2003; Zinszner et al., 1998).
1.4 This study

1.4.1 The physiological roles of the UPR

While the mechanism by which the UPR signal is transmitted from the ER to the nucleus is well characterized, the significance of the UPR pathway can be appreciated better when one investigates how the UPR-induced targets alleviate ER stress and restore ER homeostasis. This issue has been addressed previously in two elegant studies. The first study utilized DNA microarrays to determine the full complement of genes whose expression were specifically induced by the UPR (Travers et al., 2000). The second approach exploited a powerful genetic strategy to identify genes that showed genetic interactions with the UPR pathway (Ng et al., 2000). Both studies were conducted in yeast S. cerevisiae.

In S. cerevisiae, the Ire1p/Hac1p-mediated pathway is responsible for transcriptional induction of almost all UPR-target genes (Figure 1-8) (Cox and Walter, 1996; Sidrauski and Walter, 1997). Genome-wide expression analysis using DNA microarrays in conjunction with specific mutations provided the ideal solution to reveal these UPR targets. According to the microarrays expression profiling, a surprisingly large set of genes whose induction was significantly greater in wild-type than in either Δire1 or Δhac1 cells was defined (Travers et al., 2000). Among these 381 target genes (comprising approximately 6% of the total genes), 208 genes have known functions or are homologues of known genes. Of those, nearly half has established roles in the ER, the Golgi apparatus, and throughout the secretory pathway (Table 1-2) (Spear and Ng, 2001).
Therefore, the UPR can be considered a means of homeostatic control, serving to remodel the cell’s biosynthetic capacity all along the secretory pathway according to need.

Table 1-2: The diverse classes of genes regulated by the UPR. The list was extracted from Spear and Ng, 2001 (Spear and Ng, 2001).

<table>
<thead>
<tr>
<th>Function</th>
<th>Number of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secretory pathway</td>
<td>103 total</td>
</tr>
<tr>
<td>ER protein translocation</td>
<td>7</td>
</tr>
<tr>
<td>Lipid/Inositol metabolism</td>
<td>19</td>
</tr>
<tr>
<td>ER protein glycosylation</td>
<td>17</td>
</tr>
<tr>
<td>Protein folding</td>
<td>10</td>
</tr>
<tr>
<td>Protein degradation</td>
<td>6</td>
</tr>
<tr>
<td>ER-to-Golgi transport</td>
<td>11</td>
</tr>
<tr>
<td>Golgi-to-ER retrieval</td>
<td>5</td>
</tr>
<tr>
<td>Golgi glycosylation</td>
<td>6</td>
</tr>
<tr>
<td>Vacuolar targeting</td>
<td>4</td>
</tr>
<tr>
<td>Distal secretion</td>
<td>7</td>
</tr>
<tr>
<td>Cell wall biogenesis</td>
<td>11</td>
</tr>
<tr>
<td>Non-secretory pathway</td>
<td>105 total</td>
</tr>
<tr>
<td>Unknown function</td>
<td>173 total</td>
</tr>
</tbody>
</table>
Meanwhile, the complementary genetic study was conducted to identify genes that are functionally linked to the UPR pathway (Ng et al., 2000). This was accomplished by screening a bank of random mutants to isolate strains harboring mutations that require UPR activation for cell viability. Hence, the experiment revealed genes whose deficiency presumably generates cellular stress that could only be mitigated by UPR upregulation. These genes encode proteins required for protein folding, protein translocation, protein glycosylation, glycosphatidyl-inositol (GPI) anchor additions, ion transport, vesicle trafficking, membrane biosynthesis and various aspects of ERQC, such as ERAD (Ng et al., 2000). In contrast to the microarray analysis, all isolated mutants in this genetic screen were exclusively involved in some aspects of ER function. Taken together, the studies indicate that the UPR monitors the state of the ER and many aspects of ER function fall under its influence. The studies also provide a physiological link between the UPR and ERQC, showing that the UPR monitors and regulates the ERAD pathway.

Other aforementioned studies confirm this relationship by testing the corollary. Two groups studying the degradation of misfolded proteins found that by expressing ERAD substrates and by introducing mutations that disrupt the ERAD pathway, it was observed that the UPR was activated (Casagrande et al., 2000; Friedlander et al., 2000). By using specific substrates, it was shown that mutant cells defective in inducing the UPR were also impaired for ERAD function (Casagrande et al., 2000; Travers et al., 2000). Together, these studies established a regulatory circuit by which the UPR regulates a means of abrogating the toxicity of irreversibly damaged proteins.

In addition to upregulating ERAD components that directly enhance the clearance of misfolded proteins from the ER, the surprising breath of the UPR transcriptional scope
suggests that its role in ER stress tolerance may need other functions apart from ERAD. For example, UPR activation also induces specific COPII or coatamer components (Travers et al., 2000), implying that the increase in vesicle trafficking might catalyze the passage of misfolded species to the vacuole for degradation. Indeed, a recent study confirmed this idea (Spear and Ng, 2003). It showed that under ER stress, the ERAD pathway for misfolded proteins is saturable, so in order to maintain ER homeostasis, the UPR activates an ‘overflow’ pathway to export excess misfolded species to the vacuole for degradation. The importance of this ER-to-vacuole degradation pathway was revealed through the observation that in mutant strains defective in vesicular trafficking, despite retaining the ability to activate the UPR, overexpression of misfolded CPY* was toxic to these cells (Spear and Ng, 2003).

Based on information gleaned from these studies on the ER’s role in stress tolerance, the following model has been proposed (Spear and Ng, 2003). Accumulation of misfolded proteins causes the widespread loss of ER function. However, when the UPR is induced, it lessens this stress by increasing the synthesis of limiting factors such as factors involved in translocation, folding and glycosylation to assist proteins in refolding. On the other hand, to remove terminally misfolded proteins, the UPR expands QC functions including ERAD and anterograde vesicle trafficking such that under severe ER stress, when ERAD is saturated, excess misfolded proteins could take advantage of an alternative pathway to be transported to the vacuole for degradation.
1.4.2 This study

Taking a different strategy, my study also endeavors to uncover the physiological roles of the UPR in yeast since the mechanism by which the transcriptional output of the UPR restores ER homeostasis remains unclear. In this study, I addressed this question by studying the contribution of one specific target gene to ER stress tolerance. In order to answer this question, an approach that uncouples the expression of individual target gene from UPR regulation was devised. Among the UPR-induced 381 target genes, the ER chaperone, Kar2p, is perhaps the most interesting so it is first selected to be analyzed using this strategy. The reasons for choosing Kar2p as the first target to study are discussed in detail below and in the introduction of chapter 2.

1.4.3 Background of Kar2p

*KAR2* encodes an essential protein belonging to the heat shock protein (HSP) 70 family of molecular chaperones (Normington *et al.*, 1989; Rose *et al.*, 1989). Its sequence is homologous to bacterial DnaK (Scidmore *et al.*, 1993) and the mammalian BiP/GRP78 and has 67% identity to mouse BiP (Normington *et al.*, 1989). Accordingly, Kar2p has several structural features in common with BiP: a functional secretory signal sequence close to its N-terminus, a yeast ER retention signal (HDEL) at the C-terminus, and the lack of potential N-linked glycosylation sites (Rose *et al.*, 1989). Moreover, *KAR2* is regulated like BiP; both are UPR-regulated genes so that their mRNAs are synthesized at a high basal level and further increased when there is an accumulation of unfolded proteins in the ER (Kozutsumi *et al.*, 1988; Rose *et al.*, 1989). In addition, *KAR2* is also
regulated by heat shock (Rose et al., 1989). Thus, transcription of KAR2 is regulated by three independent cis-acting elements (Figure 2-1): (1) a heat shock element (HSE) containing four repeats of a conserved 5-bp recognition unit (5′-aGAAccTTCTgGAAa cTTCa-3′), (2) a pyrimidine (GC)-rich which is involved in the high level of basal expression of the KAR2 gene, and (3) a 22-bp UPRE responding to UPR activation.

Kar2p performs multiple functions in the ER, including folding of nascent polypeptides (Gething and Sambrook, 1992), refolding of denatured proteins (Simons et al., 1995), protein translocation across the ER membrane (Sanders et al., 1992; Vogel et al., 1990), and regulation of ERAD (Nishikawa et al., 2001). The diversity of functions of Kar2p is thought to rely on the intrinsic properties of the Hsp70s (for details, see pages 12 to 13: the ATP/ADP cycle) and on its recruitment of specific DnaJ cofactors. Four DnaJs of the yeast ER have been described, which are Sec63p, Scj1p, Jem1p, and Erj5p. Sec63p is an essential transmembrane protein that interacts with Kar2p to assist nascent polypeptides in translocation into the ER lumen (Brodsky et al., 1995; Corsi and Schekman, 1997; Matlack et al., 1999). Scj1 and Jem1p, two non-essential UPR-regulated lumenal proteins, are involved in Kar2p functions required for protein folding and ERAD (Nishikawa et al., 2001; Silberstein et al., 1998). In addition, Kar2p plays a direct role in nuclear membrane fusion during karyogamy, a process activated during mating (Latterich and Schekman, 1994). This function is independent of its role in translation and folding, and Jem1p has also been found to function during karyogamy in cooperation with Kar2p (Brizzio et al., 1999; Nishikawa and Endo, 1997). Erj5p, a non-essential UPR-regulated transmembrane protein, was recently identified (Carla Fama et al., 2007). It was observed that ERJ5 synthetically interacted with genes involved in
protein folding in the ER (kar2-159, ∆scj1∆jem1p) and loss of ERJ5 gene caused a constitutively-activated UPR, indicating that ERJ5 is required for maintaining the folding capacity of the yeast ER.

It has been previously proposed that not only KAR2 expression is induced by the UPR but Kar2p itself is an important regulator of the UPR (Bertolotti et al., 2000; Okamura et al., 2000). In the previous model, Kar2p binds to Ire1p in the nonstressed condition to repress the activation of Ire1p and under conditions of ER stress, Kar2p dissociates from Ire1p (presumably resulting from its sequestration onto unfolded proteins), thus triggering dimerization/oligomerization and activation of Ire1p. This Kar2p-dependent Ire1p activation is supported by several studies (Kohno et al., 1993; Okamura et al., 2000). First, the overexpression of Kar2p in yeast cells, but not other ER chaperones was sufficient to attenuate the UPR. Furthermore, Kar2p co-immunoprecipitated with Ire1p from extracts of nonstressed cells. Notably, the levels of Kar2p that were associated with Ire1p were significantly decreased in cells challenged with ER stress. Despite these findings providing correlated support for the model, it does not explain how the high molar ratio of Kar2p to Ire1p can be reconciled with the sensitivity of the UPR to subtle changes in levels of ER unfolded proteins. Moreover, in a recent study, Oikawa et al. showed that deletion of Kar2p binding site in the Ire1p (lying within amino acids 448-520 in yeast Ire1p) did not compromise the Ire1p regulation by the presence and absence of unfolded proteins (Oikawa et al., 2007). Although the precise contribution of Kar2p-Ire1p binding to UPR activation remains unclear, several lines of evidence suggest that Kar2p binding is not essential to Ire1p regulation (Credle et al., 2005; Kimata et al., 2007; Kimata et al., 2004).
Regardless of the role of Kar2p-Ire1p binding on UPR induction, there is no doubt that upon ER stress, Kar2p mobilizes to bind elevated levels of ER unfolded proteins and keeps them soluble for ER-associated protein degradation (ERAD). This raises an interesting conundrum. Since half of UPR targets encode proteins that enter the secretory pathway, Kar2p is, in fact, an essential facilitator of the UPR at its last stage, the synthesis of target proteins. How then, is it possible to activate the UPR when a key facilitator is also a target gene that is immediately needed to deal with the misfolded products of stress? In order to address this question and also to study the contribution of upregulated Kar2p to ER stress response, we selected Kar2p as the first gene to be analyzed using the novel devised approach.
Chapter 2

A strategy to specifically uncouple the expression of the KAR2 gene from the UPR

2.1 Introduction

The UPR is a stress response pathway that can be activated when unfolded or misfolded proteins accumulate in the ER lumen, a condition referred to as ER stress. In yeast, the activation of the UPR results in a set of 381 genes to be up-regulated as shown by genome-wide expression analysis (Travers et al., 2000). Half of these genes represent functions throughout the secretory pathway including protein folding and modification, protein QC, lipid synthesis, protein trafficking, and vacuolar (lysosomal) proteases. Our previous work has shown that activation of the UPR is essential for ER stress tolerance; the overexpression of misfolded carboxypeptidase Y, CPY*, is lethal in UPR-deficient Δire1 cells but benign in wild-type cells (Spear and Ng, 2003). However, the mechanism by which the transcriptional output of the UPR restores ER homeostasis remains unclear. In this study, I address this question by studying the contribution of one specific target gene to ER stress tolerance.

For a variety of reasons explained below, I chose the KAR2 gene as the first target to analyze. KAR2 encodes the molecular chaperone Kar2p/BiP, a highly conserved protein belonging to the Hsp70 family of molecular chaperones and represents a major target of the UPR. Kar2p plays multiple functions in the ER according to its recruitment to specific cofactors. Under stress, Kar2p mobilizes to bind misfolded proteins and keeps them soluble for ER-associated protein degradation (ERAD) (Nishikawa et al., 2001). Kar2p is required for karyogamy (nuclear membrane fusion during mating), for which it
received its name (Rose et al., 1989), but how it carries out this function is unknown. Interestingly, Kar2p is also required for the translocation of newly synthesized proteins into the ER and for their subsequent folding (Brodsky et al., 1995; Simons et al., 1995; Vogel et al., 1990; Young et al., 2001). That raises an interesting conundrum. Since half of UPR targets encode proteins that enter the secretory pathway, Kar2p is, in fact, an essential facilitator of the UPR at its last stage, the synthesis of target proteins. How then, is it possible to activate the UPR when a key facilitator is also a target gene that is immediately needed to deal with the misfolded products of stress?

To address this question and also to study how the upregulation of KAR2 contributes to ER stress tolerance, I have designed a strategy to uncouple the regulation of the KAR2 gene from the UPR. This method allows me to assess directly the contribution of this single gene to the regulatory response.

2.2 Design of a strategy

To determine the role of Kar2p in stress tolerance, a yeast strain specifically engineered to uncouple KAR2 upregulation by UPR was constructed. For this question, mutagenesis of KAR2 itself is undesirable since any effect observed can be indirect and be due to the loss of important housekeeping functions. An important point in this strategy is that no mutations are introduced into the open reading frame (ORF) of KAR2 so that wild-type Kar2p can be expressed to promote normal growth and function in resting cells—only its regulation is unresponsive to the UPR. Therefore, a strain was engineered to carry a KAR2 allele with its promoter modified at the unfolded protein
response element (UPRE). The UPRE is the binding site for Hac1p, the transcriptional regulator of the UPR and is necessary and sufficient to mediate the transcriptional induction by the UPR (Mori, 1992; Mori et al., 1998). Thus, by replacing the UPRE with random sequences (Figure 2-1), the expression of KAR2 may not be induced by the UPR. In addition, previous studies have shown that the disruption of the UPRE eliminates activation by the UPR without affecting basal activity (Kohno et al., 1993).
Figure 2-1: UPRE sequence in the promoter of KAR2 is replaced with a random sequence (RS) in order to uncouple expression of KAR2 from the UPR.

There are three cis-acting elements in the KAR2 promoter region, one (heat shock element [HSE]) responding to heat shock and the other (unfolded protein response element [UPRE]) responding to the presence of unfolded protein in ER. Lying between these two elements is a GC-rich region that is involved in the basal expression of the KAR2 gene. The location of each element is indicated in the diagram. To build the strain in the following content, UPRE is replaced by random sequence (RS) with the same length.
To generate the strain, a yeast strain (MS785) in which the KAR2 gene has been deleted (kar2-\(\Delta\)L148::LEU2) was initially utilized. This strain carries a URA3 plasmid with wild-type KAR2 (pMR397) for strain viability (Vogel et al., 1990). MS785 was mated with a wild-type strain to produce a heterozygous diploid. An integrating plasmid carrying a functional KAR2 gene altered with the replacement of its 22-bp UPRE with random sequences (upre\(^d\)-KAR2) was then transformed into the diploid cell. Since the diploid cell carries a functional copy of KAR2, the plasmid was dropped by counter-selection on media containing 5-fluoro-orotic acid (5-FOA). At the same time, the integrating plasmid was forced to integrate into ura3-52 locus to maintain cell viability. After tetrad dissection of the heterozygous diploid, the haploid strain, called CHY119, dependent only on upre\(^d\)-KAR2 for viability was obtained. As a control, another integrating plasmid carrying the entire functional KAR2 gene was integrated into ura3-52 locus (UPRE-KAR2), called CHY113 (Figure 2-2).
Figure 2-2: A schematic diagram representing the strategy taken to obtained CHY119 and CHY113 strains.
2.3 Characterization of UPR-uncoupled \textit{KAR2} yeast strain

Once CHY119 was obtained, the strain was characterized in order to ensure that it behaves as expected. CHY119 is designed to uncouple upregulation of \textit{KAR2} gene from UPR signal upon ER stress. Compared to wild-type cells, the only alteration in CHY119 is the replacement of UPRE in \textit{KAR2} promoter with a random sequence. Thus, CHY119 should be exactly the same as wild-type cells under non-stress condition, as wild-type Kar2p can be expressed at basal level in CHY119 to maintain its housekeeping functions. Upon ER stress, CHY119 should also be the same as wild-type cells except that now, the \textit{KAR2} gene is no longer UPR-inducible. According to these criteria, three requisite phenotypes were examined: (1) Resting CHY119 cells should grow as well as wild-type cells, (2) the replacement of UPRE in \textit{KAR2} promoter with a random sequence should disable its ability to respond to UPR signals, and (3) the impairment does not disrupt the UPR pathway itself.

2.3.1 Examination of CHY119 by cell growth

In order to determine any differences in growth between CHY119 and wild-type strains, equal amounts of wild-type (W303 strain) and CHY119 cells were collected and ten-fold serial dilutions of cell suspensions were spotted on a synthetic complete (SC) plate. Unexpectedly, CHY119 cells displayed slowed growth in the assay compared to wild-type cells (Figure 2-3A). This may be caused by the accidental introduction of mutations into the ORF of \textit{KAR2} which may interfere with Kar2p’s essential functions.
This may also be caused by other intervening factors that create stress inside the cells even under a resting condition where no extrinsic ER stress is applied.

Figure 2-3: The engineered strain grows as well as wild-type in resting conditions. (A) Equal numbers of wild-type or CHY119 cells were spotted as 10-fold serial dilutions on synthetic complete (SC) plate and incubated at 30°C until colonies were formed. (B) Total RNAs were isolated from wild-type or CHY119 cells and subjected to Northern blot analysis with probes for KAR2 mRNAs. (C) Serial dilution-spotting assay as described in (A).
To clarify this discrepancy, I first examined the sequence of the integrated \textit{KAR2} gene in the genome of CHY119. Sequencing data confirmed the entire ORF was correct and the UPRE was indeed replaced with the 20-bp random sequence (Figure 2-1). A Northern blot assay was then carried out using \textit{KAR2} probes to examine if \textit{KAR2} can be expressed properly in the CHY119. Surprisingly, the Northern blot analysis of \textit{KAR2} mRNA revealed that an additional \textit{KAR2} mRNA with a smaller molecular weight appeared in the gel apart from the full-length \textit{KAR2} mRNA (Figure 2-3B). This indicated that the ORF of the endogenous \textit{KAR2} gene in the original MS785 strain was only partially deleted by \textit{LEU2} and that a considerable amount of Kar2p existed as a truncated form which was functionally inactive. These truncated Kar2 proteins are misfolded and likely to cause stress to the cells, thus causing CHY119 cells to grow poorly even though there were no extrinsic stresses. Despite this setback, this result is encouraging as it implied that CHY119 cells were indeed sensitive to ER stress, caused by the accumulation of misfolded proteins within the ER due to their inability in upregulating \textit{KAR2} expression.

To correct this problem, the entire ORF of endogenous \textit{KAR2} gene in the CHY119 strain or the control strain (CHY113) was replaced with a \textit{KanMX} cassette using homologous recombination to produce strains CHY220 and CHY483 respectively. Now, resting CHY220 grew indistinguishably from wild-type as expected (Figure 2-3C). This confirmed that the UPR-uncoupled expression of \textit{KAR2} is sufficient for normal growth in the absence of stress.
2.3.2 The integrated *KAR2* gene with the mutant promoter loses its ability to respond to UPR signals in CHY220 strain

In order to ensure that the transcription of *KAR2* is not upregulated in response to ER stress in CHY220 strain, the mRNA level of *KAR2* in CHY220 was compared to that in wild-type cells when the cells were subjected to ER stress. To this end, CHY220 and wild-type cells were grown in the presence of two well-known extrinsic ER stressors, tunicamycin (Tm) and dithiolthreitol (DTT) to induce the UPR (Cox *et al.*, 1993; Mori *et al.*, 1993; Travers *et al.*, 2000). Tm is an N-linked glycosylation inhibitor that causes misfolding of some glycoproteins while DTT is a reducing agent that disrupts cysteine oxidation. Although the underlying mechanisms in creating ER stress are different for both reagents, these reagents can cause protein misfolding in the ER and trigger the UPR pathway.

These cells were harvested and total RNAs were isolated for a Northern blot assay using probes for *KAR2* mRNAs. As expected, the mRNA level of *KAR2* (normalized to the *ACT1* mRNAs as loading controls) in wild-type cells was much greater (12-fold or 13-fold in the presence of Tm or DTT, respectively) than UPR-deficient ∆ire1 strain, indicating an activation of the UPR in these cells (Figure 2-4A, lane 4 versus lane 6 and quantification data below; figure 2-4B, lane 4 versus lane 6 and quantification data below). The mRNA level of *KAR2* in CHY220 was similar to the level in ∆ire1 strain, confirming that *KAR2* could not be upregulated by the UPR in these cells (Figure 2-4A, lane 5 versus lane 6 and figure 2-4B, lane 5 versus lane 6).
Figure 2-4: Upregulation of Kar2p is uncoupled from the UPR in CHY220 strain. Determination of the level of KAR2 mRNA during the UPR. The UPR was induced in wild-type, ∆ire1 and CHY220 cells by the addition of either 2.5 μg/ml tunicamycin (A) or 10 mM DTT (B) for 1 hour. Total RNAs were harvested before and after treatment, and the relative abundance of KAR2 and ACT1 mRNAs were analyzed by Northern blot analysis. The same blot was probed for KAR2 mRNA, stripped, and then probed for ACT1 mRNA. The KAR2 mRNA level shown in the lower panel of (A) and (B) was quantitated and normalized to ACT1 mRNA level.
2.3.3 UPR functions normally in the CHY220 strain

In order to ensure that only the upregulation of \textit{KAR2} is impaired and the activation of the UPR is normal in the CHY220 strain, a β-galactosidase assay was carried out to examine the expression of a \textit{LacZ} reporter gene downstream of a UPRE element. A plasmid expressing the \textit{LacZ} reporter gene, driven by a crippled \textit{CYC1} core promoter fused with the UPRE, was transformed into W303 and CHY220 cells. In a cell carrying such a reporter plasmid, when Ire1p is activated upon ER stress, it results in the activation of Hac1p, which then binds to the UPRE on the plasmid, thus allowing the expression of \textit{LacZ} (Figure 2-5A) (Cox \textit{et al.}, 1993).

To test this, cells were harvested after the treatment of Tm and the induction of \textit{LacZ} expression from the reporter construct was detected using o-Nitrophenyl-beta-galactopyranoside (ONPG). ONPG is similar to lactose in structure. If β-galactosidase is present, the colorless ONPG is split into galactose and o-nitrophenyl, a yellow compound which can be detected by its absorption at 420 nm. For quantification, the β-galactosidase activity was expressed in Miller units (as \(1000 \times \frac{A_{420}}{[(t_{\text{min}})(V_{\text{ml}}) \times (A_{600})]}\)) (Guarente, 1983). As shown in figure 2-5B, the UPRE-\textit{LacZ} reporter was highly induced in CHY220 cells in the presence of Tm as indicated by the high level of β-galactosidase activity. The level of β-galactosidase activity in CHY220 cells was similar to that in wild-type cells under the same condition, indicating that apart from impairment of \textit{KAR2} upregulation by the UPR, the general UPR pathway is unperturbed in CHY220 cells.
Figure 2-5: Activation of UPR is unperturbed in CHY220 cells.  
(A) A schematic diagram depicting the construct of the UPRE-lacZ reporter gene. This construct contains a crippled CYC1 promoter upstream of LacZ and UPRE serving as a UAS. When Ire1p is activated upon ER stress, the activated Ire1p would cause the activation of Hac1p, which then binds to the UPRE on the plasmid, thus allowing the expression of LacZ gene. (Cox et al., 1993) (B) Wild-type and CHY220 strains containing a single copy of the UPRE-lacZ reporter were assayed for β-galactosidase activity (see Materials and Methods) after cells were treated with 2.5 µg/ml tunicamycin for 1 hour. The data were representative of three independent experiments and error bars reflect standard deviation (SD) of the mean from triplicate analyses.
2.4 Summary

In order to study the contribution of KAR2 upregulation to stress tolerance, I have constructed the strain CHY220 in which KAR2’s regulation is specifically uncoupled from UPR signals. This strain behaves precisely as designed as shown by the data in the previous paragraphs: (1) There is no growth defect in the resting condition, (2) the UPR pathway is normal upon ER stress, and (3) KAR2 gene is now uncoupled from the UPR. With the outcome of these critical control experiments verified, potential misinterpretations due to indirect effects can also be ruled out. The CHY220 strain may now be utilized in further experiments in order to examine the role of Kar2p in stress tolerance.
Chapter 3

The role of the Kar2p chaperone in stress tolerance at single gene analysis

3.1 Introduction

This study aims to examine the contribution of a single gene, \textit{KAR2}, to the stress tolerance. To this end, a yeast strain in which the upregulation of \textit{KAR2} has been uncoupled from the UPR has been generated (CHY220). Characterization of this strain has been carried out in order to ensure it behaved as expected. CHY220 cells grow just as well as wild-type cells and the UPR is normal in these cells. I have also established that \textit{KAR2} is not upregulated by the UPR in CHY220 in the presence of extrinsic ER stressors. The role of Kar2p may now be examined using this strain.

3.2 Elevated levels of Kar2p are required for stress tolerance

In the first instance, I first asked if elevated levels of Kar2p were required for stress tolerance. To answer the question, CHY220 cells were subjected to three forms of ER stress, Tm, DTT, and overexpression of misfolded proteins in the ER. Unlike Tm and DTT which might have pleiotropic effects, overexpression of misfolded proteins is the most direct and specific way of inducing the UPR. For this, the ORF of a misfolded protein, CPY*, was placed behind a tightly regulated \textit{GAL1} promoter (\textit{GAL1-CPY*}) for overexpression.

As shown in figure 3-1A, wild-type and control CHY438 cells carrying the integrated functional \textit{KAR2} gene grew equally well in the presence of Tm and DTT. In
contrast, CHY220 cells were sensitive to Tm but were able to tolerate the challenge by DTT as well as wild-type cells. This is of great interest since DTT leads to protein misfolding by a different mechanism. DTT disrupts cysteine oxidation and the UPR responds by increasing the expression of the oxido-reductases, such as Ero1p (Endoplasmic reticulum oxidoreductin 1p) (Frand and Kaiser, 1998; Pollard et al., 1998), while, Tm causes misfolded proteins to accumulate in the ER by blocking N-glycosylation. This implied that the upregulation of KAR2 by the UPR is not necessary in order for cells to cope with reductive stress caused by DTT. The induction of Ero1p may be sufficient for the tolerance of cells to reductive stress. On the other hand, the elevation of Kar2p levels is required for abrogating the toxicity of accumulated abnormal proteins induced by Tm treatment.

Consistent with this finding, the overexpression of misfolded CPY* was also lethal to CHY220 cells although higher levels of CPY* (two copies of GAL1-CPY* plasmids, labeled as CPY*OE in figures) were required for cells to die (Figure 3-1B). It should be noted that overexpression from two copies of GAL1-CPY* plasmids (CPY*OE) were used as an ER stressor in the subsequent experiments because of its specificity in activating the UPR.
Figure 3-1: KAR2 upregulation is required for ER stress tolerance of accumulated misfolded protein, but not for that of redox stress. 

(A) Equal numbers of wild-type and CH220 cells were spotted as 10-fold serial dilutions on synthetic complete (SC) plate, SC plate containing 0.125 and 0.25 µg/ml Tm, or SC plate containing 1 and 10 mM DTT. Plates were incubated at 30 °C until colonies were formed. (B) Equal numbers of wild-type and CHY220 cells transformed with either one copy (left panel, GAL1-CPY*) or two copies (right panel, CPY*OE) of GAL1-CPY* plasmids were spotted as 10-fold serial dilutions on the plates containing glucose to repress expression or galactose to induce expression.
3.3 Kar2p maintains its essential functions of protein translocation and folding when it becomes limiting under stress

I have established that the elevation of Kar2p levels is required for ER stress tolerance of abnormal proteins. Next, it is not understood how its activation provides this function. The simplest explanation is that the limiting amounts of Kar2p compromised its essential functions of protein translocation and folding. To test this hypothesis, the biogenesis of a model GPI-anchored glycoprotein Gas1p was monitor in CHY220 challenged by overexpression of CPY*. Gas1p is the ideal model protein since its maturation requires nearly every ER protein processing function present including protein translocation, GPI-anchor addition, N-linked glycosylation, O-linked mannosylation, chaperone-mediated folding, and disulfide bond formation. Defects in any of these steps can be detected clearly by alterations in gel mobility in a pulse-chase experiment.

The cells challenged by overexpression of CPY* were metabolically labeled with \(^{35}\text{S}\text{Cys/Met}\) for 5 minutes and chased for 15 minutes. Proteins from the cells and medium were then precipitated by trichloroacetic acid (TCA) prior to Gas1p-specific immunoprecipitation, analyzed by SDS-PAGE and visualized by autoradiography. As shown in figure 3-2A, in wild-type cells overexpressing CPY*, at 0-chase time, most of Gas1p was in the ER from, while a small fraction was in the Golgi/plasma membrane (PM) form (Figure 3-2A, lane 1). After 15 minutes, all of Gas1p appeared to be in the Golgi/PM form (Figure 3-2A, lane 2). CHY220 cells gave a similar pattern for Gas1p biogenesis (Figure 3-2A, compare lanes 3 and 4 to lanes 1 and 2), indicating that Gas1p biogenesis was entirely unperturbed in CHY220 cells under stress. This showed that essential Kar2p functions remained intact in CHY220 cells.
Figure 3-2: CHY220 cells maintain essential functions of Kar2p under stress. (A) Gas1p biogenesis was entirely unperturbed in CHY220 cells under stress. Wild-type and CHY220 cells overexpressing CPY* were pulse-labeled for 5 min with $[^{35}\text{S}]\text{Cys/Met}$ and followed by a cold chase as indicated. Endogenous Gas1p were immunoprecipitated from detergent lysates, separated by SDS-PAGE, and visualized by autoradiography. Positions of ER and Golgi/plasma membrane (Golgi/PM) forms of Gas1p are indicated. (B) The processing of Gas1p (SRP-independent substrate) and DPAP B (SRP-dependent substrate) was normal in CHY220. Wild-type and CHY220 cells overexpressing CPY* were pulse-labeled for 10 min with $[^{35}\text{S}]\text{Cys/Met}$. Endogenous Gas1p and DPAP B were immunoprecipitated from detergent lysates, separated by SDS-PAGE, and visualized by autoradiography. Positions of untranslocated (pre-), ER forms of Gas1p and untranslocated (pre-), mature (m-) forms of DPAP B are indicated.
Next, I sought to further examine the translocation function by checking two distinct translocation pathways which are distinguished by their dependence upon the signal recognition particle (SRP). SRP-dependent and SRP-independent pathways. Gas1p translocation uses the SRP-independent pathway by which cytosolic Gas1p polypeptides (65 kDa) are targeted to the translocon and subsequently translocated into ER where they are modified to form 105 kDa GPI-anchored precursors with N- and O-linked oligosaccharides. As shown in figure 3-2B (left panel), after a 10-minute pulse, an untranslocated form of Gas1p (preGas1p) was observed in the sec63-201 mutant specifically defective in SRP-independent pathway (left panel, lane1). In contrast, only the translocated form of Gas1p (ER Gas1p) was found in wild-type cells (left panel, lane2). Similarly, in CHY220 cells, only translocated Gas1p was detected, indicating that the translocation of Gas1p was normal in CHY220 cells under stress (left panel, lane3).

For the SRP-dependent pathway, I examined the biogenesis of DPAP B, a vacuolar membrane protein whose translocation can also be monitored by a shift in gel mobility due to N-linked glycosylation in the ER lumen. As shown in figure 3-2B (right panel), unlike a fraction of the untranslocated form of DPAP B (preDPAP B) seen in sec65-1 mutant, a strain specifically defective in SRP-dependent pathway, only the translocated DPAP B (mDPAP B) appeared in both wild-type and CHY220 cells (right panel, compare lanes 2 and 3 to 1) Taken together, the data suggested that both SRP-independent and SRP-dependent-pathway were functional in CHY220 cells even under stress conditions.

To date, the data have shown conclusively that protein translocation (ER Gas1p and DPAP B in figure 3-2B) and vesicular trafficking of normal cargo proteins
Golgi/PM Gas1p in figure 3-2A) are unaffected when Kar2p is limiting. The latter result implied that folding is also unaffected since unfolded Gas1p would not be transported out of the ER (Fujita et al., 2006). In order to examine the effects of limiting Kar2p on protein folding directly, experiments that would assess the folding states of proteins kinetically were performed. Most secretory proteins form intramolecular disulfide bonds. These bonds are precise and form only when the protein is properly folded. Again, Gas1p was used as a model. Gas1p has 14 cysteines that form 6 disulfide bonds. When the protein is properly folded, the molecule is more resistant to alkylation because only two free sulfhydryls exist in the folded conformation. In contrast, unfolded Gas1p is highly sensitive to alkylation (Sayeed and Ng, unpublished results) and can be detected by smears on a polyacrylamide gel when they are modified by the high molecular weight alkylation reagent, maleimide-PEG (MalPEG5000).

Cells were pulse-labeled with $^{35}$S]Cys/Met for 5 minutes, chased for 40 minutes and proteins from the cells and medium were precipitated by TCA and followed by the treatment of MalPEG5000 prior to Gas1p-specific immunoprecipitation. Subsequently, proteins were analyzed by SDS-PAGE and visualized by autoradiography. As shown in figure 3-3, in wild-type cells, the majority of Gas1p’s ER form seen at 0-chase time (Figure 3-3, lane 5) was modified by MalPEG5000 and the heterogeneous population of MalPEG5000-bound Gas1p migrated as a long smear on the gel (Figure 3-3, lane 6), whereas the Golgi form (Figure 3-3, lane 7) produced a distinct band in the presence of MalPEG5000 (Figure 3-3, lane 8) with a molecular weight which was the same as the unmodified Golgi form (Figure 3-3, compare lanes 8 to 7). As a control, the pulse-chase experiment was performed in parallel with wild-type cells under reductive conditions.
(+DTT) and the TCA precipitate pellets were treated with MalPEG5000 prior to Gas1p-specific immunoprecipitation. Because disulfide bonds could not be formed under reductive conditions, MalPEG500 modified every free sulfhydryls that exist in Gas1p to give rise to an apparent high molecular weight Gas1 protein (~195 kDa) (Figure 3-3, lanes 2 and 4).
Figure 3-3: Protein folding is unaffected in CHY220 cells under stress.

(A) Wild-type and CHY220 cells overexpressing CPY* were pulse labeled for 5 min with $[^{35}S]$Cys/Met and proteins were precipitated by TCA. The TCA pellets were washed with cold acetone to remove residual TCA and then resuspended by boiling for 10 min and vortexing often MalPEG5000 buffer (50 µl/OD$_{600}$) containing 5mM of MalPEG5000, 100mM Tris [pH 7.4] and 2% SDS. Samples were incubated on ice for 1 hour and lysates obtained were used for immunoprecipitation with anti-Gas1p antiserum.
Taken together, wild-type cells in figure 3-3 displayed standard folding kinetics of Gas1p: most Gas1p proteins in the ER form are unfolded and protein folding has been achieved in the ER prior to transport to the Golgi such that the folded Golgi form was resistant to the reagent. The same results were observed in wild-type cells challenged with CPY* overexpression (Figure 3-3, lanes 13 to 16). Interestingly, the capacity of protein folding was also unaffected in CHY220 under identical stress conditions (Figure 3-3, lanes 21 to 24), which strongly supported the previous observation that protein folding is unperturbed when Kar2p is limiting. Together with the earlier results, these data indicated that Kar2p maintains its two essential functions of protein translocation and protein folding when it becomes limiting under stress.

3.4 The primary function of elevated Kar2p levels in stress tolerance is to clear the ER of aberrant proteins.

From the previous experiments, I have ruled out the possibility that essential functions are compromised when Kar2p is limiting. Thus, the data implied that elevating Kar2p plays a direct and active role in stress tolerance. What is this role? To answer this question, Kar2p’s function in ERAD pathway was examined. Kar2p functions in ERAD by binding substrates and maintaining their solubility (Nishikawa et al., 2001). To determine whether ERAD function is compromised, CPY* turnover was analyzed following induction by the metabolic pulse-chase assay.

The cells challenged by overexpression of CPY* were metabolically pulse labeled with $[^{35}\text{S}]\text{Cys/Met}$ for 10 minutes and followed by a cold chase for various time points. Proteins were precipitated and analyzed by the same method described earlier except that
quantification of data were performed after digestion with endoglycosidase H (EndoH), an enzyme that specifically cleaves N-linked oligosaccharides. This is necessary because as previously observed for CPY* overexpressed in wild-type cells where a diffuse tail of CPY* radioactivity appears due to the extensive modification of core carbohydrates when transported to Golgi apparatus (Spear and Ng, 2003), the pattern was also seen in other testing strains overexpressing CPY* in the pulse-chase assay (data not shown). Thus, after immunoprecipitation was performed using anti-HA antibodies (for HA-tagged CPY*), the immunoprecipitated samples were digested with EndoH, which allows the CPY* population to collapse into a single species prior to SDS-PAGE analysis.

As shown in figure 3-4A, CPY* turnover was strongly compromised in CHY220 cells when compared to its turnover in wild-type cells. This was significant since turnover of excess substrate in normal cells continued unimpeded even when ERAD was compromised (Figure 3-4A, an ERAD deficient strain, Δcue1 versus wild-type). Under such conditions, the UPR activates an alternative ER-to-vacuole overflow pathway for transporting misfolded proteins out of the ER to the vacuoles to be degraded (Spear and Ng, 2003). When compared to wild-type cells, the stabilized portion of CPY* observed in a Δpep4 stain, which is deficient in proteolytic activity in the vacuole, represented the population of CPY* that utilized the ER-to-vacuole overflow pathway for its degradation (Figure 3-4A, a Δpep4 strain versus wild-type) because CPY* expression levels under this condition are sufficient to saturate ERAD and excess CPY* is transported to the vacuole to be degraded by vacuolar proteases (Spear and Ng, 2003). In comparison, CPY* was more stabilized in CHY220 cells than that in Δcue1 or Δpep4 cells, which
implied that both ERAD and ER-to-vacuole overflow pathways in CHY220 cells are
compromised. Moreover, I also found that the majority of CPY* was dependent on the
overflow pathway in CHY220 cells under this condition as the level of CPY*
stabilization in CHY220 cells was similar to what was observed in a double mutant
containing upre<sup>d</sup>-<i>KAR2</i> and <i>Δ</i><i>pep4</i> alleles (Figure 3-4A, CHY256).

Given that both pathways are impaired in CHY220 cells, I envisioned a condition
that would lead to extensive accumulation of misfolded proteins in the ER. To test this
directly, wild-type and CHY220 cells were induced for CPY* overexpression from <i>GAL1</i>
promoter in the presence of galactose, further synthesis was terminated by adding glucose,
and the fate of the protein was examined by CPY* expression shut-off assay (to check the
levels of CPY* accumulated in cells) and by indirect immunofluorescence (to check the
localization of accumulated CPY*). In the CPY* expression shut-off assay (Figure 3-4B),
CPY* was found to be rapidly cleared from wild-type cells. In CHY220 cells, however,
CPY* persisted even after hours of glucose shut-off. Most importantly, the indirect
immunofluorescence showed the accumulated proteins were observed in the ER as
demonstrated by its colocalization of the ER marker Kar2p (Figure 3-5). Taken together,
these data suggested that the primary function of elevating Kar2p levels in stress
tolerance is to clear the ER of aberrant proteins by the ERAD pathway and to export them
out of the ER.
Figure 3-4: The clearance of CPY* is compromised in CHY220 cells.

(A) Wild-type, Δcue1 (an ERAD deficient strain), Δpep4 (required for activation of most vacuolar protease), CHY220, and CHY256 (upr−KAR2, Δpep4) cells overexpressing CPY* were pulse labeled for 10 min with [35S]Cys/Met and followed by a cold chase for the duration indicated. CPY* was immunoprecipitated from detergent lysates, deglycosylated using endoglycosidase (EndoH), separated by SDS-PAGE, and visualized by autoradiography. Plots reflect three independent experiments with the SD of the mean indicated. (B) Wild-type and CHY220 cells were induced for CPY* overexpression in the presence of galactose, further synthesis was terminated by adding 2% glucose as indicated, and the level of CPY* was examined by Western blot analysis.
Figure 3-5: CPY* is accumulated in the ER of CHY220 cells. Wild-type and CHY220 cells were grown at 30°C in the media containing 2% galactose to induce CPY* overexpression for 6 hours and synthesis was terminated by adding 2% glucose as indicated. Cells fixed and permeabilized on glass slides were decorated with mouse α-HA mAb (CPY*) and α-Kar2p antibodies. Antibody complexes were bound with AlexaFluor 488 goat α-mouse and AlexaFluor 594 goat α-rabbit antibodies for visualization (CPY* in the green channel; Kar2p in the red channel).
Although data from the pulse-chase experiment and CPY* expression shut-off assay suggested CHY220 cells have a defect in transporting CPY* out of the ER, I sought to directly assess this impairment by counting the percentage of cells in a population able to transport CPY* to the vacuole. Thus, indirect immunofluorescence assay was carried out to visualize vacuolar localization of CPY* in wild-type and CHY220 cells under pep4 knockout background. The number of cells with vacuolar staining were counted and expressed as a percentage (%) of total cells counted. As shown in figure 3-6A, staining of CPY* was found at sites distinct from the ER (white arrows in ∆pep4 panel) in the majority of the cells (68.9%) in the population of ∆pep4 strain. I determined these sites to be vacuoles because non-ER CPY* colocalized with the vacuolar marker, GFP-tagged alkaline phosphatase (ALP-GFP) (Figure 3-6B). However, only 35% of CHY220 cells displayed vacuolar localization of CPY* (Figure 3-6A, white arrows in CHY256 panel), indicating that the elevated levels of Kar2p indeed play a role in assisting misfolded proteins to be transported out of the ER. Moreover, it is noteworthy, that for those wild-type cells displaying vacuolar localization of CPY*, the CPY* was concentrated in the vacuole, with little staining in the ER. In contrast, a decrease vacuole staining was present in CHY220 cells displaying vacuolar localization of CPY* (Figure 3-6A, compare ∆pep4 cells with white arrows to CHY256 cells with white arrows). This data also supported the idea that ER-to-vacuole overflow pathways in CHY220 cells are impaired.
Figure 3-6: CHY220 has a defect in transporting excess CPY* out of the ER.

(A) Δpep4 and CHY256 cells were grown at 30°C in the media containing 2% galactose to induce CPY* overexpression for 8 hours. Cells were analyzed by indirect immunofluorescence assays as in figure 3-5. White arrows indicate vacuole staining of CPY*. The number of cells with vacuolar staining were counted and expressed as a percentage (%) of total cells counted. At least 250 cells from five separate microscopic fields were counted for both strains. Results were expressed as the mean ± SD of three independent experiments. (B) Δpep4 cells carrying plasmids overexpressing CPY* were grown as in A. Cells were probed using α-HA (CPY*) and α-GFP (ALP-GFP) followed by Alexa Fluor594 goat α-mouse and AlexaFluor 488 goat α-rabbit antibodies (CPY* in the red channel; ALP-GFP in the green channel).
3.5 Summary

Studying the CHY220 strain, I discovered that Kar2p unexpectedly maintains its essential functions of protein translocation and folding when it becomes limiting under stress. Using this mechanism, it can temporarily dispense with its role in ridding misfolded proteins to facilitate the UPR. With the UPR deployed, the enhancement of quality control functions restores ER homeostasis. I also found that the primary function of elevating Kar2p levels in stress tolerance is to clear the ER of aberrant proteins and that the clearance of accumulated abnormal proteins from the ER is vital for cell survival under stress because, with unchecked accumulation of misfolded proteins in the ER under prolonged stress cells eventually die.
Chapter 4

A detailed understanding of Kar2p’s roles and their biological significance in stress tolerance

4.1 Introduction

In the previous chapter, the contributions of Kar2p to stress tolerance were analyzed using the system which was set up. I found that the primary function of elevating Kar2p levels in stress tolerance was to clear the ER of aberrant proteins. Furthermore, there were two novel findings worth noting. The first was the demonstration that simple accumulation of abnormal proteins in the ER is toxic. In contrast, the accumulation of equal or greater amounts of CPY* in the vacuole has no effect on cell growth (Spear and Ng, 2003). The second discovery revealed that Kar2p’s functions are prioritized according to their importance to the cell. Surprisingly, essential Kar2p functions of protein translocation and protein folding are completely unperturbed when Kar2p is limiting under stress. A ‘molecular triage’ mechanism for protein function, to our knowledge, has not been reported before. For this reason, the goals of chapter 4 have been broadened to obtain a greater detailed understanding of this mechanism and its biological significance.

4.2 The loss of Kar2p’s essential functions is of immediate detriment while ER stress can be temporarily tolerated when Kar2p is limiting

At first glance, the molecular triage mechanism did not make much sense. Although the importance of the essential functions is obvious, under stress, the data
showed that the clearance of misfolded proteins also becomes essential. In this view, why should the cells give housekeeping functions more weight? To resolve the conundrum, the stress response was examined in greater detail. It is well established that the UPR is required for stress tolerance. However, a lag period exists between the moment stress is elicited and when a full response can be mounted. During that period, I hypothesized that cells must preserve essential functions during the imbalances caused by the stress are tolerated until the response restores homeostasis.

To test the hypothesis, a strain that carries the temperature sensitive kar2-159 allele was examined. Unlike the upre^d-KAR2 allele, kar2-159 disrupts essential functions of Kar2p at the non-permissive temperature of 37 °C (Vogel et al., 1990). Loss of fitness after a transient loss of function would confirm my hypothesis. Indeed, that was precisely what was observed. After temperature shift to 37 °C for 1 hour, over 90% of cells died and few cells can survive beyond 2 hours (Figure 4-1A). In contrast, CHY220 cells recovered fully after exposure to CPY* stress for the same durations. The tolerance was robust enough even to survive 6 hours of stress (Figure 4-1B). These data demonstrated that the loss of Kar2p’s essential functions is of immediate detriment while ER stress can temporarily be tolerated when Kar2p is limiting. It should be noted that prolonged stress is lethal to CHY220 cells. Taken together, these experiments provided a physiological basis for why a molecular triage mechanism is an important facet of regulating Kar2p function.
Figure 4-1: The loss of Kar2p’s essential functions is of immediate detriment.
(A) Equal numbers of wild-type and kar2-159 cells shifted to 37°C (non-permissive temperature) at indicated time were spotted as 10-fold serial dilutions on the SC plate and incubated at 25°C (permissive temperature) for recovery. (B) Wild-type and CHY220 cells were induced for CPY* overexpression for the indicated time. After induction, equal numbers of wild-type and CHY220 cells were spotted as 10-fold serial dilutions on plates containing 2% glucose to terminate CPY* expression and incubated at 30°C for recovery.
4.3 Mechanism of Kar2p functional prioritization

Next, I sought to examine the underlying mechanism for Kar2p functional prioritization. Kar2p performs its different roles according to how it is recruited. In protein translocation, Kar2p is recruited to the translocon pore complex via its binding to the DnaJ homology domain of Sec63p. In ERAD, Kar2p binds directly to misfolded protein substrates. I proposed a model of tiered recruitment to explain the molecular triage mechanism. I hypothesized that under Kar2p-limiting condition, the association of Kar2p to Sec63p remains normal while recruitment to CPY* is reduced.

To examine this, Kar2p’s association with Sec63p or CPY* was analyzed by using co-immunoprecipitation assay. Cells challenged by overexpression of CPY* were harvested, lysed by mildly beating with zirconium beads using mini-bead beater and subjected to native immunoprecipitation with antisera against HA-tagged CPY*. The immunoprecipitated complex was subsequently analyzed by immunolotting against either Kar2p-specific or Sec63p-specific antibodies. For this single experiment, I transformed a plasmid expressing CPY* under a strong constitutive GPD promoter (GPD-CPY*) to induce ER stress in cells instead of using two copies of GAL1-CPY* for the first trial. An interaction between Kar2p and misfolded CPY* was detected in both wild-type and CHY220 cells under stress although the level of CPY* expressed by GPD-CPY* was lower than by GAL1-CPY* (Figure 4-2A).
Figure 4-2: The accumulation of excess CPY* in CHY220 cells is attributable to the reduced interaction between Kar2p and CPY*.

(A) Wild-type and CHY220 cells were transformed with empty vector or a plasmid overexpressing CPY* under GPD promoter. Total cell lysates were solubilized with 1% Triton X-100, cleared and immunoprecipitated with anti-HA resin. Bound proteins were eluted by boiling in SDS loading buffer, resolved by SDS-PAGE and followed by Western blot analysis with the indicated antibodies. (B) Microsomes were prepared from wild-type cells overexpressing CPY*, CHY220 cells overexpressing CPY* and Δire1 cells overexpressing CPY*. Membranes were solubilized in 1% Triton X-100 and centrifuged at 1000,000 g to separate pellet and supernatant fractions. Detergent-soluble (S), detergent-insoluble (P), and total (T) fractions were resolved by SDS-PAGE, and followed by Western blot analysis to detect CPY* and Sec61p. The extent of membrane solubilization was determined by blots for Sec61p, an integral membrane protein.
As shown in figure 4-2A, the amount of Kar2p that pulled down with CPY* was indeed reduced in CHY 220 cells when compared to wild-type cells (Figure 4-2A, compare lanes 4 to 2), indicating that the accumulation of excess CPY* in CHY220 cells is at least partly attributable to the reduced interaction between Kar2p and CPY*.

Furthermore, because the interaction of Kar2p with misfolded proteins is believed to maintain the solubility of misfolded proteins for ERAD, I wondered whether the reduced association of Kar2p with CPY* would contribute to more CPY* aggregates in CHY220 cells as compared to wild-type cells under the same condition.

To test this, microsomal membranes were prepared from wild-type and CHY220 cells overexpressing CPY*. The membranes were solubilized in nonionic detergent and followed by centrifugation in order to separate large protein aggregates from soluble proteins remaining in the supernatant. Detergent-insoluble (pellet) and detergent-soluble (supernatant) fractions were collected for Western blot analysis. To control for the procedure, Δire1 cells overexpressing CPY* were used. It has been shown that CPY* forms severe aggregates in Δire1 cells due to limiting ER chaperones (Spear and Ng, 2003). Indeed, in the experiment, CPY* from Δire1 cells were predominantly found in the pellet fraction, indicating that CPY* existed mainly as aggregates in Δire1 cells (Figure 4-2B, lane 9). In every case, Sec61p, the ER integral membrane protein, was found in the soluble fraction, indicating that the ER membrane was completely solubilized. As shown in figure 4-2B, under the same stress condition, the amount of CPY* aggregates in the pellet fraction of CHY220 cells was significantly increased when compared to that in the pellet fraction of wild-type cells (Figure 4-2B, compare lanes 6 to
3), indicating that CPY* was more likely to form aggregates in CHY220 cells. The data correlated well with the results obtained in figure 4-2A, in which CHY220 cells displayed reduced interaction between Kar2p and CPY*. This showed that reduced interaction between Kar2p and CPY* resulted in greater aggregation of CPY* in CHY220 cells. In addition, the data also supported the previous observation that the ERAD pathway is compromised when Kar2p is limiting since protein solubility is an important prerequisite for ERAD (Nishikawa et al., 2001).

Unfortunately, the association between Kar2p and Sec63p in the CHY220 strain could not be detected by the same coimmunoprecipitation assay. Currently, I am testing a variety of buffer conditions for coimmunoprecipitation experiment or trying other approaches such as chemical cross-linking to solve this problem. The use of a chemical crosslinker would allow the detection of any transient interaction between Kar2p and Sec63p.

4.4 Screening for high-copy suppressors

To better characterize physiological functions of UPR-upregulated Kar2p, we carried out a high-copy suppressor screen to identify genes that can bypass the defect imposed by limiting Kar2p. For this, CHY220 cells containing plasmids overexpressing CPY* were transformed with multicopy genomic library constructed in YEp13 plasmid and grown on galactose to induce misfolded CPY* expression. 27 viable colonies were isolated. 25 colonies were found to encode galactose regulatory genes that simply decreased the level of misfolded proteins from the GAL1 promoter and were discarded.
To date, two positive clones that did not diminish CPY* expression were identified (Figure 4-3, g-25 and g-27). The recovered plasmids were characterized by DNA sequencing analysis and the sequences obtained were then aligned to identify corresponding regions in the yeast genomic database (www.yeastgenome.org). Figure 4-4 shows the mapping region of each library insert and all genes within mapping regions are listed below. In future, restriction mapping of these library inserts will be performed to identify the ORF/gene that can bypass the defect imposed by limiting Kar2p.
Figure 4-3: Two high-copy suppressors were identified which bypassed the defect imposed by limiting Kar2p. CHY220 cells bearing plasmids overexpressing CPY* were transformed with multicopy genomic libraries constructed in YEp13 and grown on galactose to induce misfolded CPY* expression. Two positive clones (g-25 and g-27) were identified and the recovered plasmids were characterized by DNA sequencing analysis.
Figure 4-4: Mapping regions of library inserts from two positive clones.
Library g-25 mapped to chromosome XV, containing the following genes:

1. The C-terminal region of RPL20B (corresponding to aa 226-315)
   
   **Function:** Protein component of the large (60S) ribosomal subunit nearly identical to Rpl20Ap and has similarity to rat L18a ribosomal protein

2. The N-terminal region of HSD1/YOR311C (corresponding to aa 1-232)
   
   **Function:** Endoplasmic reticulum (ER)-resident membrane protein whose overproduction induces enlargement of ER-like membrane structures and suppresses a temperature-sensitive sly1 mutation

Library g-27 mapped to chromosome XII, containing the following genes:

1. RCK2/YLR248W
   
   **Function:** Protein kinase involved in the response to oxidative and osmotic stress; identified as suppressor of *S. pombe* cell cycle checkpoint mutations

2. YEF3/YLR249W
   
   **Function:** Translational elongation factor 3 which stimulates the binding of aminoacyl-tRNA (AA-tRNA) to ribosomes by releasing EF-1 alpha from the ribosomal complex

3. The C-terminal region of SSP120/YLR250W (corresponding to aa 1-172)
   
   **Function:** Protein of unknown function
Out of the genes found in the insert of library g-25, \textit{HSD1} is the likely candidate, as judged by its protein localization and function. \textit{HSD1} encodes an ER-resident membrane protein. Although Hsd1p’s specific cellular function has not been determined, it has been shown that the overexpression of Hsd1p induces the enlargement of ER-like membrane structure and suppresses temperature sensitive \textit{sly1} mutant that arrests vesicular transport from the ER to Golgi compartments at the non-permissive temperature (Kosodo \textit{et al.}, 2001). This observation provides clues as to how Hsd1p might overcome the growth defect of limiting Kar2p. Most likely, it is due to its ability in expanding the ER membrane content when overexpressed. This is an indirect effect of overexpression of a membrane protein rather than a direct effect of its function. However, the result is instructive as it indicated that the growth defect of CHY220 is due to the limited capacity of the ER. In the future, the role of Hsd1p as a high-copy suppressor will be further analyzed in detail to ascertain possible functions of Hsd1p in tolerating ER stress.

As for the insert of library g-27, it is hard to predict which gene is the candidate based on the known cellular positions and functions of those genes unless restriction mapping is performed on the insert to identify the gene whose overexpression can bypass the growth defect of CHY220 cells challenged by ER stress. There are two full length-genes expressed in the insert. One is \textit{RCK2} gene which encodes a cytosolic protein kinase involved in the response to oxidative and osmotic stress and another is \textit{REF3} gene encoding the translational elongation factor 3 which stimulates the binding of aminoacyl-tRNA (AA-tRNA) to ribosomes by releasing EF-1 alpha from the ribosomal complex. More likely, \textit{RCK2} gene would be the candidate and if it is the case, the result is very
intriguing because this may imply a cross-talk between two different stress signaling pathways.

4.5 Summary

In this chapter, my data indicated that the loss of Kar2p’s essential functions is of immediate detriment, thus providing a physiological basis for the necessity for Kar2p to prioritize its multiple functions in such a way that essential functions are maintained when it is limiting in the cell. Although a clear mechanism to explain this phenomenon has not been clarified, the reduced recruitment of Kar2p to CPY* can account for impaired degradation of CPY* under this circumstance. Additionally, a screen to identify genes that can bypass the defect imposed by limiting Kar2p was carried out. Unfortunately, no promising candidate was found in this screen. It might reflect that the loss of Kar2p upregulation cannot be compensated by the overexpression of a single gene. However, *HSD1* gene identified provided us with the instructive information; *HSD1*’s function in expanding ER membrane indicates that the growth defect of CHY220 is due to the limited capacity of the ER.
Chapter 5

Cell death by the accumulation of misfolded proteins is not by loss of cell cycle regulation

5.1 Introduction

Initial experiments showed that the inability to upregulate Kar2p expression during stress results in abundant amounts of misfolded proteins accumulated in the ER (Figures 3-4 and 3-5). Left uncleared in the ER, aberrant proteins could be dire to cells as demonstrated by the growth defect observed in CHY220 cells overexpressing misfolded CPY* (Figure 3-1). Why would this situation cause cell death?

To answer this question, I first wanted to examine if CHY220 cells escape any cell cycle regulation upon ER stress. This is because other forms of stress such as oxidative and heat stress do trigger the activation of cell cycle blocks in yeast cells as a transient protection so as to allow time for the damage to be repaired (Flattery-O’Brien and Dawes, 1998; Jenkins and Hannun, 2001). If that is also the case for ER stress, loss of this checkpoint in CHY220 could jeopardize cell viability.

In the case of mammalian cells, ER stress does trigger a checkpoint in G1 phase and the arrest is thought to provide cells with a time window to adapt and survive. This protective response is mediated by one of proximal effectors in mammalian UPR, PERK, which is an ER transmembrane protein kinase. The major function of PERK upon ER stress is to globally repress protein translation via phosphorylating the α subunit of eukaryotic translation initiation factor 2 (eIF2α), which leads to its inactivation and the reduction of translation initiation (Harding et al., 2000; Harding et al., 1999). More
recently, Brewer and Diehl further linked the protein phosphorylation pathway with the UPR-induced G1 cell cycle arrest (Brewer and Diehl, 2000). They showed that the activation of PERK also mediates translational repression of cyclin D1, promoting cell cycle arrest.

Although *S. cerevisiae* does not include a translational repression mechanism to tolerate ER stress (Spear and Ng, 2003), it would still be worthwhile to check if a similar strategy is used in yeast. With these reasons, I would like to compare the nature and kinetics of cell cycle progression in ER-stress imposed CHY220 cells to those in wild-type cells by flow cytometric analysis.

5.2 CHY220 cells under ER stress displays a similar pattern of cell cycle progression with wild-type cells

To assess cell cycle progression in CHY220 and wild-type cells upon ER stress, cells were first treated with two different concentrations of Tm, harvested at specified intervals, and analyzed by flow cytometry (Figure 5-1: 0.5 µg/ml Tm, figure 5-2: 1 µg/ml Tm). As shown in figure 5-1 and figure 5-2, following the addition of Tm, the peak corresponding to the presynthetic DNA content (1C) was considerably reduced in wild-type cells (Figure 5-1, compare b to a and figure 5-2, compare b to a) and after 4 hours of exposure to the drug, most of the cells were in the G2+M phase of cell cycle (2C) (Figure 5-1, c and figure 5-2, c). In addition, after 2 hours of Tm addition, a small percentage of cells began to acquire high amounts of DNA content (3C or 4C), which represents the process of cytokinesis in yeast cell cycle. These data indicated that Tm delayed cell cycle
progression at G2/M phase and also cytokinesis, which was consistent with previous studies (Bicknell et al., 2007; Vai et al., 1987). CHY220 cells displayed similar delay patterns in cell cycle progression to wild-type cells although both delays seemed to appear later at 4 hours of Tm addition. These results suggested that cell cycle regulation in CHY220 cells was normal during ER stress caused by Tm treatment.
Figure 5-1: CHY220 cells do not lose any cell cycle regulation in response to ER stress caused by Tm.

DNA content analysis of Tm-treated wild-type and CHY220 cells by using flow cytometry. Cells were treated with 0.5 µg/ml of Tm for the indicated time and analyzed as described in ‘materials and methods’. Histograms of fluorescence intensity versus cell numbers were shown. The first peak (indicated as 1C) represents prereplicated cells and the second peak (2C) represents replicated cells. The appearance of 3C and 4C cells is represented by a third and a fourth peak.
Figure 5-2: CHY220 cells do not lose any cell cycle regulation in response to ER stress caused by Tm.

DNA content analysis of Tm-treated wild-type and CHY220 cells by using flow cytometry. Cells were analyzed as described in figure 5-1, except for the addition of 1 µg/ml Tm to cells.
To further confirm that cell cycle progression is still unaffected in CHY220 cells during ER stress, I examined the effects of the overexpression of CPY* on the kinetics of cell cycle in wild-type and CHY220 cells. As shown in figure 5-3 (wild-type cells) and figure 5-4 (CHY220 cells), the cell cycle kinetics in CHY220 cells was similar to that in wild-type cells during the course of CPY* overexpression. Taken together, the data indicated that there were no changes in the regulation of cell cycle progression in CHY220 cells under the experimental conditions of ER stress utilized.
Figure 5-3: CHY220 cells do not lose any cell cycle regulation in response to ER stress caused by overexpression of CPY* (compared to figure 5-4). DNA content analysis of wild-type cells nonexpressing and overexpressing CPY* by flow cytometry. Cells were induced for CPY* overexpression for the indicated time and followed by analysis described in figure 5-1.
Figure 5-4: CHY220 cells do not lose any cell cycle regulation in response to ER stress caused by overexpression of CPY* (compared to figure 5-3). DNA content analysis of CHY220 cells nonexpressing and overexpressing CPY* by flow cytometry. Cells were induced for CPY* overexpression for the indicated time and followed by analysis described in figure 5-1.
5.3 Summary

In this chapter, the cell cycle regulation of CHY220 cells was examined to determine the cause of death for CHY220 cells under prolonged ER stress. For this, the cell cycle profiles of CHY220 cells exposed to two different ER stressors were examined by flow cytometric analysis and compared to wild-type cells under the same conditions. Unfortunately, no difference was detected between CHY220 and wild-type cells. Clearly, the cause of cell death when Kar2p is limiting under ER stress for prolonged periods of time is not due to the loss of cell cycle control.
Chapter 6

The importance of ridding the cells of misfolded proteins from the ER

6.1 Introduction

Countering the toxicity caused by the accumulation of misfolded proteins in the ER is vital for cell survival according to data obtained in understanding the roles of KAR2 upregulation in stress tolerance (Chapter 3). One new finding is that in the mutant defective in regulating KAR2 by the UPR (CHY220), both ERAD and the vacuolar pathways are impaired, causing significant amounts of misfolded CPY* to accumulate in the ER upon ER stress. Without the ability to clear CPY* efficiently from the ER, cells cannot survive. This phenotype is consistent with what was observed in \( \Deltaerv29 \) cells.

Erv29p is a yeast ER export receptor. It has been shown that Erv29p mediates the trafficking of misfolded CPY* from the ER to the Golgi through COPII-dependent export (Caldwell et al., 2001; Kincaid and Cooper, 2007; Spear and Ng, 2003) and that ER export is required for the efficient clearance of CPY* by ERAD (Vashist et al., 2001). Thus, in \( \Deltaerv29 \) cells, both ERAD and the vacuolar overflow pathways are blocked.

Just like CHY220 cells, \( \Deltaerv29 \) cells die when CPY* is overexpressed (Spear and Ng, 2003). In contrast, blocking ERAD or vacuolar degradation alone has no effect on cell growth under identical conditions as observed in ERAD-deficient cells or cells defective in the activation of vacuolar protease (Spear and Ng, unpublished data). Based on these results, I wondered if the ability to reduce the amounts of ER-misfolded proteins
by any means available (for CPY*, either by the ERAD or the ER-to-vacuole pathway) is essential for cells to tolerate ER stress.

Although previous studies performed in ∆erv29 (Spear and Ng, 2003) have provided correlated support for the importance of removing misfolded proteins from the ER, considering that Erv29p is responsible for exporting unknown numbers of folded proteins from the ER to their sites of function, one cannot rule out the possibility that the cell death observed in ∆erv29 cells overexpressing CPY* is due to an indirect effect of a more global disruption to the export process. In other words, the death phenotype could be the result of so-called ER overload, which is caused by the general accumulation of proteins (either misfolded or folded ones) in the ER and not due to a specific effect of the accumulation of misfolded proteins in the ER.

Therefore, in this study, I sought to directly examine whether the accumulation of aberrant proteins in the ER generate cellular toxicity by using wild-type cells. To do so, a condition where only misfolded proteins are accumulated in the ER of wild-type cells would be created first and the impact on wild-type cells would be evaluated under this circumstance. The observation of the loss of cell fitness would indicate the toxicity of ER-accumulated misfolded proteins and the importance of clearance of misfolded proteins in allowing cells to survive the resultant stress.

**6.2 The removal of misfolded proteins from the ER is essential for stress tolerance**

The degradation of CPY* being an ERAD substrate, should be mediated by the ERAD pathway when it is expressed at low levels under the threshold of ERAD
machinery. However, when misfolded CPY* is overexpressed and the ERAD machinery is saturated, excess amount of CPY* has to be exported out of the ER and transported to the vacuole for degradation (Spear and Ng, 2003). In order to generate a condition where misfolded CPY* can be accumulated in the ER, I first looked for a possible CPY* mutant variant which is incapable of leaving the ER because overexpression of such an ‘export-defective’ CPY* variant would result in the saturation of the ERAD pathway and the accumulation of excess CPY* due to its inability in exiting the ER.

From studies of Erv29p function in the export of the soluble cargo protein, glyco-pro-α-factor (gpαf), it was shown that an export signal within the pro-region of gpαf is required for its binding to Erv29p and subsequent packaging into COPII-derived vesicles (Otte and Barlowe, 2004). Given that the transport of CPY* from the ER to the Golgi is also dependent on Erv29p and COPII-coated vesicles, it is evident that a potential export signal existing in the structure of CPY* mediates its export although it has not been characterized. Thus, based on these studies, my strategy to find an ‘export-defective’ CPY* variant is to take advantage of several CPY* mutant variants that exist in the lab and examine the effects of their overexpression on wild-type cells. I would screen for CPY* variants whose overexpression promote cell death first and then further explore if the cell death phenotype results from a transport defect of CPY* variants.

CPY* is a mutant version (G255R) of the endogenous vacuolar protease, carboxypeptidase Y (CPY). It contains four N-linked glycans at positions 124, 198, 279, and 479 (denoted by the upper case letters A, B, C, and D respectively in figure 6-1). Four different CPY* mutant variants, ABCd-CPY* (glycosylated at sites A, B, and C, lower case letters designate mutant sites), abcD-CPY* (glycosylated only at site D),
abcd-CPY*-D1 (abcd-CPY* lacking amino acids region 25-117), abcd-CPY*-D2 (abcd-CPY* lacking amino acids region 116-206) were randomly selected for the first trial. These four CPY* variants, like CPY*, are still ERAD substrates [(Spear and Ng, 2005) and Kanehara and Ng, unpublished data] so their overexpression under the control of GAL1 promoter in wild-type cells would be suitable for testing the idea.

As shown in figure 6-1A, cells overexpressing various variants displayed different growth sensitivity. When compared to cells overexpressing CPY* (as a control in the experiment), cells overexpressing ABCd-CPY* grew similarly (compare rows 2 to 1). However, those overexpressing abcd-CPY*, abcd-CPY*-D1, and abcd-CPY*-D2 showed a growth defect (compare rows 4, 5, 6 to 3). This indicates that overexpression of abcd-CPY*, abcd-CPY*-D1, and abcd-CPY*-D2 but not ABCd-CPY* caused the cells to become sick. One possible reason for this, as suspected, is that these variants are defective in getting out of the ER due to the loss of recognizable export signals in their structure so that when they are overexpressed and ERAD pathway is saturated, excess misfolded proteins could not be diverted to the Golgi via the ER export machinery, thus accumulating in the ER and is toxic to the cells. If this is the case, the expression of these variants at low levels within the threshold of ERAD machinery should not be harmful to cells because misfolded proteins can be cleared by the ERAD. This is exactly what was observed in figure 6-1B. When the CPY* variant was expressed under the control of its own promoter in wild-type cells (using CPY-abcd-CPY* as an example in the figure 6-1B), it grew as well as cells expressing CPY* with the same promoter (Figure 6-1B, compare rows 2 to 1), suggesting that the CPY endogenous promoter gave lower levels of
abcD-CPY* expression and this level was still under the threshold of the ERAD machinery.

Figure 6-1: Overexpression of certain CPY* mutant variants causes a growth defect. (A) Equal numbers of wild-type cells overexpressing CPY* and wild-type cells overexpressing other four CPY* variants, ABCd-CPY*, abcD-CPY*, abcD-CPY*-D1 (abcD-CPY* lacking amino acids region 25-117) and abcD-CPY*-D2 (abcD-CPY* lacking amino acids region 116-206) were spotted as 10-fold serial dilutions on the plates containing 2% glucose or 2% galactose (B) Equal numbers of wild-type cells induced for CPY* and abcD-CPY* expression under its own promoter were spotted as 10-fold serial dilutions on the plate and incubated at 30 °C until colonies formed. The panel at left side showed schematic representation of CPY* or CPY* variants used in the assay. They were expressed either from the GAL1 promoter (A) or from the CPY promoter (B). Black dots indicate the CPY* G255R mutation, white square boxes indicate signal sequences, and orange square boxes indicate HA-tags. Glycans at positions 124, 198, 279, and 479, are denoted by the upper case letters A, B, C, and D, respectively. Lower case letters designate mutant sites.
To further confirm that the overexpression of the CPY* variant does result in the accumulation of the variant in the ER, I used abcD-CPY* as a substrate to examine the localization of excess abcD-CPY* in Δpep4 cells by indirect immunofluorescence. PEP4 is required for the activation of most vacuolar proteases, thereby making Δpep4 vacuoles deficient in proteolytic activity. Certain ERAD substrates such as CPY* may be exported out of the ER when ERAD is saturated and these substrates go to the vacuole for degradation (Spear and Ng, 2003). As expected, CPY* was found in the vacuoles of Δpep4 cells (Figure 6-2, white arrows). In contrast, abcD-CPY* was exclusively found in the ER of Δpep4 cells when overexpressed as shown by colocalization with the ER marker, Kar2p (Figure 6-2), indicating that abcD-CPY* lost its ability to leave the ER for degradation in the vacuole. The data also provided a strong support to the idea that the growth defect observed in wild-type cells with overexpressed abcD-CPY* (Figure 6-1A, GAL1-abcD-CPY*) was directly attributable to the toxicity caused by the accumulation of abcD-CPY* in the ER.
Figure 6-2: abcD-CPY* loses its ability to be transported out of the ER when overexpressed. 

Δpep4 cells overexpressing CPY* and abcD-CPY* were grown at 30 °C in the media containing 2% galactose for 8 hours. Cells fixed and permeabilized on glass slides were decorated with mouse α-HA mAb (CPY*) and α-Kar2p antibodies. Antibody complexes were bound with AlexaFluor 488 goat α-mouse and AlexaFluor 594 goat α-rabbit antibodies for visualization (CPY* in the green channel; Kar2p in the red channel).
6.3 CPY* requires potential export signals to be recognized by the ER export pathway

As the abcD-CPY* variant, compared to CPY* and ABCd-CPY*, lacks the first three glycans in its structure, it is reasonable to draw the conclusion that the first three glycan groups may somehow contribute to the formation of ER export signals for misfolded CPY* that is recognized by the export machinery. In addition, when the turnover of overexpressed abcD-CPY* was examined by pulse-chase analysis, I found that without functional export signals, the turnover of overexpressed abcD-CPY* was significantly retarded in wild-type cells (Figure 6-3A). Furthermore, using glucose shut-off to stop synthesis of abcD-CPY* after 6-hr induction also showed the inability of cells to clear excess abcD-CPY* when ER export signals were absent (Figure 6-3B)
Figure 6-3: Potential export signals are required for CPY* degradation.
(A) Wild-type cells overexpressed CPY* and abcD-CPY* were pulse labeled for 10 min with [35S]Cys/Met and followed by a cold chase as indicated. CPY* was immunoprecipitated from detergent lysates, deglycosylated using EndoH, separated by SDS-PAGE, and visualized by autoradiography. Plots reflect three independent experiments with the SD of the mean indicated. (B) Wild-type cells bearing GAL1-CPY* and GAL1-abcD-CPY* were induced for CPY* expression in the presence of galactose, further synthesis was terminated by adding 2% glucose as indicated, and the level of CPY* was examined by Western blot analysis.
6.4 Summary

In this chapter, I directly demonstrated that the clearance of misfolded proteins from the ER is essential for stress tolerance. By using wild-type cells to study the question, I ruled out any possible indirect effect coming from the utilization of mutant strains. The importance of reducing the amounts of ER-misfolded proteins also indicates that the root cause of cell death observed in CHY220 cells under ER stress is due to the toxicity caused by the inability of the cells to rid the ER of aberrant proteins when Kar2p upregulation by UPR is compromised. Accordingly, in response to ER stress, the function of UPR-elevated Kar2p plays a critical role in ER quality control, which is to reduce the toxicity of ER-accumulated misfolded proteins by removing them via both ERAD and ER-to-vacuole degradation pathways.

In addition, CPY* was shown to contain a potential export signal, which mediates its export out of the ER. This is intriguing because signaling elements within folded secretory proteins required for efficient export from the ER have been identified but not yet characterized in misfolded proteins such as CPY* (Barlowe, 2003; Belden and Barlowe, 2001; Kincaid and Cooper, 2007; Otte and Barlowe, 2004). From my preliminary data, an unidentified export signal in CPY* seems to form, even through CPY* is misfolded, which acts in an ER export event. To identify this element, Dr. Kawaguchi in the lab is proceeding with a variety of in vitro and in vivo assays to explore the underlying molecular mechanisms by using a CPY* deletion series.
Chapter 7
Materials and Methods

7.1 Plasmids used in this study

For the experiments in which cells were transformed with two copies of plasmids expressing \textit{GAL1-CPY} in chapters 2 to 6, pCB11 and pCH49 were used. pCB11 (generated by Christine Blackman) is the \textit{GAL1-CPY} fragment (\textit{GAL1/10} promoter-driven HA-tagged CPY*) cloned into pRS313 (Sikorski and Hieter, 1989) whilst pCH49 is the \textit{GAL1-CPY} fragment cloned into pRS314 (Sikorski and Hieter, 1989). pCB11 and pCH49 were constructed by releasing \textit{GAL1-CPY} as a SalI and EcoRI fragment from pES67 (Spear and Ng, 2003) and ligated into SalI/EcoRI-digested pRS313 and SalI/EcoRI-digested pRS314 respectively. For the experiment shown in the left panel of figure 3-1B, only pCB11 was transformed into cells.

For overexpressing CPY* under control of the GPD promoter in cells (Figure 4-2A), pES76 (generated by Eric Spear) was used. pES76 was constructed by digesting pDN436 (carrying HA-tagged CPY*, as described in Ng \textit{et al}., 2000) with XbaI and inserted into XbaI-digested pES27 (CEN/URA3-based YCp50 vector with the GPD promoter and ACT1 terminator).

Plasmids used in chapter 6 were described below:

\textbf{pES28} (generated by Eric Spear, as described in Spear and Ng, 2003): A plasmid containing \textit{GAL1-CPY} (HA-tagged) was constructed by digesting pDN436 (Ng \textit{et al}., 2000) with AccI, followed by treatment with T4 DNA polymerase, and then digested with
SphI. The released insert was ligated into BamHI (blunt)/SphI-digested pTS210 (YCp50 with the GAL1/10 promoter).

**pCH66:** a plasmid containing GAL1-abcDCPY* (a HA-tagged CPY* variant glycosylated only at the fourth glycan) was constructed by digesting pES147 (Spear and Ng, 2005) with AccI, followed by treatment with T4 DNA polymerase, and then digested with SphI. The released insert was ligated into BamHI (blunt)/SphI-digested pTS210 (YCp50 with the GAL1/10 promoter).

**pCH67:** a plasmid containing GAL1-ABCdCPY* (a HA-tagged CPY* variant glycosylated at the first three glycans) was constructed by digesting pES132 (Spear and Ng, 2005) with AccI, followed by treatment with T4 DNA polymerase, and then digested with SphI. The released insert was ligated into BamHI (blunt)/SphI-digested pTS210 (YCp50 with the GAL1/10 promoter).

**pDN436:** a plasmid containing HA-tagged CPY* driven by the endogenous PRC1 (CPY) promoter was described previously (Ng et al., 1990).

**pES132:** a plasmid containing HA-tagged abcD CPY* (a CPY* variant glycosylated only at the fourth glycan) driven by the endogenous PRC1 (CPY) promoter was described previously (Spear and Ng, 2005).
7.2 Strains, primers and antibodies

Yeast strains used in this study were listed in table 7-1. Primers used in this study were listed in table 7-2. Monoclonal α-HA antibodies (HA.11) were purchased from Covance Research Products. Rabbit polyclonal α-GFP antibodies were purchased from BD Biosciences Clontech. Secondary antibodies AlexaFluor 488 goat α-mouse IgG, AlexaFluor 594 goat α-rabbit IgG, AlexaFluor 488 goat α-rabbit IgG, and AlexaFluor 594 goat α-mouse IgG were purchased from Molecular Probes Inc.. Goat HRP-conjugated α-mouse IgG secondary antibodies were purchased from Pierce Biotechnology Inc.. Donkey HRP-conjugated α-rabbit IgG antibodies were purchased from Jackson ImmunoResearch Laboratories Inc.. α-Kar2p and α-Sec61p antibodies (raised in rabbits) were generously provided by Peter Walter (University of California, San Francisco, CA). α-Gas1p antibodies were raised against a GST-fusion protein containing the N-terminal amino acids 40-289 of Gas1p (generated by Davis Ng). α-DPAP B antibodies were a kind gift of Dr. Tom Steven (University of Oregon, Eugene, OR).
7.3 Generation of CHY113 and CHY119 strains (by Christine Blackman)

7.3.1 Construction of plasmids required for integration of $upr_e^d$-KAR2 allele at URA3 locus

To generate an integrating plasmid carrying the functional KAR2 gene with its 22-bp UPRE replaced by random sequences (sequences from pBR322 intragenic sequences) ($upr_e^d$-KAR2), a series of plasmid intermediates were constructed as listed below:

**pCB3:** A fragment A was amplified from genomic DNA by PCR using the forward primer N248 and the reverse primer N250. The N248 primer (5’-TTTGAATTCCATAGCGGAAACG-3’) is complimentary to a gene upstream of KAR2, TAD2 and contains an EcoRI restriction site at the 5’ end whilst the N250 primer (5’-AACATGAGAACTCGGGTGCCTGGGGAACG-3’) is complimentary to the region upstream of the UPRE in KAR2 promoter (nucleotides -150 to -132) (UPRE is located between -131 to -110) and flanked by the pBR322 sequence-AACATGAGAAC- at the 3’end. Another fragment B was amplified from genomic DNA by PCR using the forward primer N251 and the reverse primer N252. The N251 primer (5’-TGACAGCTTAAAAGTTGCTTTTTATATAAAAGGAC-3’) is complimentary to the region downstream of the UPRE of KAR2 (nucleotides -111 to -86) and flanked by the pBR322 sequence-TGACAGCTTAAAAGTTGCTTTTT at the 5’ end whilst the N252 primer (5’-AGGAACAGTAACGACGACG-3’) is complimentary to the ORF of KAR2 from nucleotides 562 to 579. The primer N250 and the primer N251 were phosphorylated by treatment with T4 DNA kinase prior to PCR.

Amplification was carried out by the PCR condition: initial denaturation ($96\,^\circ\mathrm{C}$, 1 minute) for 1 cycle, then denaturation ($96\,^\circ\mathrm{C}$, 30 seconds), annealing ($45\,^\circ\mathrm{C}$, 30 seconds) and
extension (72°C, 1 minute) for 30 cycles. Then, the EcoRI-digested fragment A and the Clal-digested fragment B were ligated into the vector pDN121 digested with the same enzymes, EcoRI/Clal, to generate pCB3. This resulting construct contained the first 90 bp of KAR2 ORF and the entire promoter region altered at 22-bp UPRE by replacing with pBR322 sequences (named as upre^KAR2 promoter).

**pCB6:** a 1,027-bp fragment between two EcoRI sites within KAR2 ORF was excised from pMR397 which carries an intact copy of the KAR2 gene [a gift from Mark D Rose, Princeton University, Princeton, NJ (Rose et al., 1989)] and ligated into EcoRI-digested pCH3 to generate pCH6. The resulting construct contained the upre^KAR2 promoter and the first 1,160 bp of KAR2 ORF.

**pCB7:** pCB6 was digested with XhoI, treated with T4 DNA polymerase, and subsequently digested with HindIII. The released 1,144-bp insert was ligated into pMR397 which was first digested with AatII, treated with T4 DNA polymerase, and then digested with HindIII to create pCB7. The resulting construct had a pMR366 vector backbone [a URA3 based low-copy-number shuttle plasmid (Botstein et al., 1979)] carrying the upre^KAR2 promoter and the first 863 bp of KAR2 ORF.

**pCB10:** a 1,220-bp fragment which contains KAR2 ORF (codon 864 to stop codon) was excised from pMR397 by digesting with HindIII and ligated into HindIII-digested pCH7 to yield pCB10. The resulting construct contained the upre^KAR2 promoter and the entire KAR2 ORF in pMR366 backbone.
pCB20: pMR397 was digested with BglII/KpnI, treated with T4 DNA polymerase, and then ligated to remove the CEN and the majority part of ARS regions for generating pCB20. The resulting construct was an integration plasmid carrying the functional KAR2 promoter and ORF in pMR366 backbone.

pCB18: pCB10 was digested with NcoI/PvuI. The released insert that contains the upre\textsuperscript{d}-KAR2 promoter and the KAR2 ORF was cloned to an integration vector generated by digesting pCB20 with NcoI/PvuI to yield pCH18. The resulting construct was an integration version of pCH10.

7.3.2 Generation of CHY119 and CHY113 strains

To generate the strain CHY119 and CHY113, MS785 strain which lacks the KAR2 gene (MAT\textalpha, kar2-1\text{VL148-}:LEU2, described in Rose \textit{et al.}, 1989) and carries pMR397 with the wild-type KAR2 gene for strain viability was mated with YJL183 wild-type (MAT\textalpha, ura3\textDelta99, leu2\textDelta1, trp\textDelta99, ade2-101\text{ochre}) to produce the heterozygous diploid. pCB18 or pCB20 was cleaved at Ncol site within URA3 gene to linearize it and transformed into the diploid cell. Transformants were later plated on synthetic complete (SC) media containing 5-fluoroorotic acid (5-FOA) (1 mg/ml) and incubated at 30\degree C. 5-FOA allowed the diploid cell to counterselect the URA3-based pMR397 plasmid, while, pCB18 or pCB20 was forced to integrate into \textit{ura3}\text{-52} locus of the diploid cell for cell viability. Two haploid strains in which pCB18 and pCB20 were integrated respectively were obtained after sporulation and tetrad dissection. Both haploid cells were
backcrossed 6 times with W303 wild-type \((\text{MAT}a, \text{ura3}-1, \text{leu2}-3, \text{his3}-11, \text{trp1}-1, \text{can1}-100, \text{ade2}-1)\) to generate CHY119 (\(\text{kar2-} \text{VL148::LEU2, ura3-52::upre}^d\)-\text{KAR2}, \text{W303 background}\)) and CHY113 (\(\text{kar2-} \text{VL148::LEU2, ura3-52::UPRE-KAR2}, \text{W303 background}\))

7.3.3 Generation of CHY220 and CHY438 strains

CHY220 and CHY438 strains were generated by replacing the entire endogenous \text{KAR2} ORF in CHY119 and CHY113 strains with a \text{KanMX} cassette using homologous recombination. A \text{KanMX} cassette was amplified from genomic DNA prepared from a heterozygous diploid with wild-type \text{KAR2} and another chromosomal copy of \(\Delta \text{kar2::KanMX}\) (from \text{KanMX} yeast ORF knock-out collection: http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html) by PCR using the forward primer CH20 (5’-AGGAACCTGGACAGCGTGTCGAA-3’) and the reverse primer CH21 (5’-CAACCTTGAAGCTTCCAGCAGC-3’). Two primers were designed which annealed to the 5’ and 3’ regions flanking the \text{KAR2} ORF respectively. After PCR

\[\text{Condition: initial denaturation (94 }^\circ\text{C, 1 minute) for 1 cycle, denaturation (92 }^\circ\text{C, 30 seconds), annealing (55 }^\circ\text{C, 45 seconds), extension (72 }^\circ\text{C, 2 minutes) for 30 cycles, a fragment containing the KanMX cassette with regions flanking the KAR2 gene was isolated and transformed into CHY119 and CHY113 strains. Transformants were selected on the synthetic medium containing 300 } \mu\text{g/ml G418 (Sigma-Aldrich) to obtain CHY220 (kar2::KanMX, ura3-52::upre}^d\)-\text{KAR2}, \text{W303 background}) and CHY438 (kar2::KanMX, ura3-52::UPRE-KAR2, W303 background).} \]
7.4 *GAL1-CPY* overexpression

Cells containing the *GAL1-CPY* gene expressed by either two copies of plasmids (pCB11 and pCH49) or one copy of plasmid (pES28, pCH66 [for *GAL1-abcDCPY*], or pCH67 [for *GAL1-ABCdCPY*]) were grown at 30°C overnight in SC medium containing the appropriate amino acids and 3% raffinose as a carbon source to early to mid-log phase. Cells were harvested, transferred into SC medium containing the appropriate amino acids and 2% galactose, and induced for CPY* overexpression for 6 hours before being processed (Figure 3-2, figure 3-3, figure 3-4A, figure 3-4B, figure 3-5, figure 4-2B [Wild-type and CHY220 cells], figure 6-3, figure 5-3, and figure 5-4) except for ∆*pep4* and CHY256 cells in figure 3-6 (carrying pCB11 and pCH49), and wild-type cells in figure 6-2 (carrying pES28 or pCH66). These cells were induced in 2% galactose for 8 hours before being processed for indirect immunofluorescence assay.

For the experiment involving ∆*ire1* cells (Figure 4-2B), cells were grown at 30°C overnight in SC medium containing the appropriate amino acids, 3% raffinose and 50 μg/ml myo-inositol. To initiate CPY* expression, cells were harvested and then transferred into medium containing 2% galactose and 50 μg/ml myo-inositol for a 6-hour induction.
7.5 Northern blots analysis

7.5.1 Preparation of RNA

Total RNA was isolated from yeast cultures as described in chapter 13 of ‘Current Protocols in Molecular Biology’. In short, cells were collected, washed once with ice-cold water, resuspended in 400 µl TES solution (10 mM Tris-Cl [pH 7.5], 10 mM EDTA, 0.5% SDS) and then 400 µl acid phenol was added. The mixture was vortexed vigorously for 10 seconds, incubated at 65 °C for another 1 hour with occasional, brief vortexing and followed by 5-minute incubation on ice. After centrifugation, the aqueous phase (top) was collected and mixed with 400 µl acid phenol. The mixture was again vortexed, incubated on ice and spun to collect the aqueous phase. This extraction step was carried out again with 400 µl of chloroform and the final aqueous phase was transferred into a new tube containing 40 µl of 3M NaOAc (pH 5.3) and 1 ml of ice-cold 100% ethanol. After centrifugation, the precipitated pellet was resuspended in 50 µl DEPC-treated H₂O.

7.5.2 Northern blot hybridization

Cells were treated with 2.5 µg/ml Tm (Sigma-Aldrich) or 10 mM DTT (Golden Biotechnology) for 1 hour and total RNA was prepared as described above. 10 to 20 µg RNA was denatured in formamide and formaldehyde, loaded on a 1.5% agarose gel containing 6.7% formaldehyde and ran in 1 X E buffer (20 mM MOPS [pH 7.0], 5 mM
NaOAc, 0.5 mM EDTA). The RNA was then transferred to Nylon membranes (Amersham Biosciences) as described by manufacturer.

A KAR2-specific probe was amplified from genomic DNA by PCR using the forward primer CH1 (5’-ACAGACTAAGCGCTGGCAAGCT-3’) and the reverse primer CH2 (5’-CAGCATGGGTAACCTTAGAGCC-3’) to obtain a 550-bp DNA fragment corresponding to 5’ end of coding sequence whilst an ACT1-specific probe was generated by PCR using the forward primer CH3 (5’-ATCGTCGTTAGACCAAGA CACC-3’) and the reverse primer CH4 (5’-CGAAGTCCAAGGCGACGTAACA-3’) to obtain a 559-bp DNA fragment corresponding to 5’ end of coding sequence. All probes were labeled with \([\alpha^{-32}P]dCTP\) using Ready-To-Go DNA-labeling kit (Pharmacia).

After transferring, the RNA was immobilized on the membrane using a UV cross-linker. The membrane was incubated in Church hybridization buffer (0.5 M Na PO4 [pH7.2], 7% SDS, 1 mM EDTA) at 65°C for more than 2 hours and subsequently probed with KAR2-specific and ACT1-specific probes (as a loading control) overnight at 65°C in the same buffer. The membrane was washed once in 1 X SSC, 1% SDS at room temperature for 10 minutes and twice in 0.5 X SSC, 1% SDS at 65°C for 10 minutes. Hybridization signals were then visualized and quantitated using a PhosphorImager and Image Quant software. For normalization, mRNA levels of KAR2 for each sample were normalized by its loading control to obtain the relative KAR2/ACT1 mRNA abundance.
7.6 Metabolic pulse-chase analysis

7.6.1 Cell labeling

The pulse-chase assay were carried out as described previously (Ng et al., 2000). Briefly, log phase cells were pelleted and resuspended in 1 ml/3 OD$_{600}$ units of synthetic complete SC medium lacking methionine and cysteine. After 30 min of incubation at the appropriate temperature, cells were labeled for 5 or 10 minutes with 35.75 μCi of $[^{35}\text{S}]$Cys/Met (PROMIX L-$[^{35}\text{S}]$, Amersham Biosciences) per A$_{600}$OD unit of cells, and the label was chased by adding excess cold Cys/Met to a final concentration of 2 M. 3 OD units were removed at the desired times and cell labeling/chase was terminated with the addition of trichloroacetic acid (TCA) to 10%.

7.6.2 Preparation of cell lysates

Cells were homogenized by the addition of 0.4 ml of 0.5-mm zirconium beads, followed by agitation in mini-bead beater cell disruptor (Biospec Products). The homogenate was transferred into a new tube and pooled with a subsequent 10% TCA bead wash. After centrifugation, the TCA precipitate pellet was resuspended in 150 μl of TCA resuspension buffer (100 mM Tris base, 3% SDS, 1mM PMSF) and heated at 100°C for 10 minutes. The sample was spun to collect the supernatant.
7.6.3 Immunoprecipitation

For immunoprecipitation, the radioactive counts were normalized by measuring TCA-precipitable counts using a LS6500 scintillation counter (Beckman coulter) and adjusting lysates for equal counts. The appropriate amount of detergent lysate (the sample with the smallest counts was set 50 µl) was added into 700 µl IPSI buffer (1% Triton X-100, 50 mM Tris [pH 7.5], 1 mM PMSF) containing 0.3 µl yeast protease inhibitor cocktail (Sigma-Aldrich) and the appropriate antiserum. After 1-hour incubation at 4 °C, the reaction was centrifuged for 20 minutes at 16,000 g and the supernatant was transferred to a new tube containing protein-A sepharose beads (Sigma-Aldrich). The tube was rotated for 2 hours, washed 3 times with IPSI buffer (0.2% SDS, 1% Triton X-100, 50 mM Tris [pH 7.5]) and once with PBS. For experiments involving overexpression of CPY* in cells, N-linked glycosylation was removed by treatment of 300 U endoglycosidase H (New England Biolabs) according to the manufacturer’s protocol at this point. Immunoprecipitated proteins were subsequently eluted from protein-A sepharose beads by boiling in SDS-loading buffer (6% SDS, 250 mM Tris [pH 6.8], 10% glycerol, bromophenyl blue) for 10 minutes, resolved on SDS-PAGE, and visualized by autoradiography.
7.7 MalPEG5000-protein folding assay

Cell labeling, homogenization and TCA precipitation was carried out as described previously. The TCA precipitate pellet was then washed once with cold acetone to remove residual TCA and resuspended in MalPEG5000 buffer (50 µl/OD_{600} unit) (5 mM MalPEG5000, 100 mM Tris [pH7.4], 2% SDS). The sample was boiled at 100 \(^{\circ}\)C for 10 minutes with occasional, brief vortexing and incubated on ice for 50 minutes and at 100 \(^{\circ}\)C for another 10 minutes. After centrifugation, the cell lysate was used for immunoprecipitation as described in pulse-chase assay. For a DTT control, the culture was treated with 10 mM DTT for 20 minutes prior cell labeling.
7.8 Western blot analysis

Cells were grown in medium containing the appropriate amino acids and carbon source. Cell lysates were prepared by TCA precipitation as described previously and resolved by SDS-PAGE. Nitrocellulose membrane (Amersham Biosciences) was prepared by wetting in chilled Buffer A (25 mM Tris, 193 mM Glycine, 0.025% SDS, 20% methanol) for 15 to 60 minutes and the electrophoresed proteins were then transferred to the pre-soaked nitrocellulose membrane in a cooling apparatus containing chilled Buffer A by applying a constant current of 300 mA for 75 minutes. When the transfer was complete, the blot was incubated in blotting buffer (5% nonfat dry milk in PBST [137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄⋅2H₂O, 2 mM KH₂PO₄, 0.1% Tween-20]) at 37 °C for 30 minutes and then probed in fresh blotting buffer containing appropriate primary antibodies (1:10,000) for 2 hours at room temperature (or overnight at 4 °C) with gentle agitation. After being rinsed once with H₂O and washed twice with PBST for 13 minutes at room temperature (or 4 X 7 minutes if the blot was probed at 4 °C overnight), the blot was incubated in PBST containing HRP-conjugated secondary antibodies (1:10,000 α-mouse IgG [Pierce Biotechnology] or α-rabbit IgG [Jackson ImmunoResearch Laboratories]). Finally, the blot was washed three times and then developed using ECL reagents (Pierce Biotechnology).
7.9 CPY* expression shut-off assay

Cells carrying either one copy (pCH66 in figure 6-3B) or two copies of GAL-CPY* plasmids (pCB11 and pCH49 in figure 3-4B) were grown overnight to ~0.5 OD\textsubscript{600} units in the SC medium containing the appropriate amino acids and 3% raffinose as the carbon source. Expression of CPY* was induced with the addition of 2% galactose for 6 hours and then the synthesis was stopped by the addition of 2% glucose. Samples were harvested by centrifugation at the indicated times and cell lysates were prepared by TCA precipitation as described previously. Lysates corresponding to 0.02 OD\textsubscript{600} units of cell equivalents were resolved by SDS-PAGE and analyzed by Western blot analysis as described earlier: blots were probed with primary antibodies (monoclonal α-HA (for CPY*) [Covance Research Products]), then with secondary antibodies (goat HRP-conjugated α-mouse IgG [Pierce Biotechnology]) and developed using ECL reagents (Pierce Biotechnology). To re-probe with α-Kar2p antibodies, the same blots were stripped by incubating it in 100 mM glycine [pH 2.5-3.0] at 55 °C and then shaking at room temperature for another 30 minutes. After stripping, the blots were re-probed with polyclonal α-Kar2p antibodies (Peter Walter, University of California, San Francisco), followed by incubation with the donkey HRP-conjugated α-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories) and finally developed using the same methods.
7.10 Analysis of protein aggregates

Wild-type cells overexpressing CPY* (pCB11 and pCH49), CHY220 cells overexpressing CPY* (pCB11 and pCH49), or Δire1 overexpressing CPY* (pES67; Spear and Ng, 2003) were grown overnight to ~0.5 OD$_{600}$ units in SC medium containing the appropriate amino acids and 3% raffinose as the carbon source. Expression of CPY* was induced for 6 hours by the addition of 2% galactose. Cells of 5 OD$_{600}$ units were collected, washed once with H$_2$O and the pellet was resuspended in 500 µl TNE buffer (50 mM Tris [pH7.4], 150 mM NaCl, 5 mM EDTA) containing 1 mM PMSF and 0.3 µl yeast protease inhibitor cocktail (Sigma-Aldrich) per OD$_{600}$ unit. Cells were disrupted by beating with 0.5-mm zirconium beads (10 X 1 minute with 5-minute intervals on ice) using a vortex mixer at full speed in the cold room. The lysate was cleared by centrifugation twice at 750 g for 5 minutes at 4 °C to remove cell debris and unbroken cells. For solubilization, Triton X-100 was added to 1% vol/vol and incubated at room temperature for 5 minutes. At this point, 50 µl of the sample (T) was saved for Western blot analysis. After ultracentrifugation at 100,000 g for 15 minutes at 4 0°C, the supernatant was removed and 50 ml portion (S) was saved for Western blot analysis. The pellet was resuspended in 450 µl of 3% SDS, 50 mM Tris [pH7.5], boiled at 100 0°C for 5 minutes and 50 µl portion (P) was saved for Western blot analysis. Sample (T), sample (S) and sample (P) were boiling in SDS-loading buffer (6% SDS, 250 mM Tris [pH 6.8], 10% glycerol, bromophenyl blue) for 10 minutes, resolved on SDS-PAGE and analyzed by Western blot analysis as described earlier: blots were probed with primary antibodies (monoclonal α-HA (for CPY*) [Covance Research Products] or α-Sec61p), then
followed by secondary antibodies (goat HRP-conjugated α-mouse IgG [Pierce Biotechnology] or donkey HRP-conjugated α-rabbit IgG [Jackson ImmunoResearch Laboratories]) and finally developed using ECL reagents (Pierce Biotechnology).
Native immunoprecipitation (IP) assay was modified from that previously described by Carvalho *et al.* (Carvalho *et al.*, 2006). Briefly, cells were grown to log phase in SC medium containing the appropriate amino acids and carbon source. Cells of 40 OD<sub>600</sub> units were harvested, washed once with H<sub>2</sub>O and resuspended in 500 µl of TBS IP buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 2 mM PMSF, 0.3 µl yeast protease inhibitor cocktail [Sigma-Aldrich] per OD<sub>600</sub> unit). The following steps were performed at 4°C. Cell disruption was carried out by beating with 0.5-mm zirconium beads (3 X 15 seconds with 5-minute intervals on ice) using mini-bead beater (Biospec Products). The cell lysate was transferred into a new tube and pooled with a subsequent 500 µl-TBS IP buffer bead wash. After centrifugation at 30,000 g for 30 minutes, the pellet was collected, resuspended in 1 ml of TBS IP buffer containing 1% Triton X-100 and incubated for 30 minutes. After centrifugation at 30,000 g for 10 minutes, the clear supernatant was added into a new tube containing 5 µl HA-probe mouse monoclonal IgG (Santa Cruz Biotechnology) and incubated for 30 minutes. 25 µl of Sepharose CL-4B (Sigma-Aldrich) was added into the tube followed by three-time wash with 1 ml of TBS IP buffer containing 1% Triton X-100 and then one-time wash with 1 ml of TBS IP buffer. Immunoprecipitated proteins were subsequently eluted from protein-A sepharose beads by boiling in SDS-loading buffer (6% SDS, 250 mM Tris [pH 6.8], 10% glycerol, bromophenyl blue) for 10 minutes and resolved on SDS-PAGE and analyzed by Western blot analysis as described earlier: blots were probed with primary antibodies (monoclonal α-HA (for CPY*) [Covance Research Products] or α-Kar2p), then followed by secondary
antibodies (goat HRP-conjugated α-mouse IgG [Pierce Biotechnology] or donkey HRP-conjugated α-rabbit IgG [Jackson ImmunoResearch Laboratories]) and finally developed using ECL reagents (Pierce Biotechnology).
7.12 Serial dilution-spotting growth assay

Cells were grown overnight in the synthetic complete (SC) medium containing the appropriate amino acids and carbon source. For those experiments in which cells were later spotted on the plate containing galactose, 3% raffinose was used as a carbon source in overnight cultures. Cell numbers for each culture were counted by OD$_{600}$ readings, pelleted and diluted to 2 X $10^6$ cells/ml in H$_2$O. Four ten-fold serial dilutions were made and 10 µl of each diluted sample was spotted on the plate with the appropriate components to test their growth. Plates were incubated at 30 °C until colonies were formed.
7.13 UPRE-lacZ assay

Cells were transformed with the pJC31 plasmid which carries the UPRE-CYC1-LacZ reporter (Cox and Walter, 1996) and then treated with 2.5 μg/ml Tm for 1 hour to induce UPR or with the same volume of DMSO as a control. Equal numbers of cells (2 to 3 A600 OD units) were collected, then washed gently with 1 ml of 1 X Z buffer (0.06 M Na2HPO4·7H2O, 0.04 M NaH2PO4·H2O, 0.01 M KCl, 0.001 M MgSO4·7H2O, 50 mM β-mercaptoethanol) and resuspended in 50 μl 1 X Z buffer. 50 μl CHCl3 and 20 μl 0.1% SDS were added and the mixture was vortexed hard for 20 seconds. Then, 700 μl of 2 mg/ml ONPG (o-Nitrophenyl-beta-galactopyranoside) (in 1 X Z buffer) was added and incubated at 30 °C for 1 to 10 minutes. Exact time of incubation should be noted. The reaction was stopped by quenching with 500 μl of 1 M Na2CO3. After a quick spin, A420 OD of supernatant was measured for ONPG. β-galactosidase activity was expressed in Miller units as 1000 X (A420) / [(tmin)(Vml) X (A600)] (Guarente, 1983).
7.14 Indirect immunofluorescence microscopy

Cells were grown to log phase in the SC medium containing the appropriate amino acids and carbon source. Formaldehyde was added directly to the medium to 3.7%. After 1.5-hour fixation at 30°C, cells were washed once with 0.1 M potassium phosphate buffer [pH 7.5] and subsequently spheroplasted by incubation in spheroplasting buffer (0.1 µg zymolyase 20T [ICN Biomedicals] in 100 µl of 0.1 M potassium phosphate buffer [pH 7.5], 1.2 M Sorbitol) for 40 to 50 minutes at 30°C to digest cell wall. The reaction was terminated by washing cells twice with 0.1 M potassium phosphate buffer [pH 7.5], 1.2 M Sorbitol. 30 µl of cell suspension was applied to a 0.1% poly-L-lysine-coated wells on a slide, incubated at room temperature for 10 minutes in a humid chamber (a Petri plate with a wet kimwipe) and then washed once with TBS buffer (50 mM Tris [pH 7.5], 150 mM NaCl). The slide was immersed in iced methanol for 6 minutes at -20°C, transferred into iced acetone for 30 seconds at -20°C and then incubated in TBS for 3 minutes at room temperature. Subsequent steps were performed at room temperature. 30 µl of TBST blocking buffer (5% nonfat dry milk in 1 X TBST [0.05% Tween-20 in 1 X TBS]) was added to each well, incubated for 30 minutes and then washed once with TBS. 30 µl primary antibodies were applied on wells for 90 minutes, washed twice with TBS, 30 µl secondary antibodies were then applied and the slide incubated in the dark for 90 minutes, followed by two TBS washes. Wells were allowed to dry and sealed with 5 µl mounting medium (PBS, 90% glycerol, 1 mg/ml p-phenylenediamine, 0.025 µg/ml DAPI). Samples were viewed on Olympus IX71 microscope (Melville, NY) equipped with a Photometrics CoolSNAP EScamera (Tucson,
Images were acquired and processed with MetaMorph software (Universal Imaging, West Chester, PA).

Primary antibodies monoclonal α-HA (for CPY*), polyclonal α-Kar2p, and polyclonal α-GFP (BD Biosciences Clontech) were diluted to 1:1000, 1:5000 and 1:500 respectively. Secondary antibodies AlexaFluor 488 goat α-mouse IgG, AlexaFluor 594 goat α-rabbit IgG, AlexaFluor 488 goat α-rabbit IgG, and AlexaFluor 594 goat α-mouse IgG (Molecular Probes) were diluted to 1:1000 for working concentrations.
7.15 A high-copy suppressor screen

CHY220 cells carrying two copies of \textit{GAL-CPY}* plasmids (pCB11 and pCH49) were transformed with a yeast genomic library based on the multicopy YEp13 vector (Lagosky \textit{et al.}, 1987). After transformation, the cells were grown in SC-His-Trp-Leu medium allowing transformants to receive complementing plasmids to bypass the growth defect imposed by overexpression of CPY* in CHY220 cells. Transformants were later plated on SC-His-Trp-Leu medium containing 2% galactose and incubated at 30 °C. Colonies grown on the plate were examined for the expression level of CPY* by pulse labeling for 10 minutes with \textsuperscript{35}S]Cys/Met, immunoprecipitating CPY*, resolving samples by SDS-PAGE followed by autoradiography. Those that displayed a decreased level of CPY* were discarded. Plasmids were recovered from positive isolates by zirconium bead disruption and purification using QIAprep® Miniprep kit (Qiagen). Plasmid amplification was performed after transformation into bacterial DH5α cells. Retransformation of the recovered plasmids into CHY220 cells carrying two copies of \textit{GAL-CPY}* plasmids was carried out to confirm complementation of growth defect on galactose plates.

For all clones, DNA sequence analysis was performed to determine the identity of inserts (DNA sequence facility, Temasek Life Sciences Laboratory, Singapore). The sequences of vector/insert junctions were obtained using the forward primer NI63 (5’-CGCTACTTGGAGCCACTATCGAC-3’) and the reverse primer NI64 (5’-ATCGGTGATGTCGGCGATAT-3’). Junction sequences were submitted to the
Saccharomyces Genome Database (www.yeastgenome.org) to obtain complete sequences of inserts and identities of ORF.

7.16 Flow cytometry

Cells of 1 OD\textsubscript{600} unit were fixed overnight at 4 \(^0\)C with the addition of 4 volumes of 95% ethanol. After being washed twice with 1 ml PBS, cells were resuspended in 500 µl PBS containing 1 mg/ml RNaseA (Roche) and incubated at 37 \(^0\)C overnight. Cells were harvested, resuspended in 50 µg/ml propidium iodine (in 500 µl PBS) (Sigma-Aldrich) and incubated at 4 \(^0\)C in the dark for 3 hours. Cells were harvested again and finally resuspended in 5 µg/ml propidium iodine (in 2 ml PBS). After mild sonication, samples were analyzed with FACSCalibur (BD Biosciences). For each histogram, 30,000 cells were analyzed.
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Table 7-2: Primers used in this study.

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Chapter 8
Discussion

The ER is the major site in the cell dedicated to synthesis, folding, and modification of cellular secretory and membrane proteins, which, collectively, represent a large fraction of the total protein output of cells. Despite the ER maintaining the optimized environment to support efficient folding (Ellgaard et al., 1999), an inevitable consequence of the large flux of proteins through the ER is that the folding process occasionally fails, thus resulting in the generation of irreversibly misfolded proteins. In response to the misfolded proteins (called ER stress), cells activate the UPR, an ER-to-nucleus signal transduction pathway, to regulate a wide variety of target genes to alleviate ER stress and restore ER homeostasis. In mammalian cells, there are at least three ER sensors that comprise the UPR. The first, IRE1, is conserved in all known eukaryotic cells and functions to promote splicing of an mRNA encoding the transcription factor, XBP1 (Calfon et al., 2002; Cox and Walter, 1996; Kawahara et al., 1997; Yoshida et al., 2001). The second, a UPR-specific transcription factor termed ATF6 (with α and β isoforms) that is synthesized as an integral ER transmembrane protein and is released by proteolytic cleavage upon UPR activation (Haze et al., 1999; Ye et al., 2000). A third sensor, PERK, attenuates general translation to reduce the load of proteins in the ER (Harding et al., 1999; Shi et al., 1998). Interestingly, one of the major UPR targets, BiP (Kar2p in yeast), seems to be involved in the regulation of all three sensors (Bertolotti et al., 2000; Okamura et al., 2000; Shen et al., 2002a).
In yeast, the UPR seems to be relatively simple with only the Ire1p/Hac1p-mediated signaling pathway required. Thus, as a model system, yeast provides a tractable model system for the study of this aspect of the pathway in the absence of other UPR branches. However, the extent of UPR transcriptional control mediated through the Ire1p branch alone is far more complex than anticipated. Whole genome-wide expression analysis has revealed that the transcriptional scope of the Ire1p/Hac1p-mediated UPR in yeast comprises at least 381 genes (~5% of the yeast genome) (Travers et al., 2000). About half of these genes encode functions throughout the secretory pathway including protein folding and modification, protein QC, phospholipid biosynthesis, ERAD, protein trafficking, and vacuolar protease. Thus far, it remains unclear the exact mechanism by which the transcriptional output of this gene cluster restores ER homeostasis. In this study, I address this question by studying the contribution of one specific target gene to ER stress response. For the first gene, I selected KAR2, which encodes the molecular chaperone Kar2p/BiP. Kar2p is a member of the Hsp70 family that plays multiple functions that include protein translocation, folding, nuclear membrane fusion and ER associated protein degradation (ERAD) in the ER. The contribution of Kar2p to the ER stress response is particularly intriguing not only because it plays a crucial role in multiple ER functions but because an interesting conundrum described earlier in the introduction of chapter 2 may be solved by this study (For details, see below).

Initially, a strain, named CHY220, was engineered to specifically uncouple KAR2 regulation from the UPR without compromising the activation of other target genes. A remarkable feature of the strain is that no mutations were introduced into the ORF of KAR2 so that wild-type Kar2p can be expressed to promote normal growth and function
in resting cells. By doing so, the main drawback of using traditional genetic methods for the determination of essential genes’ functions may be avoided. Classically, the most common method to analyze any essential gene is the creation of a temperature-sensitive mutant by mutagenesis to assess the phenotype of the organism when the gene of interest is rendered nonfunctional at non-permissive temperature. However, mutagenesis of \textit{KAR2} itself to study the question in this study is improper because any effect observed can be an indirect effect of the loss of important housekeeping functions of Kar2p. Thus, in my strategy, the only mutation introduced was located at the UPRE in promoter region of \textit{KAR2} and the integrity of \textit{KAR2} ORF was confirmed by sequencing analysis. With these backgrounds in place, any result obtained is validated.

Studying the CHY220 strain, I first found that elevated levels of Kar2p are required for stress tolerance. Even so, UPR-upregulated Kar2p is only required for abrogating the toxicity of accumulated abnormal proteins; it is not required to supplement the increased activity of oxido-reductases as demonstrated by the tolerance of CHY220 cells to DTT but not Tm. This is intriguing because it supports the idea that in response to reductive stress, the UPR upregulates the expression of the oxido-reductases, such as Ero1p, to lessen the stress. Ero1p, another UPR-induced protein, is necessary for the oxidation of disulfide bonds of proteins in the ER. It has been shown that Ero1p functions in determining cellular oxidizing capacity since the mutation of Ero1p causes hypersensitivity to DTT whereas increased Ero1p renders cells more resistant to DTT (Frand and Kaiser, 1998; Pollard \textit{et al.}, 1998). Moreover, even in the absence of a functional UPR, overexpression of Ero1p significantly increases the resistance to DTT, suggesting that Ero1p plays a major role in DTT tolerance conferred by UPR (Frand and
According to my data, the elevation of Kar2p during the course of the UPR is not involved in this process. After having established that Kar2p upregulation is required for ER stress tolerance of abnormal proteins, I first hypothesized CHY220 cells cannot survive ER stress because the limiting amounts of Kar2p compromised its essential functions of protein translocation and folding. Surprisingly, the two essential functions remain completely intact when Kar2p levels become limiting under stress. On the other hand, I also discovered that without Kar2p upregulation by the UPR, misfolded CPY* is poorly degraded. These unexpected findings provided two new perspectives. First, the primary function of elevating Kar2p levels in stress tolerance is to clear the ER of aberrant proteins. The second view is Kar2p functions are prioritized according to their importance in the cell. This strategy may be essential during the lag between the moment stress is elicited and when a full response can be mounted. During this period, the front burner is to ensure proper maturation of UPR target proteins to fully activate the UPR. Since half of the UPR targets have to enter the ER for their maturation, cells must preserve protein translocation and folding functions. Given that Kar2p levels are limiting at this point, Kar2p may temporarily reduce its quality control functions in ridding misfolded proteins to facilitate the UPR. Thereafter, with the UPR deployed, the enhancement of quality control functions restores ER homeostasis. The importance of this ‘molecular triage’ mechanism was proven (Figure 4-1), as a temporary loss of essential functions was lethal. In contrast, cells can fully survive a temporary disruption of Kar2p’s quality control functions.
The underlying mechanism regarding Kar2p functional prioritization is still unclear. The simplest explanation is that when Kar2p levels become limiting, the association of Kar2p to Sec63p (for translocation function) remains normal while the recruitment to CPY* is reduced (for quality control function). Indeed, I observed the Kar2p levels pulled down by CPY* was greatly reduced in CHY220 cells when compared to that in wild-type cells under the same condition. Unfortunately, in this study, the binding of Kar2p to Sec63p has not been determined because of technical problems. In addition, there are other more complicated explanations for this phenomenon. For example, under Kar2p-limiting condition, binding of Kar2p to all its cofactors is generally reduced. However, in order to constantly maintain the operation of essential functions, cells have evolved specialized molecular mechanisms to ensure their activation. These essential functions can somehow be maintained at much lower requirement in terms of the levels of Kar2p involved, or there are other mechanisms in cells ready to be deployed for such a condition.

Previous efforts in studying the effects of ER stress in yeast through expression of misfolded CPY* showed that the UPR expands quality control functions including ERAD and ER-to-vacuolar pathways to rid the ER of CPY* (Spear and Ng, 2003). Under mild ER stress, ERAD is first used to degrade CPY* while under more severe ER stress, when ERAD is saturated, an alternative pathway to the vacuole is utilized to degrade excess CPY*. This is also the case for another soluble ERAD substrate, the Z variant of α1-antitrypsin (or alpha 1-protease inhibitor, A1Pi) termed A1PiZ (Kruse et al., 2006b). These misfolded proteins may represent a subset of ERAD substrates that can exit the ER for degradation by other routes apart from degradation by ERAD. Overall, because of the
potentially catastrophic results of aberrant protein accumulation in the secretory pathway, it is not surprising that the cell possesses alternative trafficking schemes to remove unwanted proteins. However, the extent of these alternative schemes and how they are regulated are not clear. In the present study, I showed that the elevated Kar2p levels play an active role in facilitating CPY* degradation through both pathways. Probably, Kar2p does not simply maintain misfolded proteins in a soluble form for retro-translocation from the ER and degradation by ERAD (Nishikawa et al., 2001) but also preserves misfolded proteins in a certain conformation (with export signal exposed) for recognition by Erv29p and degradation by the ER-to-vacuole degradation pathway (see below for details).

In the CHY220 strain defective in inducing KAR2 expression by the UPR, both ERAD and ER-to vacuole degradation pathways are compromised, resulting in substantial amounts of misfolded CPY* accumulating in the ER, and eventually, cell death. Some findings are worth noting. The first is the demonstration that the site, rather than the extent, of accumulation is more important for the manifestation of CPY* toxicity, as simple accumulation of CPY* in the ER is lethal while in Δpep4 cells, overexpressing similar amounts of CPY* is benign, presumably because the accumulation occurs in the vacuole instead of the ER (Spear and Ng, 2003). Additionally, the data also suggested that efficient disposal of misfolded proteins by any means available seemed to be essential for stress tolerance. Previous experiments using Δerv29 cells (defective in the ER to the Golgi transport) provided a correlated support for this notion. It showed that Δerv29 cells are highly sensitive to overexpression of CPY* because excess CPY* fails to traffic out of the ER for degradation, thus accumulating in the ER (Spear and Ng,
2003). However, since Erv29p is also needed for exporting unknown numbers of folded proteins from the ER to their sites of action, one cannot rule out the possibility that the observed results on \( \Delta \text{erv29} \) cells is due to an indirect effect of a general breakdown in this process. In this study, the importance of removing excess misfolded protein from the ER was formally substantiated by utilizing wild-type cells for analysis. Overexpression of the CPY* variant that has lost its ability to exit the ER causes wild-type cells to die while the expression of full-length CPY* and other variants that could be exported at the same levels can be tolerated in the cells (Figure 6-1). Additional studies demonstrated that the accumulation of export-defective CPY* variants in the ER may underlie the cell death seen in wild-type cells (Figure 6-2 and figure 6-3), presumably because accumulated damaged proteins would interfere with essential ER functions, thus generating cellular toxicity to the cells. Taken together, the data formally demonstrated that the reduction of the levels of misfolded proteins in the ER is essential for stress tolerance and that the primary function of the UPR-elevated Kar2p is to alleviate this toxicity by facilitating the degradation of misfolded proteins by ERAD and ER-to-vacuole degradation pathways to ensure cell survival.

These findings also lend further support to a model proposed by Kincaid and Cooper (Kincaid and Cooper, 2007). Erv29p interacts with wild-type CPY (wtCPY) and the binding is required for efficient trafficking of wtCPY from the ER to the Golgi (Belden and Barlowe, 2001; Caldwell et al., 2001). Kincaid and Cooper later found that although termed misfolded, CPY* maintains the ability to bind Erv29p and exits the ER via the same mechanism as correctly folded wtCPY. Furthermore, when a defined ER export signal (the di-acidic motif present in the cytosolic domain of the yeast membrane-
spanning protein Sys1p) is appended to CPY*, this signal can promote CPY* to exit from the ER and be transported to the vacuole for degradation. Together, the data suggested that anterograde transport of misfolded proteins may utilize the same machinery responsible for exporting correctly folded proteins. By extension, they proposed a model that intact ER export signals are formed in CPY*, despite CPY* being in a misfolded state and mediate its trafficking from the ER. This model, however, still lacks direct confirmation. In the present study, I showed that indeed, the absence of a potential export signal in CPY* may prevent it from leaving the ER, which strongly supported the idea that some ERAD substrates, such as CPY*, although it is degraded primarily by ERAD, they, possessing an intact export signal, maintain the ability to exit the ER via Erv29p, resulting in a portion of misfolded proteins being exported from the ER for degradation. The molecular mechanisms underlying the ER export pathway required for the trafficking of misfolded proteins would be interesting to study in the future. One could extent the analysis from this study to explore more aspects regarding this pathway, such as to identify the actual export signal(s) in CPY*, examine the components involved in this pathway, and investigate the basis for the interplay between ERAD and ER exit, i.e., whether both pathways compete for the binding of misfolded proteins.
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VITA

Chia-Ling Hsu

EDUCATION:

• M. S. 2001, Institute of Biopharmaceutical Sciences, National Yang-Ming University, Taipei, Taiwan, R.O.C. (with Dr. Jing-Jer Lin, Screening and characterization of proteins that interact with the N-terminal 1-252 amino acids of Saccharomyces cerevisiae Cdc13p by yeast two-hybrid system).

• B. S. 1999, Department of Biology, Tunghai University, Taichung, Taiwan, R.O.C.

AWARD:

• Certificate of Academic Honors Tunghai University (1998)
• The Academic Award of Tunghai University (1999)

PUBLICATION: