Disulfide Bond Formation: Identifying Roles of PDI Family Thiol Oxidoreductases and ER Oxidant Pathways

by

Lori Ann Rutkevich

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Biochemistry
University of Toronto

© Copyright by Lori Ann Rutkevich 2012
Disulfide Bond Formation: Identifying Roles of PDI Family
Thiol Oxidoreductases and ER Oxidant Pathways

Lori Ann Rutkevich
Doctor of Philosophy
Department of Biochemistry
University of Toronto
2012

Abstract

Protein disulfide isomerases (PDIs) catalyze the oxidation and isomerization of disulfide bonds in proteins passing through the endoplasmic reticulum (ER). Although as many as 20 enzymes are classified as PDI family members, their relative contributions to protein folding have remained an open question. Additionally, Ero1 has been characterized as the ER oxidase that transfers oxidizing equivalents from oxygen to PDI enzymes. However, knockout mice lacking the mammalian Ero1 isoforms, Ero1Lα and Ero1Lβ, are viable, and the role of other potential ER oxidases in maintaining an oxidative ER environment is now an important issue. By systematic depletion of ER PDI family members and potential ER oxidases and assessment of disulfide bond formation of secreted endogenous substrates, I have outlined the functional relationships among some of these enzymes. PDI family member depletion revealed that PDI, although not essential for complete disulfide bond formation in client proteins, is the most significant catalyst of oxidative folding. In comparison, ERp57 acts preferentially on glycosylated substrates, ERp72 functions in a more supplementary capacity, and P5 has no detectable role in formation of disulfide bonds for the substrates assayed. Initially, no impact of depletion of Ero1 was observed under steady state conditions, suggesting that other oxidase systems are working in parallel to
support normal disulfide bond formation. Subsequent experiments incorporating a reductive challenge revealed that Ero1 depletion produces the strongest delay in re-oxidation of the ER and oxidation of substrate. Depletion of two other potential ER oxidases, peroxiredoxin 4 (PRDX4) and Vitamin K epoxide reductase (VKOR), showed more modest effects. Upon co-depletion of Ero1 and other oxidases, additive effects were observed, culminating in cell death following combined removal of Ero1, PRDX4, and VKOR activities. These studies affirm the predominant roles of Ero1 in ER oxidation processes and, for the first time, establish VKOR as a significant contributor to disulfide bond formation.
Acknowledgments

This work was completed with the support of a great number of people to whom I am gratefully indebted. First, I thank my supervisor, Dr. David Williams who has been an amazing mentor over the years. I have so appreciated his approachability, his creative thinking, and his commitment to his craft. For the opportunities he gave to stretch beyond myself and grow professionally, I will always be thankful. I also thank the members of my supervisory committee, Dr. John Glover and Dr. Allen Volchuk; their input and advice in the course of this project were invaluable. It was with Dr. Glover that I also first worked as a teaching assistant at UofT, and I am grateful for the experiences that opened to me as a result. I have valued the instruction and friendship of lecturers Ahlia Khan-Trottier, Sian Patterson, and Roula Andreopoulos as we worked and taught together.

In my time in the Williams lab, there have been many people I have had the privilege to work alongside. I would first thank Myrna Cohen-Doyle who made the lab run smoothly and was an incredible source of support, both personally and technically as we washed beads and poured innumerable gels side-by-side. Graduate students Achim Brockmeier and Dan Chapman were fantastic discussion partners early and later, respectively, in my time in the lab and I have valued their input and encouragement. Master’s graduate Breanna Ireland was so generous in her time and suggestions as I started in the lab and I am so glad that our friendship has grown to last beyond our time in the lab. I also wish to thank Ulf Brockmeier, Pawel Stocki, and Ronnie Lum, post-doctoral fellows of my time in the Williams lab. Each of them contributed to the completion of this work through sharing their expertise and advice and productive discussions of data and future experiments. I am also thankful to Nikko Torres, whom I had the privilege to teach in third year and then co-mentor in a summer and fourth-year project. Through his contagious enthusiasm, he made the mentoring experience so rewarding and I am proud to see him excelling in his own graduate work now. To the members of the Biochemistry Graduate Student’s Union executive, I am grateful for their dedication to supporting students and making the graduate experience an adventure for all. It was an honour to work alongside each member over the years.
I am truly thankful to several individuals who never set foot in the lab and patiently listened to my explanations of the highs and lows of my research project. These friends and family helped me to maintain perspective and perseverance over the years with encouragement and support. I am so grateful to my parents, Marilyn and Wendell Beach for their love and confidence in me, even as they didn’t know where this science-kid came from, and to my brother Michael who joined me in the science adventure and spoke my lingo. To Betty Beach I am indebted for a listening ear and for teaching me other creative outlets. To Ken and Dorothy Davis, who have been my inspiration and reminders of the greater purpose in all I undertake, I am forever thankful. And to my husband, Tim, who has walked with me on every step of this journey, his immense love, support, patience, and perspective have been invaluable.

S.D.G.
Table of Contents

Acknowledgments ........................................................................................................ iv
List of Tables ................................................................................................................ ix
List of Figures ............................................................................................................... x
Chapter 1 ...................................................................................................................... 1
  1.1 Overview ............................................................................................................... 2
  1.2 ER Protein Folding ............................................................................................... 2
  1.2.1 Classical Chaperones ....................................................................................... 4
  1.2.2 Lectin Chaperones .......................................................................................... 6
  1.2.3 Folding Catalysts .............................................................................................. 9
  1.3 ER Protein Misfolding ......................................................................................... 10
  1.3.1 Unfolded Protein Response .......................................................................... 11
  1.3.2 ER Associated Degradation .......................................................................... 13
  1.4 Disulfide Bonds in the ER .................................................................................... 16
  1.4.1 Thiol Chemistry ............................................................................................. 18
  1.4.2 The Protein Disulfide Isomerase Family ....................................................... 19
  1.4.3 Oxidation of PDIs .......................................................................................... 33
  1.5 Rationale & Approach ....................................................................................... 42
Chapter 2 ...................................................................................................................... 44
  2.1 Cell Lines and antibodies ................................................................................... 45
  2.2 Human PDI cDNA isolation and mutagenesis ................................................... 45
  2.3 RNA interference ............................................................................................... 46
     2.3.1 Constitutive ERp57 Knockdown ................................................................. 46
2.3.2 Single target knockdown ................................................................. 46
2.3.3 Combinatorial knockdowns ............................................................ 47
2.4 Western Blots ...................................................................................... 50
2.5 UPR induction and RT-PCR ................................................................. 50
2.6 Confocal Microscopy .......................................................................... 51
2.7 Warfarin inhibition of VKOR & growth curves ..................................... 51
2.8 Metabolic labeling and immunoisolations .......................................... 51
  2.8.1 Standard protocol ........................................................................... 51
  2.8.2 Radiolabeling following reductive (DTT) challenge ....................... 52
  2.8.3 Substrate trapping and sequential immunoprecipitations ............. 54
2.9 Assessment of glutathione levels ......................................................... 54
  2.9.1 Sample preparation ....................................................................... 54
  2.9.2 Colourimetric assay ....................................................................... 55
Chapter 3 .................................................................................................. 56
  3.1 Introduction ........................................................................................ 57
  3.2 Effective knockdown of PDI family members without observable UPR induction .... 58
  3.3 Identification of PDI substrates .......................................................... 60
  3.4 Individual knockdown of PDI family members ................................... 61
  3.5 Combinatorial knockdown of PDI family members ........................... 67
  3.6 Discussion .......................................................................................... 74
Chapter 4 .................................................................................................. 80
  4.1 Introduction ........................................................................................ 81
  4.2 Both PRDX4 and VKOR support cell growth and viability in the face of Ero1 depletion .......................................................... 82
  4.3 VKOR contributes to an oxidizing ER environment under conditions of Ero1 and PRDX4 deficiency ...................................................... 85
List of Tables

Table 1.1  The Protein Disulfide Isomerase Family

Table 2.1  siRNAs used in this study

Table 3.1  Summary of oxidative folding defects accompanying single and combined PDI family member knockdown
List of Figures

Figure 1.1 The Hsp70 cycle
Figure 1.2 Lectin chaperone-mediated folding
Figure 1.3 The Unfolded Protein Response
Figure 1.4 ER Associated Degradation
Figure 1.5 Thiol disulfide exchange reactions
Figure 1.6 PDI domain architecture and structure
Figure 1.7 Domain organization and structures of PDI, ERp57 and ERp72
Figure 1.8 Ero1 oxidation of PDI
Figure 1.9 Known and putative ER oxidant pathways
Figure 2.1 Standard single knockdown protocol
Figure 2.2 Labelling and immunoprecipitation flow
Figure 2.3 The glutathione colourimetric recycling assay
Figure 3.1 Efficacy of PDI family member knockdown and assessment of UPR
Figure 3.2 Identification of PDI substrates
Figure 3.3 Effect of PDI knockdown on oxidative folding of secretory proteins
Figure 3.4 ERp57 depletion delays oxidative folding of glycosylated substrates
Figure 3.5 Combined knockdown of PDI and ERp57 substantially impairs protein folding
Figure 3.6 Misfolded transferrin is degraded by the proteasome in PDI/ERp57-depleted cells
Figure 3.7 Effect of combined PDI and ERp72 knockdown on oxidative folding of albumin
Figure 4.1  Effect of Ero1, PRDX4, QSOX1 and VKOR depletion on cell growth and viability

Figure 4.2  Effects of Ero1, PRDX4, QSOX1 and VKOR depletion on the oxidative folding and secretion of albumin and on ER redox environment

Figure 4.3  Ero1 is the predominant activity responsible for oxidative recovery following DTT treatment

Figure 4.4  Combinatorial knockdowns reveal that PRDX4 and VKOR contribute to recovery following reductive challenge

Figure 4.5  Pathways contributing to oxidative protein folding

Figure 5.1  Unglycosylated transferrin persists upon Ero1 or PDI depletion

Figure 5.2  CypB+C impact on PDI substrate oxidation an ER redox balance
Chapter 1

Introduction:
Disulfide Bond Catalysis in the Endoplasmic Reticulum
1.1 Overview

Disulfide bond formation between cysteine residues is a key covalent interaction in protein folding. Occurring in the specialized environment of the endoplasmic reticulum (ER), this key modification is catalyzed by a number of enzymes that are the focus of this thesis. The protein disulfide isomerase (PDI) family of thiol oxidoreductases is the enzyme family responsible for catalysis of disulfide bond formation, breakage, and rearrangement. For delivery of disulfide-generating capability to the ER, a number of enzymes that drive ER oxidant pathways are also active. This thesis describes the results of assessing the functional relationships among these enzymes.

In this Introduction, ER protein folding will first be outlined to provide the framework in which disulfide formation takes place. Focusing in on some specifics of thiol chemistry, the enzymes of interest, the protein disulfide isomerases, will be addressed. Our current understanding of the founding family member, protein disulfide isomerase, will be detailed, including its structure, functions, and roles in physiology. Additional members of the PDI family will then be introduced, with particular attention to ERp57, ERp72, and P5. In the final section, the enzymes of the ER oxidant pathways, which prime PDI family members for disulfide formation, will be addressed. The best-characterized enzyme of this group, ER oxidoreductin (Ero1) will be described fully and its effect on PDI and the redox status of the small molecule glutathione will be illustrated. Other ER oxidant pathways and their interaction with the PDI family will also be detailed. With this background in place, the questions to be addressed in this thesis and the approach and rationale taken to answer these questions will be outlined.

1.2 ER Protein Folding

Proteins targeted to the secretory pathway first enter this pathway via the endoplasmic reticulum (ER). In this specialized organelle, polypeptides encounter a range of folding factors and undergo a number of post-translational modifications. Successful exit from the ER is generally subject to a degree of quality control such that only those proteins attaining correct folding and modification are released. Further modification can occur in the Golgi before sorting to final destinations such as the cell surface, extracellular secretions, or lysosomes.
Initial directing of a polypeptide to the secretory pathway takes place by recognition of a hydrophobic signal sequence, usually at the N-terminus, on the translating nascent chain. Binding of the signal recognition particle (SRP) sends the ribosome-nascent chain-SRP complex to the SRP receptor at the ER membrane (High, 1995) and the ribosome-nascent chain is then passed on to the Sec61 translocon, an ER membrane pore. Thus, translocation of the growing polypeptide into the ER takes place cotranslationally. In addition to translocation, the nascent polypeptide can undergo a number of other changes cotranslationally including signal sequence cleavage, insertion into the membrane (for membrane proteins; reviewed in (Cross et al., 2009)), Asn-linked glycosylation (Hebert et al., 2005), folding (Woolhead et al., 2004), disulfide bond formation (Chen et al., 1995), and, in certain circumstances, even degradation (Brodsky and Fisher, 2008). It is expected, then, that in some instances protein folding may influence co- or posttranslational modifications while in other cases, co- or posttranslational modifications may influence protein folding (Braakman and Bulleid, 2011).

Although a protein in isolation possesses in its primary structure all the information required for its three-dimensional fold (Anfinsen et al., 1961), the situation faced by a translocating chain is substantially different. Firstly, translation rate (on average 4 to 5 residues per second (Braakman et al., 1991)) occurs far more slowly than in vitro folding and variations in translation rate can greatly influence the final conformation reached by the nascent chain (Frydman et al., 1999; Evans et al., 2008). Secondly, translocation of the polypeptide does not happen in isolation, but rather translation tends to take place on polysomes such that identical polypeptide sequences are simultaneously made in a spatially confined region, increasing the potential for aggregation (Betts and King, 1999; Liu et al., 2001). This is especially notable in cases where partnering residues are found distant from one another in the primary structure; the first partner, for example a hydrophobic residue or a cysteine residue, could interact with a neighbouring polypeptide chain since the second partner has not yet been translated, generating hydrophobic or disulfide linked aggregates, respectively. Thirdly, to overcome this problem, the region surrounding the translocon is populated with ER chaperones (reviewed in (Hebert and Molinari, 2007)) which, although they limit conformational freedom and often slow the rate of folding, also prevent this unproductive aggregation from taking place.
1.2.1 Classical Chaperones

Classical chaperones may be defined as proteins that assist folding of nascent chains by preventing aggregation of unfolded chains by cycles of binding and release, but that are not part of the final polypeptide functional structure, nor imparting structural information. Furthermore, molecular chaperones are increasingly thought to retain folding intermediates in proximity to other folding assistants until their native state is reached (Hebert and Molinari, 2007). Among the classical chaperones, the ER has one member of the Hsp70 family, Grp78 or BiP (Haas and Wabl, 1983), along with several cochaperones of the Hsp40 family (Hendershot, 2004). There is also an abundant member of the Hsp90 family, Grp94 (Lee, 2001), and a member of the Hsp100 family, TorsinA (Breakefield et al., 2001). Although they are not transcriptionally induced upon temperature stress, these ER chaperones are upregulated in response to ER stress conditions (reviewed in (Schroder and Kaufman, 2005) and discussed further in Section 1.3.1).

The Hsp70 chaperone BiP plays a number of important roles in the ER. It maintains the ER permeability barrier by blocking the luminal side of inactive translocons (Alder et al., 2005) and acts as a ratchet to assist translocation of nascent chains (Matlack et al., 1999). In protein folding, it prevents immature protein aggregation by low affinity binding to extended hydrophobic domains, typically with alternating aromatic and hydrophobic residues (Blond-Elguindi et al., 1993) at BiP’s C-terminal peptide binding domain. As shown in Figure 1.1, substrate binding at this C-terminal domain is regulated by the N-terminal ATPase domain; upon ATP hydrolysis, a lid on the peptide binding domain (Fig. 1.1, light pink) clamps shut to generate a tight-binding state. This ATP hydrolysis, as well as delivery of substrate to BiP is activated by the Hsp40 cofactors possessing J-domains (ER J-proteins). Substrate release is dependent on exchange of the ADP in the tight-binding state for ATP to return to the open state and this exchange is assisted by the nucleotide exchange factors, BAP (Chung et al., 2002) and Grp170 (Steel et al., 2004). The binding and release of non-native client proteins to and from BiP slows folding, but improves overall folding efficiency by avoiding non-productive aggregates. Newly synthesized proteins will often encounter and interact with BiP first as they exit the translocon (Hammond and Helenius, 1994; Molinari and Helenius, 2000; Wang et al., 2005a), but BiP can also interact later in a protein’s folding pathway and has been found to be central in a multichaperone complex for bringing a number of folding assistants in close proximity to act on a folding intermediate (Meunier et al., 2002).
Figure 1.1 The Hsp70 Cycle. Starting at (1), a non-native client protein such as a folding nascent chain is bound to J-proteins and recruited to BiP (2). Upon release of the J-protein and ATP hydrolysis (3), the “lid” of the substrate binding domain (light pink) closes for tight binding. The association of a nucleotide exchange factor (NEF; 4) stimulates the release of ADP (5) and the re-binding of ATP (6) to open the lid. Release of the NEF and substrate (7) occurs, allowing the substrate to carry out folding attempts. If folded, the substrate will no longer be bound by BiP, but if unsuccessful in its folding, the substrate can be re-bound and enter the BiP cycle again to prevent aggregation of the intermediate.

(adapted from (Kampinga and Craig, 2010))
The ER Hsp90 family member, Grp94 is highly abundant and is thought to act on client proteins downstream of BiP (Melnick et al., 1994), suggesting that it prefers more maturely folded intermediates. ATP binding and hydrolysis induce conformational changes in Grp94 to modulate its activity and it has been recently implicated in both folding processes as well as in disposal of misfolded proteins (reviewed in (Eletto et al., 2010)). The role of Torsin A is also less understood. It is thought to partition between the ER and the nuclear envelope (Naismith et al., 2004), but has also recently been implication in the degradation of misfolded proteins (Nery et al., 2011; discussed further in section 1.3.2).

1.2.2 Lectin Chaperones

One of the most common modifications to occur in the ER is the glycosylation of specific residues to generate glycoproteins. At least 80% of proteins passing through the ER are modified by covalent addition of preassembled glycans composed of Glc3Man9GlcNAc2 to the side chains of asparagines found in Asn-X-Ser/Thr motifs. This N-linked glycosylation is carried out by the oligosaccharyl transferase (OST) complex (Parodi et al., 1972). Situated in the membrane adjacent to the translocon (Chavan et al., 2006), the multi-subunit OST adds the glycan cotranslationally, once the consensus sequence has emerged about 12 amino acids into the ER (Nilsson and von Heijne, 1993). Two of the terminal glucose residues are rapidly and cotranslationally trimmed off, first by glucosidase I and then by glucosidase II. The remaining monoglucosylated side chains are specifically recognized by another class of ER chaperones, the lectin chaperones calnexin (Cnx) and calreticulin (Crt) (reviewed in (Rutkevich and Williams, 2011)).

Cnx is a type I membrane protein composed of a globular domain that houses its glycan-binding lectin site, as well as an extended proline-rich hairpin arm domain that interacts with the thiol oxidoreductase, ERp57 (Schrag et al., 2001; Leach et al., 2002). As shown in Figure 1.2A, the binding of nascent glycoproteins to Cnx brings them into proximity to this important folding factor (see section 1.4.2.2). Crt is the soluble parologue of Cnx and its composite NMR (Ellgaard et al., 2001) and crystal (Kozlov et al., 2010d; Pocanschi et al., 2011) structures reveal a similar lectin-binding site in its globular domain and a slightly shorter arm domain that maintains its ERp57-binding capacity. In addition to their sugar-mediated binding to folding
Figure 1.2. Lectin chaperone-mediated folding. (A) Cnx and Crt (green) interact with the thiol oxidoreductase ERp57 (dark blue) to bring a folding polypeptide intermediate (light blue) into proximity of the folding factor. Lectin-site residues are shown in red. (B) The Cnx/Crt cycle: Cnx or Crt interact with monoglucosylated substrates via sugar and/or polypeptide sites. Near-native glycoproteins are re-glucosylated by UGGT, but terminally misfolded glycoproteins are targeted for degradation (Rutkevich and Williams, 2011).
polypeptides, there is increasing evidence that both Cnx and Crt possess a specific polypeptide-binding site, that recognizes non-native folding conformers in a manner similar to classical chaperones (Brockmeier and Williams, 2006; Ireland et al., 2008; Brockmeier et al., 2009). Those nascent chains that have N-linked glycosylation sites close to their N-terminus rapidly and cotranslationally interact with Cnx, even bypassing interaction with BiP in some cases (Molinari and Helenius, 2000). When a nascent glycoprotein is bound to Cnx or Crt, however, folding is arrested and it is during the “off” phase that attempts at folding are carried out. Upon release from Cnx or Crt, the final glucose is removed by glucosidase II, preventing reassociation with the lectin chaperone. As shown in Figure 1.1B, if folding takes place successfully in this off phase then the protein can move along the secretory pathway. However, some substrates require multiple rounds of association with these lectin chaperones before their correct fold is found (Solda et al., 2007). The re-glucosylation of the Man$_9$GlcNAc$_2$ is performed by the enzyme UDP-glucose:glycoprotein glucosyltransferase (UGGT) and results in the Cnx-binding form of the glycan. Thus UGGT is a folding sensor in this cycle, recognizing exposed hydrophobic patches, and notably, will only reglucosylate if the intermediate is nearly-native; grossly misfolded intermediates are not acted upon and are targeted instead for degradation (Section 1.3.2; (Caramelo et al., 2003; Caramelo et al., 2004).

If Cnx/Crt function is impaired in cells, for example by inhibition of the glucosidases that generate the monoglucosylated glycan, there is more rapid folding, but it is frequently incorrect, leading to more aggregation and loss of quality control of incompletely folded substrates (Jackson et al., 1994; Hebert et al., 1996; Vassilakos et al., 1996; Molinari et al., 2004). In addition, the loss of Cnx or Crt in knockout mice results in cells viable in culture, but embryonic lethality in the case of Crt (Mesaeli et al., 1999) and neurologically abnormal and smaller mice in the case of Cnx (Kraus et al., 2010). The importance of the Cnx/Crt cycle is linked to how dependent a given nascent chain is to the cycle for its productive folding; in some cases alternative ER folding pathways support normal nascent chain folding when the Cnx or Crt cycle is removed (e.g. (Allen and Bulleid, 1997)), while in other cases, productive glycoprotein folding is significantly impaired (Vassilakos et al., 1996; Molinari et al., 2004).

By these knockout and inhibition studies the roles of Cnx and Crt in their binding cycle have been increasingly well defined. Rounds of interaction with Cnx/Crt, either via lectin mediated binding or by polypeptide-based interaction, result in general slowing in the folding of
the polypeptide intermediate, but improve the overall efficiency of the process. This is due to the sequestration of the nascent chain from aggregation with other folding intermediates (Helenius and Aebi, 2004). The Cnx/Crt cycle also retains misfolded substrates in the ER until their native state is reached, thereby ensuring quality control of the proteins exiting the folding-proficient environment of the ER (reviewed in (Hebert and Molinari, 2007)). The recruitment of ERp57 by Cnx/Crt has also been shown to facilitate disulfide formation and folding in glycoproteins utilizing the Cnx/Crt cycle both in vitro (Zapun et al., 1998), and in cells (Zhang et al., 2006; Jessop et al., 2009a).

In addition to working on glycoproteins in general folding, the Crt/ERp57 pair is also vital in a specialized ER complex required for loading high affinity peptides onto Major Histocompatibility Complex Class I (MHC-I) proteins. MHC-I traffics to the cell surface where it presents peptide to cytotoxic T-cells, reporting on the presence of viral infection or of tumor-associated factors requiring an immune response. MHC-I acquires peptides within the Peptide Loading Complex (PLC), which consists of MHC-I bound to Crt/ERp57, another protein tapasin, and an ABC peptide transporter known as TAP (reviewed in (Chapman and Williams, 2010)). Importantly, ERp57 associated with Crt also interacts with tapasin, which greatly stabilizes the PLC and assists in peptide loading (Dong et al., 2009); discussed further in Section 1.4.2.2).

1.2.3 Folding Catalysts

In addition to the classical and lectin chaperones that generally slow folding, but increase its efficiency, a polypeptide translocated into the ER encounters a number of folding catalysts that speed up reactions that would otherwise occur too slowly, namely disulfide bond formation and rearrangement and the cis-trans isomerization of proline residues. Disulfide formation will be covered in detail in Section 1.3.

Proline isomerization is carried out by a family of enzymes known as the peptidyl-prolyl isomerases (PPIs) (Fischer et al., 1984; Fischer et al., 1989). Although most peptide bonds in native proteins are found in the trans conformation, X-P peptide bonds can be found in both cis and trans conformations and the switch from one to the other is a rate-limiting step in some protein folding pathways (Kiefhaber et al., 1990). It can also serve as an activation/inhibition signal in natively folded proteins as significant conformational changes can accompany the cis-
trans switch. NMR studies have revealed the structural consequences of proline isomerization in a number of native proteins including HIV-1 capsid protein and interleukin-2 tyrosine kinase (reviewed in (Andreotti, 2003). Furthermore, the PPI Pin1 has been characterized in its ability to carry out specific proline isomerization of select target proteins to regulate a number of cellular processes including cell cycle, immune response, and neuronal differentiation (Lu and Zhou, 2007). The search for other PPI-mediated functional switches is beginning to reveal the importance of this regulation mechanism in cell signalling autoinhibition, gene expression, and ion channel dynamics (Lu et al., 2007).

The mammalian ER contains a number of PPIs including three members of the FK506-binding proteins (FKBPs), as well as cyclophilin B (CypB). Although in vitro work has shown the ability of these enzymes to speed folding of client proteins (e.g. (Jansen et al., 2012)), evidence of their role in cells is indirect. For example, the FKBPs are largely characterized as interacting with BiP and BiP substrates (Davis et al., 1998; Wang et al., 2007a), while CypB has been found both in large multichaperone complexes with BiP and ERp72 (Meunier et al., 2002; Zhang and Herscovitz, 2003), as well as with Cnx and Crt via the same interaction site used by ERp57 (Kozlov et al., 2010b). Since many nascent chains passing through the ER, as well as resident proteins of the secretory pathway, possess cis-prolines, it is expected that these ER PPIs carry out their isomerization and may gain access to their substrates via these chaperone interactions.

1.3 ER Protein Misfolding

Although the ER contains chaperones and folding catalysts to assist protein folding, as outlined above, misfolding of nascent polypeptides is still common and the ER has developed various means for sensing and coping with a higher unfolded protein load. These processes are known as the unfolded protein response (UPR) and ER associated degradation (ERAD). Their activation occurs routinely in physiological processes such as during conditions of high secretory activity (e.g. in plasma cells and pancreatic beta cells; (Wu and Kaufman, 2006), under hypoxic conditions (Gess et al., 2003), or when dealing with a mutated and misfolding protein (reviewed in (Schroder and Kaufman, 2005)). Activation of these pathways is also induced artificially by various means of perturbing ER homeostasis including treatment with drugs to deplete ER calcium, preventing glycoprotein entry into the Cnx/Crt cycle, or sustained reduction of the ER.
1.3.1 Unfolded Protein Response

The UPR is a collection of signalling pathways that sense ER folding capacity and communicate changes in this capacity to gene expression programs (reviewed in (Ron and Walter, 2007)). In general, upon activation, translational control and mRNA decay reduce the ER protein folding load, the ER membrane itself is expanded to accommodate higher protein loads (Schuck et al., 2009), ER folding and degradation machinery is upregulated to increase folding and disposal capacities, and, if homeostasis cannot be attained, apoptosis is activated (reviewed in (Schroder and Kaufman, 2005)).

In metazoans, there are three branches of the UPR that have been characterized, defined by their ER transmembrane signalling components. As summarized in Figure 1.3, these are IRE1 (the only branch conserved in yeast), PERK, and ATF6, each of which registers ER stress in the ER lumen and transmits that signal across the membrane to eventually produce b-ZIP transcription factors for UPR target gene activation. In all cases, initial evidence indicated that these signalling proteins are maintained in an inactive state by association with BiP, but when unfolded proteins accumulate in the ER, competition for BiP shifts the ER BiP population away from the UPR sensing proteins and permits their activation (Bertolotti et al., 2000; Shen et al., 2002; Liu et al., 2003). However, more recent evidence with IRE1 mutants unable to bind BiP showed that regulated UPR activation still occurred (Kimata et al., 2004) and, in agreement with recent IRE1 crystal structures (Credle et al., 2005; Gardner and Walter, 2011), supported a new model in which IRE1 and PERK bind directly to unfolded proteins. In the case of ATF6, inactivation by BiP may be additionally augmented by glycan-dependent interaction with Crt (Hong et al., 2004) and redox sensing via intra- and intermolecular disulfide bonds in its luminal domain (Walter and Ron, 2011).

In the case of IRE1, its activation by unfolded proteins leads to its dimerization and oligomerization, transautophosphorylation, and activation of its cytosolic ribonuclease function. Cleavage of the mRNA for the UPR-specific transcription factor, XBP1, by IRE1 generates a spliced XBP1\textsuperscript{s} mRNA and its translation product is a potent transcription factor. XBP1\textsuperscript{s} upregulates the expression of a number of genes including those for ER chaperones, lipid synthesis, ERAD, and ER redox enzymes (reviewed in (Schroder and Kaufman, 2005)). Interestingly, recent evidence has also suggested that the metazoan IRE1 is capable of lowering
Figure 1.3 The Unfolded Protein Response. The three branches of the UPR are shown: (A) IRE1 signalling generates cleavage of Xbp-1 mRNA and the downstream effects of the Xbp-1 protein. (B) PERK signalling results in translational control to decrease ER protein load. (C) ATF6 signalling relies on controlled proteolysis in response to stress and downstream upregulation of UPR target genes.

Adapted from (Walter and Ron, 2011)
ER protein load by clipping of ER-localized non-XBP1 mRNAs. Termed RIDD (regulated IRE1-dependent decay; (Hollien and Weissman, 2006; Hollien et al., 2009), this promiscuous cleavage by IRE1 is currently thought to be linked to a possible gradient of IRE1 activation, where RIDD occurs early in UPR sensing to lower protein load without triggering a full transcriptional program change.

The other early UPR signalling pathway is that mediated by PERK. Like IRE1, PERK dimerizes and autophosphorylates upon activation, but its only defined role is to specifically phosphorylate eIF2α. The downstream effect of this phosphorylation is general translational attenuation to reduce folding load, but also specific expression of the transcription factor ATF4. The genes upregulated by ATF4 include XBP1 (the target of IRE1) and the transcription factor CHOP. The target genes of CHOP include GADD34, to release translational attenuation by dephosphorylation of eIF2α, redox enzymes, and proteins involved in signalling cell death. It is thought that only upon extended UPR, due to unresolved ER stress, do CHOP levels become sufficiently high to trigger apoptosis (reviewed in (Schroder and Kaufman, 2005)).

Finally, the ATF6 branch is unique in that binding by BiP retains this transmembrane protein in the ER. Upon ER stress and BiP release, the ATF6α isoform is trafficked to the Golgi where it encounters two specific proteases that clip a cytosolic transcription factor domain, ATF6(N) from its membrane tether. Upon reaching the nucleus, ATF6(N) activates UPR genes such as those for XBP1, ER chaperones, and lipid synthesis (Schroder and Kaufman, 2005).

1.3.2 ER Associated Degradation

Another means of maintaining ER protein homeostasis (as reviewed in (Roth and Balch, 2011)), is to dispose of terminally misfolded proteins. The machinery required for ERAD is upregulated in the IRE1 branch of the UPR, but degradation of ER proteins occurs constitutively and, in some cases, may attenuate the need for a UPR. The three main stages of ERAD are the recognition of misfolded substrates for targeting to the ERAD pathway, retrotranslocation from the ER to the cytosol, and ubiquitylation and degradation by the proteasome (Vembar and Brodsky, 2008). The process of ERAD is not only important for proteostasis in healthy cells, but also because it is a frequent target of viral proteins in host immune evasion. For example, proteins of the human cytomegalovirus highjack ERAD machinery to promote degradation of
MHC-I molecules to avoid detection by cytotoxic T-cells (as reviewed in (Chapman and Williams, 2010)).

Called the central organizers of ERAD (Figure 1.4), the E3 ubiquitin ligases of the ER are responsible for catalysis of cytosolic ubiquitylation of substrate proteins by their RING finger domain (Carvalho et al., 2006). Embedded by a number of transmembrane domains, E3s coordinate the ERAD machinery on both sides of, and within, the membrane (Carvalho et al., 2010). On the cytosolic side, membrane proteins, as well as luminal ERAD targets retrotranslocated via Derlins and/or Sec61, are extracted via the AAA ATPase, p97, N-deglycosylated by peptide N-glycanase (PNGase), and delivered to the proteasome. The upstream events of recognition and delivery of substrate to the E3 complexes are, however, far more complex (Denic et al., 2006). Studies in yeast indicated that misfolded ERAD substrates are directed to different E3 complexes depending on the location of the domain that is misfolded; luminal and membrane misfolding sites resulted in targeting to the E3, Hrd1p, while substrates with cytosolic misfolding sites were directed to the Doa10p E3 complex (Carvalho et al., 2006). The metazoan version of Hrd1p is known (Hrd1) and well characterized, but a definitive counterpart of Doa10p has remained elusive. A number of E3 accessory proteins are involved in recognizing and bringing substrate proteins to the E3 complex.

The best-characterized recognition signals that a nascent chain is terminally misfolded and requires targeting to ERAD involve those utilizing changes in glycosylation state. As described in Section 1.2.2, deglucosylation and reglucosylation of the terminal glucose residue of N-linked glycoproteins mediates their access to the Cnx/Crt cycle. In competition with UGGT, mannosidases begin to clip mannose residues from the Man\(_9\)GlcNAc\(_2\) glycan. Importantly, this is followed by slower removal of a specific mannose residue by EDEMs, generating a key signal for degradation. Since the generation of this signal is relatively slow compared to productive folding processes, it is thought that native proteins avoid this trimming to escape ERAD. Other important E3 accessory proteins SEL1L, OS9, and XTP3-B are involved in recognition of mannose-trimmed glycans, although glycan-independent interactions have also been reported (reviewed in (Smith et al., 2011)). Nonglycosylated substrate recognition and delivery is less clear, but the chaperone BiP, as well as an ER transmembrane protein HERP, have been suggested for these functions (Okuda-Shimizu and Hendershot, 2007). Additional processing
Figure 1.4  ER Associated Degradation. (1) Newly synthesized glycoproteins interact with Cnx and/or Crt and ERp57 for productive folding and disulfide formation. If the correct fold is attained, the native protein is released for secretion (2). Non-native polypeptides are retained in the Cnx/Crt cycle by UGGT (3). If terminal mannose residues are cleaved (yellow glycans) to become signals for further mannose cleavage by EDEMs (4; red glycans) then the polypeptide is directed to ERAD. For membrane-bound ERAD substrates with the misfolding signal in the ER lumen (5L_M) more than one pathway is available, but for soluble ERAD substrates (5L_S) the OS-9, SEL1L, HRD1 pathway is used. The identity of the dislocation pore is still debated (as indicated by “?”), but following retrotranslocation, degradation is carried out by the proteasome.

Adapted from (Bernasconi and Molinari, 2011).
that occurs prior to retrotranslocation and degradation is the reduction of disulfide bonds. PDI (Sakoh-Nakatogawa et al., 2009) as well as another family member, ERdj5 (Cunnea et al., 2003; Dong et al., 2008), have been implicated in this function and, importantly, ERdj5 has been found to interact with EDEMs as well as BiP by its J-domain (Ushioda et al., 2008).

The folding of polypeptide chains, and the results of their misfolding, are impacted by a number of protein networks such as BiP and its associated chaperones and catalysts, as well as Cnx/Crt and ERp57. Both productive polypeptide folding and ERAD-mediated unfolding are intimately linked to the formation and breakage of disulfide bonds when the folding intermediate possesses cysteine residues. These processes are carried out and scrutinized by various members of the PDI family of thiol oxidoreductases, often in concert with other chaperone, signalling, or degradation complexes. It is to this family and the reactions catalyzed that focus will now be directed.

1.4 Disulfide Bonds in the ER

In the ER, disulfide bonds are formed (oxidation), broken (reduction), and rearranged (isomerization). The most common means of carrying out these reactions is by thiol-disulfide exchange using cysteine/cystine pairs, in which the oxidized protein becomes reduced (Figure 1.5A, white oval) and the reduced protein becomes oxidized (Figure 1.5A, grey rectangle), as electrons are transferred from the reduced protein to the oxidized protein. One can think of this net change either as transfer of the disulfide bond “forward” to the originally reduced protein or as transfer of electrons “backward” to the originally oxidized protein. When one of the proteins acted upon is designated the substrate (S), then the net oxidation, reduction or isomerization of the substrate protein requires a specific redox state of its thiol oxidoreductase catalyst (E) (Figure 1.5B) and this redox state is determined by the chemical characteristic of the thiol oxidoreductase, as well as the environment in which it is found. The ER thiol oxidoreductases such a PDI possess CXXC motifs that carry out these exchanges. In order for a PDI family member to act as an oxidase, the CXXC motif must be oxidized. For reduction or isomerization activities, the CXXC motif must be reduced. Additionally, for reformation of the CXXC disulfide, PDIs must be acted upon by an oxidant to remove two electrons (Figure 1.5C).
**Figure 1.5.** Thiol disulfide exchange reactions. (A) Attack by the thiolate anion of the reduced partner (grey) onto the disulfide bond of the oxidized partner (white) generates a mixed disulfide intermediate. Resolution of the mixed disulfide by the other cysteine of the originally reduced partner (grey) completes the exchange of the disulfide bond. However, an escape pathway occurs if the newly free cysteine belonging to the white protein attacks the disulfide bond, such that no disulfide exchange occurs (reverse arrow at *). (B) When the CXXC of a thiol oxidoreductase enzyme (E) is oxidized, it can donate its disulfide to the substrate (S) protein to carry out net oxidation, but E becomes reduced in the process. When E is reduced, it can carry out reduction of S, and E becomes oxidized. For isomerization, E is reduced, but no net change in disulfide content in E or S takes place. (C) CXXC motifs used for oxidation reactions can be reoxidized by ER oxidant pathways.
1.4.1 Thiol Chemistry

A cysteine residue bears a thiol group in which the sulfur atom is found in the -2 oxidation state. The oxidation of two thiol groups forming a disulfide bond increases the oxidation state of both sulfur atoms to -1 and two electrons and two protons are transferred to the oxidant. Although molecular oxygen is thermodynamically capable of carrying out this oxidation, it is kinetically slow and is not observed in vivo (reviewed in (Mamathambika and Bardwell, 2008)). Rather, proteins such as PDI and peptides such as oxidized glutathione, GSSG, transfer their disulfides to substrate proteins. Other oxidized forms of cysteine thiol groups are formed by reaction of cysteines with reactive oxygen species (ROS) and are found in vivo. These include cysteine sulfenic acid (\(-\text{SOH}\)), used in intracellular signaling (Poole et al., 2004) and intermediate in the peroxiredoxin cycle (Section 1.4.3.2) and cysteine sulfenic acid (\(-\text{SO}_2\text{H}\)), found as an intermediate in cysteine metabolism and a potential agonist of some cellular receptors (Stipanuk et al., 2006).

In thiol exchange reactions, nucleophilic attack on the existing disulfide must take place. Although thiol groups are nucleophiles, the thiolate anion (\(-\text{S}^-\)) is a much stronger nucleophile. Thus thiol exchange reactions can be effectively arrested by acidification of the environment to prevent deprotonation of thiols, or conversely, modulation of a thiol pKa by its immediate environment will shift the equilibrium of ionization to alter the relative reactivity of a given cysteine (reviewed in (Hatahet and Ruddock, 2009)). The pKa of cysteine residues is typically cited as 8.3 (the pH where the concentration of protonated and deprotonated forms is equal), but this is greatly influenced by its microenvironment. For example, a neighbouring positive charge would stabilize the thiolate anion and effectively lower the cysteine pKa while a neighbouring negative charge would be destabilizing and raise the pKa. This is exemplified in the case of glutathione, where the local negative charge on this tripeptide results in a higher measured pKa at 8.75 and in PDI, where local environment lowers the pKa of its N-terminal active site cysteines to less than 5.5 (Hawkins and Freedman, 1991); discussed further in Section 1.4.2.1). The effect of microenvironment is an important consideration, not only for the thiol exchange catalyst, but also for the polypeptide substrate since the reactivity of its cysteines may be determined by neighbouring residues (Hatahet & Ruddock, 2009) and may change with alterations in its intermediate folding conformations.
1.4.2 The Protein Disulfide Isomerase Family

To catalyze thiol-disulfide exchange reactions, cells use enzymes of the protein disulfide isomerase (PDI) family. PDI itself is the founding family member and was one of the first folding catalysts reported (Venetianer and Straub, 1963); (Goldberger et al., 1963). PDI is able to carry out oxidase, reductase, and isomerase activities, but a number of other PDI family members are found in the mammalian ER that seemingly overlap these functions. Although relatively few substrates have been examined in detail, it appears that disulfide isomerization is a slow process that may be rate-limiting in the folding of certain proteins. For example, in vitro studies on the oxidative folding of bovine pancreatic trypsin inhibitor revealed that partially disulfide-bonded intermediates become kinetically trapped during folding and that isomerization of disulfides was the main process responsible for the >1000-fold enhancement in folding rate observed in the presence of PDI (Weissman and Kim, 1993).

The published sequence of rat PDI (Edman et al., 1985) showed regions homologous to thioredoxin and, using the criterion of thioredoxin-like domains, additional PDI family members were characterized soon after (Freedman et al., 1994). Today, as many as 20 proteins have been assigned to the mammalian PDI family (Table 1.1) based on ER localization and the presence of at least one thioredoxin-like domain (Kozlov et al., 2010c). Remarkably, most of these family members are ubiquitously expressed with only PDILT (van Lith et al., 2005) and PDIp (Desilva et al., 1996) showing tissue specificity for testes and pancreatic cells, respectively. As shown in Table 1.1, however, domain arrangements among PDI family members can vary greatly. The catalytic activity is localized in the $\alpha$ and $\alpha'$ domains and catalytic activity centers on a pair of cysteine residues within a CXXC motif that is able to alternate between disulfide and dithiol forms (Appenzeller-Herzog and Ellgaard, 2008). For some PDI family members, this motif has cysteine to serine substitutions. Additional thioredoxin domains without catalytic activity are termed the $b$ and $b'$ domains (Kemmink et al., 1997), and a number of PDI family members possess only these domains and therefore do not actually participate in thiol disulfide reactions. In PDI and a few other family members, a linker region between domains has been characterized as the $x$-linker region ($x$) and each of the TMX family members have a transmembrane domain. As described below, the structures and roles of each domain in PDI have been investigated.
Table 1.1 The Protein Disulfide Isomerase Family

<table>
<thead>
<tr>
<th>Protein</th>
<th>Domains</th>
<th>Active site</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDI</td>
<td>$abb'xa'$</td>
<td>CGHC, CGHC</td>
<td>O, R, I, C</td>
</tr>
<tr>
<td>ERp57</td>
<td>$abb'xa$</td>
<td>CGHC, CGHC</td>
<td>O, R, I</td>
</tr>
<tr>
<td>ERp72</td>
<td>$a^aabb'xa'$</td>
<td>CGHC, CGHC, CGHC</td>
<td>O, R, I</td>
</tr>
<tr>
<td>P5</td>
<td>$aa'b$</td>
<td>CGHC, CGHC</td>
<td>O, R, I</td>
</tr>
<tr>
<td>ERp44</td>
<td>$abb'$</td>
<td>CRFS</td>
<td>Ero1 binding</td>
</tr>
<tr>
<td>ERdj5</td>
<td>$Ja''{}a''{}ba^oa'a$</td>
<td>CSHC, GGCD, CPPC, CHPC, CGPC</td>
<td>R</td>
</tr>
<tr>
<td>PDlp</td>
<td>$abb'xa'$</td>
<td>CGHC, CTHC</td>
<td>O, R, I, C</td>
</tr>
<tr>
<td>PDILT</td>
<td>$abb'xa'$</td>
<td>SKQS, SKKC</td>
<td>Ero1 binding</td>
</tr>
<tr>
<td>PDlr</td>
<td>$ba^oa'a$</td>
<td>CSMC, CGHC, CPHC</td>
<td>O, R, I</td>
</tr>
<tr>
<td>ERp46</td>
<td>$a^oa'a$</td>
<td>CGHC, CGHC, CGHC</td>
<td>?</td>
</tr>
<tr>
<td>ERp18</td>
<td>$a$</td>
<td>CGAC</td>
<td>O, VKOR binding</td>
</tr>
<tr>
<td>hAG2</td>
<td>$a$</td>
<td>CPHS</td>
<td>?</td>
</tr>
<tr>
<td>hAG3</td>
<td>$a$</td>
<td>CQYS</td>
<td>?</td>
</tr>
<tr>
<td>TMX</td>
<td>$at$</td>
<td>CPAC</td>
<td>R, VKOR binding</td>
</tr>
<tr>
<td>TMX2</td>
<td>$ta$</td>
<td>SNDC</td>
<td>?</td>
</tr>
<tr>
<td>TMX3</td>
<td>$abt$</td>
<td>CGHC</td>
<td>O</td>
</tr>
<tr>
<td>TMX4</td>
<td>$at$</td>
<td>CPSC</td>
<td>O, R, VKOR binding</td>
</tr>
<tr>
<td>TMX5</td>
<td>$at$</td>
<td>CRFS</td>
<td>?</td>
</tr>
<tr>
<td>ERp29</td>
<td>$b$</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>ERp27</td>
<td>$bb'$</td>
<td>-</td>
<td>?, ERp57 binding</td>
</tr>
</tbody>
</table>

A great number of PDI family members are apparently concurrently found in the mammalian ER and the puzzle of why so many are present, particularly when PDI itself is capable of carrying out all three redox functions, is a question that is yet unsolved. PDI family members could conceivably specialize in certain functions or they may recognize distinct folding conformers and so display substrate specificity. In the following sections, those PDI family members shown to have oxidase, reductase, and isomerase activity, namely PDI, ERp57, ERp72, and P5, will be examined in more detail to reveal what is currently known about the structural basis of their action and substrate recognition.

1.4.2.1 PDI

The structure of yeast PDI reveals a U-shaped orientation of its four thioredoxin-like domains in an \textit{abb'xa'} configuration (Figure 1.6A, B; (Tian \textit{et al.}, 2006), but no full length mammalian PDI structure has yet been solved. Although mammalian PDI and some other PDI family members can functionally replace yeast PDI (Gunther \textit{et al.}, 1993), human and yeast PDI exhibit only 29.1\% sequence similarity, with the greatest differences at the \textit{bb'} domains. Furthermore, human PDI is not glycosylated while yeast PDI is highly glycosylated (Hatahet and Ruddock, 2009). However, a number of structures of individual and coupled domains of mammalian PDI (Kemmink \textit{et al.}, 1996); (Denisov \textit{et al.}, 2009) (Kemmink \textit{et al.}, 1997; Nguyen \textit{et al.}, 2008), as well as small x-ray scattering studies of full length human PDI (Li \textit{et al.}, 2006), have provided an ensemble of structural data of the mammalian enzyme that reveals strong similarity to the yeast crystal structure.

For the catalytic \textit{a} and \textit{a'} domains, the active site CXXC motif lies near the start of the second alpha helix of the thioredoxin fold (\textit{βαβαβαβαβ}) with the N-terminal cysteine of the motifs (human PDI: Cys53 and Cys397) relatively more solvent exposed than the C-terminal cysteine (Cys56 and Cys400). Functionally, this placement has been linked to differences in the relative reactivities of the N- and C-terminal cysteines. The N-terminal cysteines are more active in nucleophilic attack as their pKa’s are significantly lower (Hawkins and Freedman, 1991) and the C-terminal cysteines are less active as their pKa’s are significantly higher (Lappi \textit{et al.}, 2004) than typical cysteine residues. The stabilization of the thiolate anion at the N-terminal cysteines is thought to be a function of the dipole moment of the alpha helix (Kortemme and Creighton, 1995), the His residues (55, 399) within the CXXC motifs, and, as shown by NMR of the \textit{a}
Figure 1.6 PDI domain architecture and structure. (A) The domain arrangement of PDI is shown (abb’xa’) with residue numbers based on the mature human PDI and (B) the yeast crystal structure. Colouring of the domains and ribbon diagram match such that the a domain is in orange, the b domain is in dark blue, the b’ domain is in light blue, the x-linker is in black, and the a’ domain is in yellow. The C-terminal extension, housing the ER retention signal KDEL is also shown in red. In both the a and a’ domains, the active site cysteines are highlighted in green. The eye shown at (*) indicates the direction of viewing for the structure in (C): The human b’ domain of PDI is shown with stick representations of the hydrophobic amino acids required for substrate binding.

Adapted from (Gruber et al., 2006) (A, B) and (Denisov et al., 2009) (C)
domain, electrostatic stabilization by Glu47, Lys57, and Lys81 (Kemmink et al., 1996). The activity of the C-terminal cysteine has been proposed to be modulated by dynamic shift of conserved basic residues into the vicinity of the active site. In the case of PDI, and many other PDI family members (Ellgaard and Ruddock, 2005), Arg120 has been shown by molecular dynamic simulations to perform this function (Lappi et al., 2004), effectively reducing the pKa of the C-terminal cysteine to 6.1. Its ionization is required when the PDI active site needs to be oxidized as it resolves the mixed disulfide between PDI and its oxidant (recall Fig. 1.5C). In support of this model, mutation of this residue results in oxidation-deficient PDIs (Lappi et al., 2004). The proton transfer that occurs upon ionization of the active site cysteines is also thought to be supported by a relay of charged-pair residues that are conserved among PDI family members (Ellgaard and Ruddock, 2005). Although not conserved in yeast PDI (Tian et al., 2006), these residues have been shown to be important for the activity of thioredoxin (Dyson et al., 1997). It is uncertain how frequently the C-terminal cysteines attack a PDI-substrate mixed disulfide to result in the unproductive “escape pathway” (Fig. 1.5A), but mutation of the CXXC motifs to CXXA or CXXS results in stabilization of the mixed-disulfide intermediate by eliminating the escape pathway.

In the catalytically active domains, there are two other features that are conserved among PDI family members. One is a conserved proline residue (61, 405) in helix-2 that is structurally important (Tian et al., 2006) and the second is a cis-proline residue (100, 441) found at the start of $\beta$4 and adjacent to the active site. When the cis-Pro is mutated to alanine in yeast PDI, catalytic activity is lost (Tian et al., 2006), but when mutated to threonine in human PDI family member TMX, substrates become disulfide trapped (Matsuo et al., 2009), suggesting that this cis-Pro may play a role in substrate release. Since substrates are trapped, this mutation may prevent the ionization (and thus lower the activity) of the C-terminal cysteine, inhibiting the escape pathway (Fig. 1.5A) and prolonging CXXC-substrate interaction. Interestingly in an enzyme of bacterial thioredoxin superfamily, DsbA, the residue just preceding this cis-Pro has been found to be critical in modulating the ionization and activity of the N-terminal cysteine of DsbA (Ren et al., 2009), but no corresponding mutational analysis of PDI family members have yet been carried out.

The b' domain appears to be the main site for substrate binding and for the reported chaperone functions of PDI (Klappa et al., 1998a; Horibe et al., 2004). The initial crystal
structure of yeast PDI revealed a twisted U-shaped conformation (Tian et al., 2006; Fig. 1.6B). The two catalytic domains point towards the middle of the “U” and hydrophobic residues on the interior face of the “U” within the $b'$ domain, as well as in other domains, mediate binding to non-native protein substrates (Byrne et al., 2009; Denisov et al., 2009). In a second crystal structure, yeast PDI assumes a boat-like conformation with the $b$ and $b'$ domains forming a relatively rigid base and the $a$ domain exhibiting significant mobility (Tian et al., 2008). This suggests some flexibility in PDI which presumably expands the range of substrate molecules that can be accommodated in the binding cavity. Indeed, restricting the flexibility of the $a$ domain by introducing interdomain disulfides impairs catalytic activity (Tian et al., 2008). Subsequent NMR structures of the PDI $b'$ (Byrne et al., 2009), $bb'$ (Denisov et al., 2009) and $b'x$ (Nguyen et al., 2008) segments illustrate that the $b'$ domain comprises a relatively large binding site with a number of redundant residues that recognize a wide range of unfolded and relatively hydrophobic substrates rather than a particular sequence (Byrne et al., 2009). In fact, direct interactions between a hydrophobic pocket in the $b'$ domain of PDI spanning residues 240-320 and the amphipathic peptides mastoparan and somatostatin, as well as unfolded RNase A, have been documented recently in NMR titration studies (Denisov et al., 2009). Additionally, the ligand binding ability of the $b'$ domain is thought to be modulated by the $b$ domain (Byrne et al., 2009) and access to this site is thought to be gated by the $x$-linker region (Nguyen et al., 2008), particularly Met356, Leu360, and Trp364. Although the precise regulation of the proposed $x$-linker movement is unknown, NMR and small angle x-ray scattering studies (Nakasako et al., 2010; Serve et al., 2010; Wang et al., 2012a) have indicated that domain movements, particularly at the $a'$ domain, are redox-regulated, as suggested in earlier studies on the PDI-dependent unfolding of cholera toxin (Tsai et al., 2001; Tsai and Rapoport, 2002). The domain movement of the $a'$ domain relative to the $abb'$ domains is speculated by Hatahet and Ruddock (2009) to induce conformational change of the bound substrate for the purpose of gaining access to buried substrate cysteines. However, no additional experimental evidence has yet been reported to support this hypothesis.

The advancement of PDI domain structures has further aided our understanding of the contribution of PDI’s domains to its functions. For example, although both $a$ and $a'$ domains are capable of carrying out thiol disulfide exchange reactions, their activity in isolation is relatively poor compared to that observed for the full length molecule in vitro (Darby et al., 1998). Rather,
any combination of domains that included the \( b' \) domain with a catalytic domain (i.e. \( abb' \) or \( b'xa' \)) was the minimal arrangement required for efficient action on substrate. The \( b' \) domain is clearly key for the recognition of substrate and likely for its correct positioning relative to the active sites. In addition to the thiol disulfide exchange reactions carried out by PDI, it has also been shown to possess chaperone activity (Wang and Tsou, 1993). Via the hydrophobic groove on its \( b' \) domain, PDI interacts with non-native folding polypeptides and it has been shown to assist the refolding of substrates with (Puig and Gilbert, 1994; Winter et al., 2002) and without (Cai et al., 1994; Song and Wang, 1995) disulfide bonds. Although this function may be merely the result of opportunistic association of exposed hydrophobic patches, a recent study has highlighted the functional relevance of PDI’s chaperone activity as its substrate binding ability, but not its catalytic activity was essential for promoting virally-induced degradation of MHC-I (Lee et al., 2010). Working in concert with the \( b' \) domain, a reconstitution study has lately implied that the \( a' \) domain may have greater catalytic activity than the \( a \) domain (Wang et al., 2009). The basis of this difference is uncertain as the chimeric proteins used (e.g. \( abb'xa' \)) controlled for differences in the spatial proximity between the primary substrate recognition site and a catalytic domain, or to the proposed domain movements described above that could allow the \( a' \) domain more flexibility in the substrates it could access. Further functional assessment of the cooperation among PDI domains is needed to clarify these issues.

In both catalytic domains, the oxidase and isomerase activities of PDI have been well-characterized \textit{in vitro} (Lyles and Gilbert, 1991b, a), but each of these activities require opposite starting redox statuses of the active site cysteines. To act as an oxidase, the active site cysteines must be oxidized (disulfide bonded) and to act as an isomerase (or reductase) the cysteines are themselves reduced (dithiol; recall Fig. 1.5B). The form of PDI that is found in cells at steady state may indicate the preferred function that PDI is carrying out. In yeast, PDI is found predominantly in the oxidized form at steady state (Frand and Kaiser, 1999), but mammalian PDI has been detected in both forms (Appenzeller-Herzog and Ellgaard, 2008), although the majority appears to be in the reduced form (Mezghrani et al., 2001). These steady state assessments could be interpreted to indicate that yeast PDI acts more readily in oxidation reactions, while mammalian PDI is more likely to work as an isomerase or reductase. However, the \textit{in vivo} activities of both oxidation and isomerization by PDI have been confirmed in yeast (Laboissiere et al., 1995; Kulp et al., 2006), and in mammalian systems (Bulleid and Freedman, 1988). It is
likely that oxidizing or reducing equivalents (i.e. disulfide bonds or electrons for mammalian or yeast PDI, respectively) are rapidly transferred to and from PDI as needed and that PDI is indeed functional in both disulfide bond formation and isomerization in vivo. The question of how significant PDI’s role is in comparison to other PDI family members is still under consideration.

In S. cerevisiae there are five PDI family members of which only PDI is essential. Deletion studies revealed little functional interchangeability within yeast PDI homologues and underscored the predominant role of PDI in catalyzing oxidative folding of substrates since only PDI was capable of supporting native folding of the model substrate, carboxypeptidase Y (Norgaard et al., 2001). In contrast, the mammalian PDI family includes many members that have been shown to possess PDI-like activity (Table 1.1; (Alanen et al., 2006a).

The efficiency with which PDI carries out its catalytic functions in vitro, and its means of direct substrate interaction via hydrophobic patches, suggest that it will interact with a wide variety of substrates. However, direct studies of endogenous substrate proteins in mammalian cells have been relatively limited to date. The paradox of PDI’s importance, as evidenced by the problem of maintaining cell viability when PDI is constitutively depleted (Park et al., 2006), versus the possibility of functional overlap with PDI family members (Table 1.1), is likely at the root of the challenge in characterizing a greater collection of specific PDI-substrate functional roles. An example of this issue is the currently conflicting evidence over whether PDI (Kang et al., 2009) or ERp57 (Zhang et al., 2006; described further below) is required for the folding of MHC-I heavy chains. Among the reported roles specific to PDI (and not other family members), this enzyme has been shown to affect the production of infectious rotavirus particles (Maruri-Avidal et al., 2008) and there is reported involvement of PDI in the oxidative folding of thyroglobulin (Di Jeso et al., 2005), Semliki forest virus (Molinari and Helenius, 1999), immunoglobulins (Roth and Pierce, 1987), and interferon-gamma (Vandenbroeck et al., 2006). Furthermore, transient PDI knockdown experiments have implicated this enzyme in facilitating cholera toxin retrotranslocation from ER to cytosol whereas the related family members ERp57 and ERp72 either had no effect or an opposing effect, respectively (Forster et al., 2006). A recent PDI knockdown study also indicated a role for PDI in the specific retention of proinsulin in pancreatic β-cells (Rajpal et al., 2012). Effective, yet non-cytotoxic depletion of PDI, as well as other PDI family members, and the assessment of oxidative folding of many substrates would
be revealing to address the challenges associated with determining the functional importance of this key thiol oxidoreductase.

1.4.2.2 ERp57

Among PDI members that most closely resemble the domain structure of PDI, the best characterized is ERp57. This PDI family member shares a high degree of similarity with PDI, particularly in the quantified activities of its catalytic domains (Frickel et al., 2004), but, unlike human PDI, is unable to substitute for yeast PDI in \textit{pdi1} deletion strains (Gunther et al., 1993). Its substrate-binding \textit{b}’ domain has diverged such that it interacts with the ER lectin-chaperones calnexin (Cnx) and calreticulin (Crt) (Oliver et al., 1997; Oliver et al., 1999). The crystal structure of ERp57 \textit{bb}’ domains (Kozlov et al., 2006) revealed that ERp57 lacks the hydrophobic patch on the \textit{b}’ domain present in PDI (Fig. 1.7A). Instead, the exterior face of its \textit{b}’ domain contains a cluster of positively charged residues (Fig. 1.7B, magenta) that interact with a negatively charged region at the tip of the arm domains of Cnx and Crt (Fig. 1.2A). The affinities of the interaction have been quantified at a K\text{d} of 26 µM for Cnx (Kozlov et al., 2006) and 10 µM for Crt (Frickel et al., 2002).

Since Cnx and Crt are largely glycoprotein-specific chaperones, ERp57 has optimized access to folding glycoproteins through this interaction (reviewed in (Ellgaard and Frickel, 2003)). Indeed, ERp57 has a well established specificity for Asn-linked glycoproteins that begins cotranslationally (Molinari and Helenius, 1999, 2000) and that is abrogated when its interactions with Cnx and Crt are disrupted (Zapun et al., 1998; Jessop et al., 2009a). ERp57 knockout mice are embryonic lethal, suggesting an important role for ERp57 in development (Garbi et al., 2006), but cell lines derived from these mouse embryos are viable and show no obvious ER redox defect. Studies in which ERp57 has been depleted through RNA interference or gene knockout have established that ERp57 is required for the efficient oxidative folding of mouse MHC class I heavy chains (Zhang et al., 2006), influenza hemagglutinin (Solda et al., 2006), clusterin, β1 integrin (Jessop et al., 2007), and progranulin (Almeida et al., 2011).

There are, however, reports of ERp57 acting directly on select substrates independently of a requirement for Cnx or Crt, namely in the uncoating of Simian Virus 40 by ERp57-mediated disulfide isomerization of capsid proteins (Schelhaas et al., 2007) and the ERp57-catalyzed
Figure 1.7. Domain organization and structures of PDI, ERp57 and ERp72. Show in cartoon representation are: (A) yeast PDI structure (PDB 2B5E), (B) ERp57 structure (PDB 3F8U) and (C) composite model of ERp72, in which solved domain structures are positioned on an ERp57 scaffold. Catalytic cysteines are shown in orange. Substrate and protein partner binding sites are highlighted. ERp57-bound tapasin (cyan) is shown in ribbon representation. Residues forming hydrophobic patches on catalytic domains and the substrate-binding surface on the $b'$ domain of PDI (human substrate binding site mapped onto yeast PDI) are shown in blue. The ERp57 residues that interact with calnexin are shown in magenta. Images generously provided by Dr. G. Kozlov, McGill University (Rutkevich and Williams, 2011).
folding and assembly of MHC Class I molecules (Zhang et al., 2009). Additionally the loading of high affinity peptides onto MHC Class I proteins (Peaper and Cresswell, 2008) via direct interaction of ERp57 with tapasin, a protein that assists in this peptide-loading function (Wearsch and Cresswell, 2007), has yielded insight into the possible mechanism of substrate recognition in these cases. In a crystal structure of the ERp57-tapasin complex, hydrophobic patches adjacent to the ERp57 active sites mediate interaction with tapasin (Fig. 1.7B; (Dong et al., 2009). These regions are conserved in PDI, ERp57 and ERp72 and suggest new location for substrate interaction other than at non-catalytic domains (Rutkevich and Williams, 2011). Clearly, structural information reveals that PDI and ERp57 access substrate differently via their $b'$ domains, but it is still uncertain to what degree these two family members functionally overlap.

1.4.2.3 ERp72

The PDI family member, ERp72, contains three catalytic and two non-catalytic domains (Mazzarella et al., 1990) and, although it shares high sequence similarity with ERp57, it is not able to bind to Cnx or Crt (Kozlov et al., 2009). Structural studies on domain groupings of ERp72 (Kozlov et al., 2009; Kozlov et al., 2010a) have revealed that its $b'$ domain possesses a negatively charged region in lieu of the positively charged interaction surface on ERp57, and so no interaction between Cnx or Crt and ERp72 has been observed. Rather, the substrate binding site on ERp72 is thought to occur at the hydrophobic regions bordering the active sites on its catalytic domains (Fig. 1.7C; (Kozlov et al., 2010a; Rutkevich and Williams, 2011).

Interestingly, there is no interaction between ERp72 and tapasin (Dong et al., 2009). The relatively rigid position of the $a^0$ domain and N-terminal extension of ERp72 is thought to block the binding site on the $a$ domain utilized by tapasin when binding to ERp57, and generally limit ERp72’s protein interaction partners (Kozlov et al., 2010a).

Limited information is available concerning a functional relationship between ERp72 and ERp57. ERp72 was unable to substitute functionally for ERp57 in the oxidative folding of MHC class I molecules (Zhang et al., 2006) but it did replace ERp57 in mixed disulfide complexes with several glycoprotein substrates when ERp57 was either eliminated or its recruitment to substrates by Cnx/Crt impaired by treatment with a glucosidase inhibitor (Solda et al., 2006; Jessop et al., 2009b). The structural basis for this overlap may be due to their common hydrophobic regions near the catalytic sites of both ERp57 and ERp72 (Fig. 1.7B, C).
Additionally, ERp72 has been implicated in the folding of thyroglobulin (Menon et al., 2007) and apolipoprotein B (Linnik and Herscovitz, 1998). However, when CXXA mutations were made in the catalytic domains of ERp72 to trap substrate-ERp72 mixed-disulfide intermediates, there was relatively poor success (Jessop et al., 2009b), potentially indicating a narrow substrate specificity for ERp72.

Many instances of reported ERp72 involvement in disulfide exchange activities are found when ERp72 is in a complex with a number of other ER chaperones and folding factors (Linnik and Herscovitz, 1998; Meunier et al., 2002) including BiP, Grp94, PDI, and cyclophilin B. This suggests that ERp72 may also encounter substrates indirectly via recruitment through these interactions. In support of this hypothesis a recent ER-localized membrane yeast two-hybrid screen carried out by Jansen and coworkers also characterized the specific interaction between ERp72 and cyclophilin B. These authors showed that in vitro folding of immunoglobulin G was accelerated in the presence of the ERp72/cyclophilin B complex and mapped the interaction between the two enzymes to a distinct acidic region at the N-terminus of ERp72 and a lysine-rich region of cyclophilin B (Kd of 3.6 µM; (Jansen et al., 2012). Other novel interaction partners for ERp72 revealed by this study include peroxidredoxin 4 (PRDX4), a known ER oxidant (introduced in Section 1.4.3.2) and the redox-sensitive selenoprotein, Sep15 (Jansen et al., 2012). The latter protein has been found associated with UGGT and proposed to play a role in ER quality control (Korotkov et al., 2001). The functional significance of these interactions remains to be more fully characterized.

1.4.2.4 P5

Although discovered many years ago, P5 (ERp5, CaBP1) remains a poorly understood member of the PDI family with one non-catalytic and two catalytic domains (Rupp et al., 1994). Its catalytic domains have been shown in vitro to be capable of carrying out thiol disulfide exchange reactions (Kramer et al., 2001; Kikuchi et al., 2002; Alanen et al., 2006a). It has been found in a large multi-enzyme complex that also includes PDI, ERp72, and BiP (Meunier et al., 2002). Only recently has it been implicated in a direct interaction with BiP, possibly mediating its substrate selection (Jessop et al., 2009b). Indeed, in CXXA substrate trapping studies, P5 was found to interact with a wide range of targets in a semi-permeabilized cell translation system, but proteomic identification of endogenous substrates in intact cells was unsuccessful (Jessop et al.,
The structural basis of P5-substrate or -BiP interactions is currently unknown and it is also uncertain whether P5’s primary function is that of an oxidase, isomerase or reductase. The recent ER interaction study identified interaction of P5 with EDEM (Jansen et al., 2012), supporting the possibility that P5 may be recruited to ERAD machinery for reduction of disulfide bonds. This is further supported by P5 knockdown studies in the Williams Lab that led to ERAD escape of MHC-I molecules in HCMV-infected cells (D. Chapman, personal communication). If such a difference in function is correct, then P5 would be expected to exhibit minimal functional overlap with PDI family members carrying out oxidase and isomerase activities.

1.4.2.5 Other PDI family members

Among the remaining PDI family members, only a few have been characterized in any detail and most have significant differences from PDI in their domain architecture and active site motifs (Table 1.1). Discovered in 2002, ERp44 has an \textit{abb}' domain arrangement and the active site motif is CXXS (Anelli et al., 2002). Its crystal structure (Wang et al., 2008) gave precedent for the x-linker gating of the \textit{b}' domain in PDI and it is best known for its functions in ER retention of the ER oxidase Ero1 (Anelli et al., 2003); see Section 1.3.3.1) and of the inositol 1,4,5- triphosphate receptor type I (Higo et al., 2005). It may also have a specific role in late-stage folding quality control or subunit assembly as it is found in the folding pathways of other multi-subunit substrates including adiponectin (Wang et al., 2007b) and IgM (Anelli et al., 2007).

ERdj5 is the other well-characterized ubiquitous PDI family member. It is unique in that it contains four catalytic domains and a J-domain shown to interact with BiP (Cunnea et al., 2003). Notably, three of its four \textit{a}-like domains have a CXPC motif, similar to thioredoxin, and its calculated reduction potential reveals that it has the greatest capacity to act as a reductase among PDI family members. It is consistent with these characteristics then, that ERdj5 is upregulated in response to ER stress and that it has been found to play a role in reducing the disulfides of ERAD-targeted substrates (Dong et al., 2008; Ushioda et al., 2008; Riemer et al., 2009).

PDIp and PDILT are the tissue-specific family members, found predominantly in the pancreas and testes, respectively. PDIp closely resembles PDI and is able to bind to peptides derived from a number of pancreas-synthesized proteins (Klappa et al., 1998b), although its
exact physiological role is uncertain. PDILT is also similar to PDI in domain arrangement, but its catalytic motifs are SXXS and SXXC so disulfide exchange activity is not expected. It has been found to interact with Ero1α (van Lith et al., 2005) and a lectin chaperone (van Lith et al., 2007), but most recently its role in quality control of a sperm membrane protein has been highlighted (Tokuhiro et al., 2012).

Both PDIr and ERp46 possess three catalytic domains and are regulated by androgen and hypoxic conditions, respectively (Hatahet and Ruddock, 2009), but although their functions remain largely unknown, ERp46 has been reported to have a role in regulating insulin content in β cells (Alberti et al., 2009). Also with unknown physiological function, ERp18 is a single active domain family member, but substrate trapping studies indicated it was able to share a number of substrates with ERp46 and ERp72 (Jessop et al., 2009b). More recently added PDI family members, hAG2 and hAG3, are also small single catalytic domain proteins, but their active motifs are unusual: CPHS and CQYS, respectively (Hatahet and Ruddock, 2009), likely restricting them to reductase or isomerase activities, and only hAG2 has a reported role in intestinal mucus production (Park et al., 2009).

There are five PDI family members that possess a transmembrane domain: TMX, TMX2, TMX3, TMX4, and TMX5. While TMX has been found with calnexin at mitochondria-associated membranes (Lynes et al., 2011), it has also been shown to co-immunoprecipitate with both calnexin and MHC-I (Matsuo et al., 2009). The TMX-MHC-I interaction was further stabilized upon inhibition of glycosylation suggesting a role for this family member when ERp57 cannot access folding glycoproteins via Cnx/Crt. TMX3 has been characterized as having the most PDI-like activity (Haugstetter et al., 2007), and its substrate specificity is speculated to be broad, yet limited to membrane-proximal folding events. The oxidoreductase activity of TMX4 has also been verified (Roth et al., 2009), but the active domain of TMX2 is thought to be on the cytosolic side of the membrane (Hatahet and Ruddock, 2009).

The PDI family members ERp29 and ERp27 possess only non-catalytic domains and have no thiol disulfide exchange capabilities. ERp29 has been shown to influence the folding of thyroglobulin in complex with BiP and Grp94, likely in a chaperone-like manner (Sargsyan et al., 2002), although a general chaperone activity for this family member has not yet been characterized (Hubbard et al., 2004). However, in support of a chaperone function, the
overexpression of ERp29 promoted the trafficking of both wild type and the ΔF508 mutant of cystic fibrosis transmembrane conductance regulator (CFTR) to the plasma membrane (Suaud et al., 2011). Notably, ERp29 has also emerged as a potential biomarker in cancer treatments (Zhang and Richardson, 2011), as its higher expression is associated with reduced tumorigenesis. ERp27 has only one reported function, binding to ERp57 at the Crt binding site on its b’ domain (Alanen et al., 2006b), but the significance of this function is unknown.

1.4.3 Oxidation of PDIs

When net oxidation of substrate is carried out by PDI family members possessing the classic CXXC motif, the active site cysteines shuttle from an oxidized disulfide state to a dithiol state as the disulfide is donated to the substrate protein (Hatahet and Ruddock, 2009). In order for the PDI family member to catalyze another oxidation reaction, the active site must be re-oxidized to the disulfide state. In vitro data showing the efficient oxidation of PDI by oxidized glutathione (GSSG) initially suggested that this small molecule was responsible for regenerating oxidized PDI (Lyles and Gilbert, 1991a), but it did not explain the ultimate source of oxidizing equivalents to the ER. The enzymes responsible for carrying out this re-oxidation have been the subject of much study over the past decade.

1.4.3.1 Ero1-driven oxidation of PDI

Fueling the search for an ER oxidant protein was the finding that deletion of gshl in yeast, thereby eliminating glutathione, was not lethal and that disulfide bond generation proceeded normally (Grant et al., 1997). In contrast, upon its discovery the ER oxidoreductin protein (Ero1p) in yeast was shown to be essential for disulfide bond formation (Frand and Kaiser, 1998; Pollard et al., 1998). Its catalytic cycle was elegantly elucidated to reveal disulfide transfer between cysteine pairs (Figure 1.8). By accepting oxidizing equivalents from molecular oxygen via its bound flavin adenine dinucleotide (FAD) (Tu and Weissman, 2002; Gross et al., 2004), the O₂ molecule is reduced to H₂O₂ (Gross et al., 2006) and a disulfide is formed between the “active site” cysteines of Ero1p. Another pair of Ero1p cysteines on a flexible loop accepts the disulfide bond from the active site to form the “shuttle disulfide”. And lastly, the shuttle disulfide is transferred to oxidize yeast PDI, and not newly synthesized proteins or small
**Figure 1.8** Ero1 oxidation of PDI.  (A) In the catalytic mechanism of Ero1, thiolate anion attack by the reduced PDI onto the disulfide bonded shuttle cysteines of Ero1 (1; red loop) results in an oxidized PDI CXXC motif and a reduction of the shuttle cysteines (2).  The electrons from the shuttle cysteines are then passed on to the active site cysteines (black loop) to reduce their disulfide bond (3).  The electrons are then passed on to the FAD cofactor and then to molecular oxygen (4), generating one molecule of \( \text{H}_2\text{O}_2 \), but also re-forming the disulfide bond between the active site cysteines.  (B) Proposed model of the Ero1α-PDI complex with the solved human \( \text{bb}' \) domains of PDI in green and the \( a \) and \( a' \) domains positioned putatively.  The FAD cofactor is shown in the red and yellow spheres, the position of the CXXC motifs on PDI’s catalytic domains are shown in the larger orange spheres, and the location of the Ero1 shuttle loop is indicated.  The inset highlights the interactions between the protruding β-hairpin of Ero1α (pink) and the \( b' \) domain of PDI (green) and residues crucial for this interaction are represented in stick diagrams.

Adapted from (A; Sevier and Kaiser, 2008) and (B; Araki and Inaba, 2012)
molecules like reduced glutathione (GSH), (Frand and Kaiser, 1999; Tu et al., 2000; Sevier and Kaiser, 2006). Another important feature illuminated by the yeast Ero1p is its regulation by two other cysteine pairs which form the “regulatory disulfides”. When these intrachain disulfide bonds are formed, the flexible loop is tethered, effectively impeding the movement of the shuttle disulfide from the active site to the PDI recipient and shutting Ero1p off (Heldman et al., 2010). Reduction of these disulfides takes place step-wise; the Cys143-Cys166 disulfide is reduced first and then reduction of Cys150-Cys295 is the critical step for full activation of Ero1p. Reduced PDI is responsible for this activation so that the action of Ero1p is redox controlled and takes place only when disulfides are required (Sevier et al., 2007). The regulation of Ero1p is desirable as a pool of reduced PDI must also be maintained for the isomerization of incorrect disulfides and to minimize the production of the reactive oxygen species (ROS) H₂O₂ (Shimizu and Hendershot, 2009).

The mammalian isoforms of Ero1p, Ero1α (Cabibbo et al., 2000) and Ero1β (Pagani et al., 2000), have also been shown to carry out this function, oxidizing PDI and possibly other PDI family members such as ERp57 and ERp72 (Jessop et al., 2009b; Appenzeller-Herzog et al., 2010; Schulman et al., 2010). The regulatory cysteines in Ero1α and Ero1β approximate those in Ero1p with a few exceptions. In Ero1α, the two cysteines of the shuttle disulfide are alternatively bound to regulatory cysteines when the enzyme is inactive. In the inactive state, disulfides exist between Cys94 – Cys131 and between Cys99 – Cys104 and in the active state the disulfide switches to the shuttle cysteines, Cys94 – Cys99 and the regulatory cysteines Cys104 and Cys131 are reduced (Appenzeller-Herzog et al., 2008; Baker et al., 2008). Interestingly, the activated form of Ero1α shows higher affinity for PDI than the inactive form (Masui et al., 2011), supporting evidence for the tight regulation of Ero1α in cells as a threshold concentration of reduced PDI will first have to be reached before Ero1α activation. In contrast the regulation of Ero1β has been shown to be looser. An accessory regulatory disulfide is found between Cys100 – Cys262, and the main regulatory disulfide between Cys90 – Cys130 is reduced upon activation and the shuttle disulfide is formed between Cys90 – Cys95 (Wang et al., 2011). Since Cys95 remains reduced and unpaired in the inactive state, it may be responsible for autonomous activation of Ero1β and its looser regulation.

The recent crystal structure of Ero1α also illuminated important features of Ero1α interactions (Inaba et al., 2010). Most notably, the specific interaction between a protruding β-
hairpin, harbouring a critical Trp272 residue, of Ero1α with the hydrophobic substrate-binding surface of the PDI b’ domain was shown (Fig. 1.8B; (Masui et al., 2011). This interaction is supplemented by electrostatic interactions (Inaba et al., 2010) and specifically oxidizes the C-terminal a’ domain of PDI (Baker et al., 2008) which can immediately transfer the disulfide to the a domain (Araki and Nagata, 2011). Although this structure revealed the binding mode of the FAD, the pathway to allow access of molecular oxygen is still unclear and additional conformational changes in Ero1s are expected (reviewed in (Araki and Inaba, 2012)).

In addition to differences in the rigidity of their redox regulation, Ero1α and Ero1β also differ in their tissue distribution, transcriptional regulation, and localization at specific ER microenvironments. The insulin-producing β cells of the pancreas have the highest levels of Ero1β expression, maintained by the transcription factor PDX1 (Khoo et al., 2011). Ero1β is also upregulated early in ER stress by the ATF6 pathway (Adachi et al., 2008) where its looser regulation would allow more rapid disulfide generation. In contrast, Ero1α is more ubiquitously expressed and is a specific target of the transcription factor HIF1α in response to hypoxia (Gess et al., 2003) or hypoglycemia (reviewed in (Ramming and Appenzeller-Herzog, 2012)). Upon ER stress, Ero1α is upregulated by CHOP later in the unfolded protein response (Marciniak et al., 2004) and its ER localization changes from mitochondrial associated membranes (MAMs) to the bulk ER (Gilady et al., 2010). In addition to likely providing oxidizing equivalents to the ER, this distribution change has recently been interpreted as critical to stress-induced apoptosis in cells. The inositol 1,4,5-triphosphate receptor (IP3R)-mediated calcium shuttling from ER to mitochondria and the cytosol is required for the apoptotic cascade (Decuypere et al., 2011), and IP3R (type 1) is enriched at MAMs, inactivated by reversible binding to ERp44 (Higo et al., 2005). Since ERp44 also binds specifically to Ero1α, when Ero1α levels increase in response to stress, it is now thought to sequester ERp44 away from IP3R, allowing activation of apoptosis (Anelli et al., 2012).

Despite the central roles of Ero1α and Ero1β in the ER, and the essential function of Ero1p in yeast, combined Ero1α- and Ero1β-deficient mice are viable. Furthermore, disulfide bond formation occurred normally during IgM assembly and was only partially impaired during insulin biogenesis (Zito et al., 2010a). In addition, when Ero1αβ-deficient fibroblasts were exposed to a strong reductive challenge, recovery of ER redox homeostasis exhibited only a modest delay compared to wild-type cells (Appenzeller-Herzog et al., 2010). These findings
suggest the existence of Ero1-independent pathways for the formation of disulfide bonds and several candidates have been proposed that are defined by their central enzymes: peroxiredoxin IV (PRDX4), quiescin-sulphhydryl oxidase (QSOX1), and vitamin K epoxide reductase (VKOR) (Fig. 1.9; reviewed in (Bulleid and Ellgaard, 2011)).

1.4.3.2 Peroxiredoxin 4

The Ero1 oxidation of disulfides generates one molecule of hydrogen peroxide per disulfide bond formed. Unchecked ROS accumulation leads to oxidative stress in the cell so many pathways exist for its breakdown or regulated reaction. Tavender and coworkers identified a member of the peroxiredoxin family, peroxiredoxin 4 (PRDX4) that efficiently carries out this function in the ER (Tavender et al., 2008). Using hydrogen peroxide (Tavender and Bulleid, 2010b), PRDX4 oxidizes an active site cysteine to sulfenic acid. Although H₂O₂ is capable of reacting with cysteine residues in general (Karala et al., 2009), it is observed to be relatively selective in proteins, with the so-called peroxidatic cysteines of the peroxidredoxins showing particular sensitivity to oxidation (Wood et al., 2003). The sulfenic acid on the peroxidatic cysteine then reacts with a cysteine on another PRDX4 subunit to form an interchain disulfide bond (Cao et al., 2011). This disulfide bond can be donated to thioredoxin domain-containing proteins such as the PDIs.

PRDX4 has one peroxidatic cysteine (Cys124) and one resolving cysteine (Cys245) per subunit and, with increasing H₂O₂ concentration, 2 interchain disulfides can be formed between each dimer. In addition, the crystal structure of PRDX4 revealed that PRDX4 forms stable decamer rings, regardless of its oxidation state, via a novel N-terminal loop, oriented towards the centre of the ring. Cys51, found on this loop, forms interchain disulfides between (and not within) dimer pairs to covalently link 5 pairs in the decamer, although its presence is not essential to oligomerization (Wang et al., 2012b). The formation of the Cys124 – Cys245 disulfide has also been shown to induce local unfolding in dimers and may have a regulatory role as the conformational change confers resistance of the peroxidatic cysteines to hyperoxidation of the sulfenic acid to sulfinic acid (Cao et al., 2011). Notably, only the sulfenic acid is resolvable to a disulfide by another cysteine, whereas the hyperoxidized sulfinic acid must be reduced by a sulfiredoxin (of which none have been identified in the secretory pathway) and further oxidation
Figure 1.9 Known and Putative ER Oxidant Pathways. The well-characterized pathway of delivering oxidizing equivalents to the ER via Ero1 is shown as the disulfide is transferred from Ero1 (yellow) to PDI (red) to substrate (green). In the PRDX4 pathway (blue), H$_2$O$_2$ is scavenged and reduced to H$_2$O to generate inter-chain disulfides (one is shown) between two PRDX4 subunits. This disulfide bond may be donated to PDI or other PDI family members for productive oxidative folding of substrates. In the QSOX1 pathway (orange), an FAD-mediated reduction of O$_2$ generates H$_2$O$_2$ and a disulfide bond that QSOX1 donates to PDIs (or potentially directly to substrate). In the VKOR pathway (pink), the vitamin-K dependent catalysis of γ-carboxylation and subsequent recycling of an oxidized form of vitamin-K to the reduced form of vitamin-K generates a VKOR disulfide bond that can also be donated to PDI or other PDI family members.
to sulfonic acid results in an irreversibly inactivated subunit (reviewed in (Kakihana et al., 2012)).

*In vitro* studies have shown that some, but not all, PDI family members can be oxidized by PRDX4 (Tavender *et al.*, 2010) and that PRDX4 is capable of catalyzing RNaseA oxidative folding in an H$_2$O$_2$- and PDI-dependent manner (Zito *et al.*, 2010b). In vivo evidence supports these observations, as shown by the ability of mammalian PRDX4 to rescue the lethality of Ero1p deficiency when expressed in a temperature-sensitive Ero1p mutant yeast strain (Zito *et al.*, 2010b). Furthermore, depletion of PRDX4 by RNA interference exacerbated the phenotype of Ero1α+β null mouse embryo fibroblasts, resulting in decreased cell growth, hypersensitivity to reducing agents, a more reduced ER redox balance and impaired collagen secretion (Zito *et al.*, 2010b). Like mammalian Ero1, PRDX4 is not essential since PRDX4 knockout mice are viable and fertile (Iuchi *et al.*, 2009).

1.4.3.3 QSOX

In yeast, the sulfhydryl oxidase, Erv2p was shown to rescue the lethality due to the loss of Ero1p (Sevier *et al.*, 2001). In higher organisms, the homologous proteins are members of the quiescin sulfhydryl oxidase (QSOX) family (Coppock *et al.*, 1998). QSOX1 and QSOX2 (also known as SOXN) are found in mammalian cells with different tissue distribution (QSOX2 expression is very weak in liver, lung, and skeletal muscle). Since QSOX2 has been reported predominantly at the plasma membrane and nuclear membrane (Wittke *et al.*, 2003), and QSOX1 is expressed highly in secretory cells (Thorpe *et al.*, 2002), focus has shifted to QSOX1 as a potential supplier of oxidizing equivalents to the ER. QSOX1 exists in two forms based on differential membrane splicing; QSOX1a is the full length form, predicted to be tethered to the membrane by a single-pass transmembrane domain and QSOX1b is the shorter, soluble variant. Both isoforms contain two thioredoxin domains linked to an Erv2-like domain that possesses oxidase activity (reviewed in (Sevier, 2012). Like Ero1, QSOX1 is a flavoprotein that couples the formation of disulfide bonds to the reduction of molecular oxygen to produce H$_2$O$_2$ (Kodali and Thorpe, 2010). However, whereas Ero1 oxidizes PDIs, *in vitro* studies have shown that QSOX1 shuttles disulfides from its Erv2 domain to one of its thioredoxin domains and then to a broad range of substrates, with high efficiency to reduced proteins and more slowly to small molecules like DTT and GSH (Hoober *et al.*, 1999). Indeed, a recent in vitro study on the
substrate preferences of QSOX1 suggested that loosely folded intermediates, rather than compact
globular proteins were acted upon for inter- or intradisulfide generation. However, the basis for
this discrimination is unknown (Codding et al., 2012). In addition to the cysteine pair in the N-
terminal thioredoxin domain and the cysteine pair adjacent to the FAD in the Erv-domain,
QSOX orthologs have a highly conserved third cysteine pair at the C-terminus. A crystal
structure (Alon et al., 2010) and mutational studies (Heckler et al., 2008) have shown that this
pair is not required for catalytic activity of QSOX, suggesting a possible site of regulation.

In vivo evidence for QSOX1 activity comes primarily from its ability to complement the
Ero1 deletion when overexpressed in yeast (Chakravarthi et al., 2007), but physiological
substrates for QSOX1 have not yet been identified in mammals. Overexpressed QSOX1
localizes mainly to the Golgi apparatus in Chinese hamster ovary cells (Chakravarthi et al.,
2007), although it has also been reported in the ER (Thorpe et al., 2002) and extracellularly
(Amiot et al., 2004). However, given the rapid kinetics with which QSOX1 can introduce
disulfides in vitro (~ 1000 per minute; (Hoober et al., 1999), there may be sufficient levels in the
ER at steady state to catalyze disulfide formation in PDIs or folding proteins.

1.4.3.4 VKOR

Human VKOR is an ER membrane protein that catalyzes the reduction of vitamin K
epoxide to vitamin K hydroquinone, a cofactor important for \( \gamma \)-carboxylation of glutamate
residues (from Glu to Gla) in proteins such as blood-clotting factors (Jin et al., 2007), although
the exact mechanism of this reduction is uncertain. Bioinformatic analysis revealed that there
are four cysteines conserved across all known VKOR homologues (Goodstadt and Ponting,
2004); in human VKOR these are Cys43, Cys51, Cys132, and Cys135. The Cys132 and Cys135
make up a CXXC motif that is oxidized during vitamin K epoxide reduction (Wajih et al., 2005)
and early studies suggested that the Cys43 – Cys51 pair was dispensable for VKOR activity
(Rost et al., 2005; Jin et al., 2007). The C\(^{132}\)XXC\(^{135}\) pair was anticipated to require a redox
partner, but conflicting topology models questioned whether it was proximal to the cytosol or ER
lumen to access this partner. It was only recently that the localization (Li et al., 2010) and role
(Rishavy et al., 2010) of the Cys43 – Cys51 pair as ER luminal shuttle cysteines was definitively
demonstrated, revealing a relay of electrons from an ER redox partner to Cys43 – Cys51 to
Cys132 – Cys135 (Rishavy et al., 2010) and raising the possibility that oxidized VKOR could donate a disulfide to PDI family members (Schulman et al., 2010).

Using an in vitro microsomal system, it was shown that VKOR-coupled oxidation of RNaseA was dependent on the presence of PDI and that a complex containing VKOR and PDI could be detected (Wajih et al., 2007). Subsequent substrate trapping experiments suggested that other members of the PDI family, namely TMX, TMX4 and ERp18, are the preferred interaction partners of VKOR (Schulman et al., 2010). However, a direct impact of VKOR on oxidative folding or on ER redox status in cells has not yet been demonstrated.

1.4.3.5 Alternative oxidant pathways

Other potential protein- and small molecule-mediated pathways for generating disulfide bonds de novo exist in the ER. The glutathione peroxidases GPX7 and GPX8 also scavenge H$_2$O$_2$ (Toppo et al., 2008), reside in the ER, and may have a physical association with Ero1α (Nguyen et al., 2011). In vitro studies have also revealed that these enzymes efficiently oxidize PDIs (Nguyen et al., 2011), but their reactivity towards hydrogen peroxide is considerably lower than PRDX4 (Winterbourn, 2008). In addition to the systems in place to scavenge H$_2$O$_2$, this small molecule itself has been shown, in vitro, to have the capacity to introduce disulfide bonds in proteins (Karala et al., 2009), but this is expected to be relatively minor as the PRDX-mediated oxidation occurs much faster (reviewed in (Bulleid and Ellgaard, 2011)). However, it is of interest to note that H$_2$O$_2$ may enter the ER via the NADPH-dependent oxidase, NOX4 (Chen et al., 2008) or by leakage from the mitochondrial electron transport chain (Yang et al., 2007) in addition to its generation by Ero1 (and QSOX1). Finally, dehydroascorbate (DHA) can be transported into the ER and formed in the ER (Csala et al., 2010) and has been shown to have the capacity to directly oxidize PDI (slowly) and substrate proteins (rapidly) (Saaranen et al., 2010), but the physiological relevance of this observation has not yet been determined.

1.4.3.6 Glutathione disulfide

A final player to consider in the reoxidation of PDI family members is the tripeptide glutathione (γ-Glu-Cys-Gly). Found in the cytosol as well as the ER, the sulphydryl group on the cysteine residue allows thiol redox reactions to take place. Importantly, the reduced form, GSH can dimerize to form glutathione disulfide, GSSG. In the cytosol, the ratio of reduced to
oxidized glutathione is approximated at 300:1, while in the ER this ratio is thought to approach 3:1 (Hwang et al., 1992). Since GSSG is unable to cross the ER membrane (Banhegyi et al., 1999), the shift in the GSSG:GSH ratio in the ER is attributed to the activity of the ER oxidant pathways, particularly Ero1. In this model then, GSH imported from the cytosol brings reducing equivalents into the ER to balance the action of Ero1 (Cuozzo and Kaiser, 1999; Molteni et al., 2004). Indeed, depletion of cellular GSH by inhibition of its synthesis revealed that it plays a role in reduction and isomerization in the ER, reactions that require PDI CXXC motifs to be in their reduced states (Jessop and Bulleid, 2004; Chakravarthi et al., 2006).

GSSG formed in this redox balance is not merely an unproductive byproduct of overactive Ero1. Rather, GSSG is quite capable of donating its disulfide bond to folding proteins (Bass et al., 2004) and is routinely used to regenerate oxidized PDIs in in vitro experiments (Hatahet and Ruddock, 2009). In a landmark study, Appenzeller-Herzog and coworkers (Appenzeller-Herzog et al., 2010) showed that the ER GSSG:GSH ratio is tightly controlled; when Ero1α was over-activated by reductive challenge the measured recovery in GSSG:GSH overshot steady state levels, but was quickly corrected. As this corrective effect was found to be dependent on PDI, these authors proposed that excess GSSG was used by PDI to drive its re-oxidation. While GSSG is not a de novo source of oxidizing equivalents in the ER, the processes by which it is generated can indirectly lead to the reoxidation of PDI, and likely other PDI family members as well (reviewed in (Appenzeller-Herzog, 2011)).

1.5 Rationale & Approach

A large number of thiol oxidoreductases of the PDI family have been identified and are collectively thought to carry out appropriate oxidation, isomerization and reduction of disulfide bonds in the ER. Individual functions of many PDI family members have been biochemically characterized in vitro, and several have been assigned to cooperative folding networks, but their relative contributions to substrate-level folding has remained an open question. Previous work profiling substrates by the use of CXXA trap mutants has been invaluable in initiating our understanding of substrate selection in cells for a subset of PDI family members (ERp57, ERp18, ERp72, ERp46, and P5; Jessop et al., 2007; Jessop et al., 2009a; Jessop et al., 2009b). However, this approach suffers from limitations that are effectively addressed in this thesis. By using the CXXA trap mutants, one is only able to observe the substrate selection of a PDI family member
when the active site is reduced (that is, acting as a reductase or isomerase). Since the formation of the active site disulfide is prevented by the mutation, there is no corresponding data available on substrate specificity under oxidation conditions. In addition, this method was unable to identify substrates of PDI, which is of great interest to uncover. The question of whether PDI family members exhibit substrate specificity or appreciable overlap is still unknown.

Starting with the hypothesis that PDI family members exhibit substrate specificity in their action, I studied the functional relationships among PDI family members that have the capacity for oxidation, reduction, and isomerization activity by investigating the impact of their depletion on the oxidative folding of a common set of endogenous substrates (Chapter 3). Through PDI depletion, I addressed the question of whether there was functional redundancy in disulfide exchange reactions active in the ER or if PDI activity was paramount. By assessing the oxidative folding of multiple substrates following PDI depletion, I began to assign their relative need for PDI activity. Knockdown of ERp57, ERp72, and P5, alone, and in combination with PDI, revealed which of these family members were key to productive oxidative folding, as well as how substrates were shared (or not) among family members. Functional ranking of these PDI family members on a common set of endogenous substrates was carried out to illuminate to what degree either common or novel substrate selection was utilized in cells. In addition, the use of many PDI family members within the ER may reflect differences in which redox functions (oxidation, isomerization, or reduction) each enzyme is specialized to carry out. Thus, the apparent use or disuse of some PDI family members in substrate oxidation provided a means of revealing which family members may be dedicated to which redox activities.

The upstream sources of oxidizing equivalents to the ER and PDI family members has also recently undergone an expansion in the number of proposed players. Available evidence indicates that Ero1 and PRDX4 play important roles in these processes, but the involvement of other candidate oxidant pathways is unknown in vivo. I sought to address the hypothesis that alternative oxidant pathways supplemented the action of Ero1 and PRDX4. To start these investigations, I assessed the outcome of the systematic depletion of Ero1α+β, PRDX4, QSOX1, and VKOR in cells with or without an additional reductive challenge (Chapter 4). This permitted a ranking of the use of known and putative ER oxidant pathways in human hepatoma cells. These experiments revealed the importance of some pathways and the disuse of other pathways for ER oxidation in cells.
Chapter 2

Materials & Methods
2.1 Cell Lines and antibodies

Human hepatoma HepG2 cells, gifted by Dr. K. Adeli (University of Toronto), were maintained in high glucose DMEM containing 10% fetal bovine serum (FBS), glutamine, and antibiotics. This was supplemented with either 1 µg/mL puromycin (Bioshop) or 200 µg/mL hygromycin B (Invitrogen) for HepG2 cells stably expressing ERp57 shRNAmir or mutant PDI, respectively.

Antibodies used in this study (p-polyclonal, m-mono-clonal, R-rabbit, M-mouse, G-goat, Sh-sheep): anti-PDI (pR) and -ERp72 (pR; AssayDesigns), anti-P5 (pR; Affinity Bioreagents), anti-BiP (mM; BD Biosciences), anti-CHOP (pR; Thermo), anti-actin (mM; Chemicon), anti-albumin (pR), -transferrin (pG), and -α1-antitrypsin (pR; Sigma), anti-α-fetoprotein (pR), -α2-HS-glycoprotein (pSh), -Ero1α (mM) and -PRDX4 (mM; Abcam), anti-GAPDH (mM; Millipore), and anti-QSOX1 (pR; Proteintech Group). Rabbit antisera directed against calnexin, calreticulin and ERp57 have been described previously (Danilczyk and Williams, 2001; Zhang et al., 2006; Ireland et al., 2008). The 12CA5 anti-HA (mM) was provided by Dr. Tania Watts (University of Toronto). Rabbit antisera directed against Ero1β (Zito et al., 2010a) and VKOR (Rishavy et al., 2010) were gifts of Dr. David Ron, University of Cambridge, Cambridge, UK and Dr. Kathleen Berkner, Cleveland Clinic, Cleveland Ohio, respectively. Horseradish peroxidase-conjugated secondary antibodies against mouse, rabbit, and goat (Jackson Immunoresearch) were used for Western blots. Cy5-conjugated anti-rabbit and Alexa488-conjugated anti-goat secondary antibodies (Jackson Immunoresearch) were used for confocal immunofluorescence.

2.2 Human PDI cDNA isolation and mutagenesis

mRNA was isolated from HepG2 cells and then cDNA was produced using the RevertAid™ H Minus kit (Fermentas). Using this as template, cDNA encoding PDI with an HA tag inserted after position D499, 5 residues upstream of the C-terminal KDEL sequence, was amplified using primer pair PDI-forward: 5’ - ATATATAAGCCTTGCGGCCCCGCCCACCatgCTGCGCCGCGCTCTGCTGTGCC - 3’ and PDI-reverse-1: 5’ - AGCGTAATCTGGAAACATCGTATGG-GTAATCGTCTCTCCATGTCTGGCTC - 3’. These primers encode 5’ NotI and HindIII sites (underlined), a Kozak sequence (italics), start codon (lower case) and the HA-tag (bold). This PCR product was used as template in a second PCR with primer pair PDI-forward and PDI-reverse-2: 5’-ATATATGGATCCtaCAGCTCATCTTTCACAGCTTTCCTGATCACGCT-
AATCTGGAACATCGTATGGGTA-3’ to amplify the complete PDI cDNA and encode the remainder of PDI following the HA-tag (bold) including the stop codon (lower case) and a BamHI site (underlined). The product was digested with HindIII and BamHI and ligated into pBluescript+ (Stratagene), producing pBS-PDI<sup>WT</sup>.

To generate the double mutant PDI-C56A, C400A (PDI<sup>CXXA-HA</sup>), a modified QuikChange™ mutagenesis protocol was performed (Zheng et al., 2004) using plasmid pBS-PDI<sup>WT</sup> as template and the following primer pairs: C56A-forward: 5’-GTGTGGCCACgcCAAaGCTtTGCCCCCTGAGTATGCAAAAG-3’ and C56A-reverse: 5’-CAGGGGCAaAGCtTTGgeGTGGCCACACCAAGGGGCATAG-3’; and C400A-forward: 5’-GTGTGGTCACgcCAAACAGTTGGCTCCCATTTG-3’ and C400A-reverse: 5’-CACTGTATT-TGgeGTGACCACACCACCATGGGGCATAG-3’ (mutagenic nucleotides in lower case, mutated codons underlined, silent restriction site in italics). As performed by Dr. U. Brockmeier, PCR was carried out with 5% DMSO and “hotstart” with Pfu polymerase (Fermentas) with the following cycling conditions: 3 min at 95°C, (1 min at 95°C, 1 min at 55°C, 11 min at 68°C)<sup>16</sup> cycles, and 30 min at 68°C. The constructs encoding PDI<sup>WT-HA</sup> and PDI<sup>CXXA-HA</sup> were subcloned into plasmid pQCXIH (Clontech) using NotI/BamHI sites. Recombinant Moloney virus packaged with the respective pQCXIH plasmids was used to infect HepG2 cells (Ireland et al., 2008), with stable transformants selected in 200 µg/mL of hygromycin B.

2.3 RNA interference

2.3.1 Constitutive ERp57 Knockdown

For constitutive knockdown of ERp57, recombinant Moloney virus packaged with pSM2c-ERp57shRNAmir (Cat. No.V2HS_33075, SH2238-D-8; OpenBiosystems) was used to infect HepG2 cells as described above. Transformants were subjected to a second infection with virus as well as clonal selection to maximize knockdown efficiency. PDI family double- and triple-knockdowns were carried out in this cell line using siRNAs as described below.

2.3.2 Single target knockdown

Stealth Select™ siRNAs (Invitrogen), were used for single and combinatorial knockdowns. The proprietary “Stealth” modification reduces off-target effects by preventing the sense-strand from participating in RNAi activity and also prevents RNAi mediated cellular
stress, particularly interferon responses (data not shown) for increased confidence that phenotypes observed are specific to target knockdown. Stocks of siRNA (20 µM) specific for PDI, ERp57, ERp72, P5, Ero1α, Ero1β, PRDX4, QSOX1a/b, or VKOR, or a negative non-targeting control (see Table 2.1), were prepared in RNase-free water. The protocol to achieve optimal transfection efficiency and knockdown in HepG2 cells is summarized in Figure 2.1. The day before transfection, low passage HepG2 cells (< passage 30) were plated at a density of 1.5 x 10^5 cells/mL in P100 dishes to achieve approximately 2.5 x 10^5 cells/mL the following day. On the day of transfection, 490 µL of Opti-MEM (Invitrogen) medium was added per well to 6-well plates. Next, 4 µL of stock siRNA was added to each well, mixed by gentle pipetting, and incubated for at least 5 min at room temperature. Oligofectamine™ (6 µL; Invitrogen) was then added to each well, mixed briefly by tilting the plates, and complexes were incubated for 20 min at room temperature. To complete the “reverse transfection” (i.e. suspended cells added on top of complexes), HepG2 cells, previously plated on Day 0 to control their density, were trypsinized, counted, pelleted, and resuspended in DMEM + Gln (no antibiotics, no FBS) such that 2.5 x 10^5 cells in 1.5 mL could be added to each well (final volume: 2 mL, final [siRNA]: 40 nM). After 4 h at 37°C, the medium was supplemented with 1 ml DMEM + Gln and 30% FBS (final FBS concentration: 10%). Following knockdown for 3 d, the cells were trypsinized, pooled, and resuspended to again add 2.5 x 10^5 cells per well in 1.5 mL DMEM + Gln for a second transfection with fresh complexes using the same targeting siRNA. Since the cell number increased over the first 3 days, the total number of wells transfected was greater for the second transfection (e.g. three 6-well plates on day 4 in Fig. 2.1). Following an additional 2 d, cells were processed by trypsinization and pooling for reseeding in the desired plates and analysis the following day. The final pooling of cells effectively eliminated plate-to-plate variation in knockdown efficiency and allowed application-specific control of cell density (e.g. pooling a sufficient number of wells for a desired 1 x 10^6 cells per 60 mm dish). In addition to single-knockdown studies, this same protocol was followed when constitutive ERp57 knockdown (2.3.1) cells were used for co-depletion of ERp57 with either PDI, ERp72, or P5.

2.3.3 Combinatorial knockdowns

For co-depletion of PDI and ERp72 or PDI and P5 in either control cells or constitutive ERp57 knockdown cells, the same protocol was followed as listed above, but siRNA treatment of ERp72 or P5 took place the day following PDI siRNA treatment (i.e. days 2 and 4) and total
Table 2.1 siRNAs used in this study

<table>
<thead>
<tr>
<th>Targeted Protein</th>
<th>Catalogue Number (Invitrogen)</th>
<th>Sequence (anti-sense 5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDI</td>
<td>HSS143215</td>
<td>ggaagaacuuugaagacguggcuu</td>
</tr>
<tr>
<td>PDI</td>
<td>HSS143217</td>
<td>caaggagaaccugcuggacuuuaau</td>
</tr>
<tr>
<td>ERp57</td>
<td>HSS142316</td>
<td>gacacugcaagagacuugcaccuga</td>
</tr>
<tr>
<td>ERp57</td>
<td>HSS142317</td>
<td>cccauuagcaaagguugauugcaca</td>
</tr>
<tr>
<td>ERp72</td>
<td>HSS114334</td>
<td>gaaucguugauuacaugacagca</td>
</tr>
<tr>
<td>ERp72</td>
<td>HSS114335</td>
<td>acacuuucacacagaaaaagaaaaa</td>
</tr>
<tr>
<td>P5</td>
<td>HSS115409</td>
<td>caaggcagaagugauagauuacaaaaaa</td>
</tr>
<tr>
<td>P5</td>
<td>HSS115410</td>
<td>gcacugcugggaugcuacaguau</td>
</tr>
<tr>
<td>Ero1α</td>
<td>HSS112176</td>
<td>aaugguaacuuggaagaagcccc</td>
</tr>
<tr>
<td>Ero1β</td>
<td>HSS116219</td>
<td>gca uuugaagagaaaaacauu</td>
</tr>
<tr>
<td>PRDX4</td>
<td>HSS195324</td>
<td>caguugaucagauggagaaauuu</td>
</tr>
<tr>
<td>QSOX1a/b</td>
<td>HSS155902</td>
<td>accuggaaucugacugcucuacau</td>
</tr>
<tr>
<td>VKOR</td>
<td>HSS187093</td>
<td>gagagaaccagcgagagacaccagg</td>
</tr>
<tr>
<td>Negative Control (Med GC)</td>
<td>12935-300</td>
<td>Proprietary</td>
</tr>
</tbody>
</table>
Figure 2.1. Standard single knockdown protocol. HepG2 cells were treated twice with siRNA and Oligofectamine by reverse-transfection for optimal knockdown efficiency on Day 6.
knockdown time was extended to 7 d. For co-depletion of Ero1α and Ero1β, cells were treated with a mixture of Ero1α and Ero1β siRNAs on day 1 (20 nM final concentration each), then only Ero1α on day 4 and only Ero1β on day 5 (40 nM final concentration) for analysis on day 7. When a third target, VKOR or PRDX4, was knocked down, the transfection protocol was carried out as follows: Day 1 – 20 nM each Ero1α and Ero1β siRNAs; Day 2 – 40 nM VKOR or PRDX4 siRNA; Day 4 – 30 nM Ero1α + 20 nM VKOR or PRDX4 siRNA; Day 5 – 30 nM Ero1β + 20 nM VKOR or PRDX4 siRNA and analysis on day 7. For the Ero1α+β & QSOX1 knockdown, the transfection protocol used was: Day 1 – 20 nM each of Ero1α and Ero1β siRNA; Day 3 – 40 nM QSOX1 siRNA; Day 4 – 30 nM Ero1α + 10 nM QSOX1 siRNA; Day 5 – 30 nM Ero1β + 10 nM QSOX1 siRNA for analysis on day 7.

### 2.4 Western Blots

For immunoblot analysis of control and knockdown cell lysates, SDS-PAGE and transfer onto polyvinylidene difluoride (PVDF; Millipore) membranes was carried out. Blocking in 5% skim milk in PBS-T (phosphate buffered saline + 0.1% Tween-20) took place at room temperature for 30 min. Following washing of the membrane in PBS-T, incubation of the membrane in primary antibody was carried out at room temperature for 1 hour with rocking. Washing of the membrane in PBS-T was performed again and 1 hour incubation in secondary antibody was performed. After a final washing, the membrane was treated with chemiluminescence solution and exposed to film for multiple exposures. Films were scanned and quantified by densitometry using Quantity One software (Biorad).

### 2.5 UPR induction and RT-PCR

Positive controls for UPR induction were carried out by treating HepG2 cells with either 0.5 or 5 µg/mL tunicamycin (Sigma) dissolved in DMSO or an equivalent volume of DMSO only (control) overnight at 37°C. Cells were split for immunoblot analysis and for RNA isolation. Total RNA was prepared using an RNeasy spin column kit (Qiagen) and was then subjected to PCR to amplify Xbp-1 using the One-Step RT-PCR kit (Qiagen) and human Xbp-1 amplification primers: 5’-GGAGTTAAGACAGCGCTTGG-3’ and 5’-GAGATGTTCCTGGAGGGGTGA-3’. The PCR conditions used were as follows: 35 min at 50°C, 15 min at 94°C, (1 min at 94°C, 1 min at 60°C, 1 min at 72°C) 30 cycles, and 10 min at 72°C.
2.6 Confocal Microscopy

Cells were plated on Cell-Tak-treated coverslips (BD Biosciences) in P35 dishes for 8 hours, medium removed and cells gently washed with PBS. For 30 min cells were fixed in 4% paraformaldehyde, washed with 100 mM glycine for 5 min, and permeabilized for 1 min with 0.2% Triton X-100. Staining for calnexin or transferrin was carried out by inverting each coverslip onto parafilm spotted with 200 µL PBS containing primary antibody at a concentration of 1:250, washing, and then secondary antibody at a concentration of 1:500. Washed coverslips were mounted onto slides using Fluorescence Mounting Medium (Dako Cytochemistry), sealed with clear nail polish, and dried in the dark under a heavy object. Immunofluorescence was observed by confocal microscopy at the University of Toronto Microscopy Imaging Lab using a Zeiss LSM510 upright laser scanning microscope.

2.7 Warfarin inhibition of VKOR & growth curves

For inhibition of VKOR using warfarin, cells were re-plated in medium containing 50 µM warfarin (Wallin et al., 1990) Sigma) dissolved in DMSO and incubated overnight at 37 °C. Control cells were treated with DMSO only. Cell growth and viability were monitored from day 4 onwards by trypsinizing and suspending cells in 1.5 ml of medium, counting and determining percentage of viable cells by trypan blue exclusion.

2.8 Metabolic labeling and immunoisolations

2.8.1 Standard protocol

For pulse-chase radiolabeling experiments, HepG2 cells in 60 mm dishes were starved for 30 min with Met-free RPMI 1640 and radiolabeled for 3 min in 1 mL of medium containing 0.1 mCi of [35S]Met (>1000 Ci/mmol; Perkin Elmer). The cells were chased for various durations in RPMI 1640 supplemented with 1 mM Met. The chase was terminated by placing cells on ice and then free thiols were alkylated by incubating on ice for 3 min in 2 mL of PBS supplemented with 20 mM N-ethylmaleimide (NEM; Sigma). Cells were then lysed at 4 °C in 1 mL RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate (DOC), 0.1% SDS, and protease inhibitors) containing 20 mM NEM and subjected to serial immunoisolations with 30 µg anti-albumin, anti-transferrin, anti-α-fetoprotein, anti-α-2-HS-glycoprotein and anti-α-1-antitrypsin antibodies. Media samples collected from 30 min chase
times onward were also subjected to immunoisolation. In each case, following rocking for 2 h at 4°C, immune complexes were recovered by incubation for 1 h with 30 µL of protein-A agarose beads (GE Healthcare). Bead-bound complexes were washed 4 times with 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 0.025% DOC, 0.01% SDS. Samples were then split equally for elution in SDS-PAGE sample buffer, either lacking or containing 40 mM DTT, and analyzed by SDS-PAGE (10% gels). Radioactive proteins were detected either by Storm phosphorimaging (Molecular Dynamics) and quantified with ImageQuant software (GE Healthcare) or by fluorography onto X-ray film and quantified by densitometric analysis using Quantity One software (BioRad).

For expression of a secretory protein’s status as % Oxidized, % Reduced or % Secreted, the total recovered signal was summed from densities calculated for reduced, partially oxidized, oxidized, glycan modified (post ER), and secreted forms of substrate. The sum of oxidized, glycan modified (and oxidized), and secreted (and oxidized) forms of substrate, divided by the total signal generated the % Oxidized values. The fully reduced form of substrate that matched the +DTT mobility control included on every gel was used to calculated the % Reduced values. The signal recovered from collected chase medium at each time point divided by the total signal constituted the % Secreted value.

2.8.2 Radiolabeling following reductive (DTT) challenge

In experiments coupling pulse-chase radiolabeling with a reductive DTT challenge, HepG2 cells in 60 mm dishes were starved in 2 mL Met-free RPMI for 25 min, then 1 mL of Met-free RPMI containing 15 mM DTT (Bioshop) was added per dish (5 mM final DTT concentration) and incubation was continued for another 5 min. Cells were subsequently pulse radiolabeled for 3 min in 1 mL of Met-free media containing 0.1 mCi of [35S]Met and 5 mM DTT. The chase and DTT washout were carried out by removing labeling medium and replacing with 3 mL of medium supplemented with 1 mM Met. Alkylation with NEM, lysis, immunoisolation and analysis was carried out as described above. Comparison of both labeling protocols is illustrated in Figure 2.2.
Figure 2.2. Labelling and immunoisolation flow for experiments with (grey) and without (white) reductive challenge. The DTT treatment and washout was utilized to measure re-oxidation of the ER as well as substrate folding. Oxidative folding of substrates observed at later time points is apparent on non-reducing SDS-PAGE by maturation to a greater mobility oxidized form (1), as well as post-ER glycan modifications generating higher bands (2), and secretion to the chase medium (3).
2.8.3 Substrate trapping and sequential immunoprecipitations

For sequential immunoprecipitation of mixed disulfide complexes between PDI<sup>CXXA</sup>-HA and substrates, HepG2 cells expressing mutant PDI or the viral vector were pulsed for 15 min with 50 µCi/plate of [<sup>35</sup>S]Met, incubated with PBS containing 20 mM NEM and lysed as outlined above. Immunoprecipitation was carried out using anti-HA antibody and protein-A agarose with immune complexes eluted and disrupted by incubation at 40°C for 1 h in 0.2% SDS in PBS (pH 6.8). The eluates were adjusted to 2% NP-40, 5 mg/mL gelatin and 50 µM HA peptide (Genscript) in PBS (pH 6.8) prior to a second round of immunoprecipitation with anti-substrate antibody. Immune complexes were analyzed by reducing SDS-PAGE (10% gel) and visualized by fluorography.

2.9 Assessment of glutathione levels

2.9.1 Sample preparation

For experiments involving the assay of glutathione levels following reductive challenge, cells in 35 mm dishes were treated with 1 mL of medium containing 5 mM DTT for 8 min. The DTT-containing medium was removed, cells were quickly washed with 1 mL of medium lacking DTT and then cells were incubated with 2 mL of fresh medium for the indicated times.

Cellular levels of oxidized glutathione (GSSG) and total (oxidized plus reduced) glutathione (G<sub>tot</sub>) were quantified using a modified version of the glutathione reductase recycling assay described by Rahman <i>et al</i> (Rahman <i>et al</i>, 2006). Briefly, following various knockdowns and/or reductive challenge with DTT, HepG2 cells were washed in PBS, scraped from the plate in 40 µL of PBS, transferred into ice-cold microfuge tubes containing 10 µL of 5% 5-sulfosalicyclic acid and vortexed immediately. Following centrifugation at 4°C for 5 min at 12000 x g, the supernatant fraction was transferred to new microfuge tubes and diluted with 330 µL of 0.1 M sodium phosphate buffer, pH 7.4 containing 1 mM EDTA (NaPE buffer). To assay G<sub>tot</sub>, 50 µL of sample was added directly to the wells of a 96-well plate. To measure GSSG, 130 µL of each sample was first mixed with 6 µL of 2-vinylpyridine (2-VP, Sigma) which reacts with GSH selectively (or 6 µL of NaPE buffer for G<sub>tot</sub> samples) and incubated for 1 h at room temperature. A 50 µL aliquot was then applied to the wells of the 96-well plate.
2.9.2 Colourimetric assay

To initiate the glutathione recycling assay (Fig 2.3), all wells were mixed with 100 µL of reaction mixture (for one plate, freshly prepared: 2.8 mL of 1 mM Ellman's reagent (5,5’-dithiobis(2-nitrobenzoic acid)), 3.75 mL of 1 mM NADPH, 20 units of glutathione reductase, 5.85 mL NaPE buffer; all reagents from Sigma) and absorbance was read continuously at 405 nm for a total of 5 min. Initial reaction rates were compared to those obtained with a standard curve spanning the range from 0-500 pmol GSSG.

Figure 2.3. The glutathione colourimetric recycling assay. (A) Oxidized glutathione, GSSG, is reduced to two molecules of GSH by glutathione reductase, requiring NADPH as electron donor. The GSH generated is substrate for reaction with DTNB (5,5’-dithiobis(2-nitrobenzoic acid)) in (B). (B) GSH reacts with DTNB to release one molecule of TNB (5-thio-2-nitrobenzoic acid) and forms a mixed disulfide with another molecule of TNB. Glutathione reductase resolves this mixed disulfide to regenerate GSH for another reaction with DTNB and releases another molecule of TNB. The absorbance of TNB was monitored at 405 nm ($\lambda_{\text{max}} = 412$ nm). The total glutathione levels measured are the result of the GSH in the system directly entering the reaction in (B) plus the GSSG that is first converted to GSH as shown in (A) and then undergoes the reaction shown in (B). Treatment with 2-VP effectively removes GSH from the system so that GSSG levels only are measured as the glutathione disulfide passes through reaction (A) and then reaction (B).
Chapter 3

Functional relationship between protein disulfide isomerase family members during the oxidative folding of human secretory proteins

This chapter was originally published as:


Acknowledgements:

Mutagenesis of PDI and lab development of the Moloney virus packaging system were carried out by Dr. Ulf Brockmeier. Some washing of immunoisolated protein complexes was performed by Myrna Cohen-Doyle, lab technician.
3.1 Introduction

The formation, isomerization, and reduction of disulfide bonds in the ER is catalyzed by thiol oxidoreductases of the protein disulfide isomerase (PDI) family. Assignment of as many as 20 proteins to the mammalian PDI family is based on the presence of at least one thioredoxin-like domain, with catalytic activity determined by the presence of a pair of cysteine residues within a CXXC motif that is able to alternate between disulfide and dithiol forms (Appenzeller-Herzog and Ellgaard, 2008; Hatahet and Ruddock, 2009). The family’s founding member, PDI, is able to carry out all thiol exchange functions. It has a U-shaped structure characterized by a hydrophobic interior, largely provided by the $b'$ domain, that serves as an interaction surface for non-native folding intermediates (Tian et al., 2006). However, other family members have been shown to be capable of carrying out these functions as well (Alanen et al., 2006a). Of these, the best characterized is ERp57 which shares a high degree of similarity with PDI, but its substrate-binding $b'$ domain has diverged such that it interacts with Cnx and Crt (Oliver et al., 1999; Kozlov et al., 2006). The mechanism of substrate recognition of other family members, such as ERp72 and P5, has remained elusive. Additionally, the question of why so many PDI family members reside in the ER is still open. I hypothesized that PDI family members possess substrate specificity in their action and that some family members may be dedicated for differing redox activities. The goal of this study was to test for such functional differences.

Previous substrate trapping studies revealed distinct substrate profiles for each PDI family member but with considerable overlap (Jessop et al., 2009a; Jessop et al., 2009b). Most notable was a strong preference for glycoprotein substrates by ERp57, a wide range of substrates for P5 and a surprising absence of identifiable substrates for PDI. Since in trapping studies the functional role of individual family members cannot be assessed and substrates undergoing PDI member-catalyzed oxidative folding are not detected, I undertook a systematic knockdown of multiple PDI family members in mammalian cells and report the functional consequences on the oxidative folding and intracellular trafficking of a number of endogenously expressed substrates, including albumin, $\alpha$-fetoprotein, transferrin, $\alpha_2$-HS-glycoprotein, and $\alpha_1$-antitrypsin. By high efficiency depletion of PDI, ERp57, ERp72 and P5, either alone or in various combinations, the relative importance of these family members in oxidative folding was evaluated and their functional overlap was assessed.
3.2 Effective knockdown of PDI family members without observable UPR induction

Since attempts have failed to obtain mammalian cell lines that constitutively express very low levels of PDI (Park et al., 2006), a transient RNAi knockdown approach was used to assess the functions of PDI family members in human hepatoma cells. For high efficiency knockdown, it was necessary to carry out the knockdown over a period of 6 days with two transfections of the targeting siRNA. Cell growth was equivalent in both control and knockdown conditions after the first transfection, but following the second application of siRNA a slowing of growth rate was observed in cells treated with targeting siRNA compared to cells treated with control siRNA cells (~75% of control rate for PDI knockdown and ~85% for knockdown of other PDI family members). However, no accompanying loss of cell viability or impairment of protein translation was observed (data not shown).

Using the two-step transfection method, >95% knockdown efficiency was attained for PDI, ERp57, ERp72, and P5 using either of two independent siRNAs (Fig. 3.1A). HepG2 cells constitutively expressing shRNA targeting ERp57 (ERp57sh) also exhibited knockdown of ERp57 comparable to that observed with siRNA. These cells facilitated experiments in which both ERp57 and a second or third PDI family member were depleted in combination. Knockdown efficiency also exceeded 95% in the various combination knockdowns (Fig. 3.1A). No off-target knockdown was observed for other PDI family members, and in no case was compensatory upregulation of remaining family members observed.

We anticipated that depletion of PDI family members might induce the unfolded protein response (UPR). Surprisingly, there was no increase in the expression of BiP, PDI or CHOP, representing middle and late stages of the UPR, in any of the single or multiple knockdowns (Fig. 3.1A) (Wu and Kaufman, 2006). Additionally, there was no alteration in the levels of Cnx or Crt. To monitor the IRE-1 pathway directly, we tested for Xbp-1 splicing (Yoshida, 2007) and the spliced product was not observed in any of the knockdown conditions compared to cells treated with tunicamycin as a positive control (Figure 3.1B). Similarly, there was no evidence of PERK pathway activation as measured by phosphorylation of eIF2α (data not shown).

We further tested whether PDI-depleted cells might be more susceptible to externally
Figure 3.1. Efficacy of PDI family member knockdown and assessment of UPR. (A) PDI family members were depleted from HepG2 cells by RNAi over a period of 6 d. Indicated single or multiple knockdowns were carried out transiently using two independent targeting siRNAs, designated (1) and (2), or in some instances using cells constitutively expressing shRNAmir targeting ERp57 (ERp57\textsuperscript{sh}). Equivalent amounts of cell lysates were immunoblotted for PDI family members, ER chaperones, CHOP and actin. Images shown are a composite from various experiments, all of which included actin as a loading control. Exposures were adjusted to an equivalent density of the actin control. Data are representative of a minimum of 5 replicate knockdowns. (B) Following the indicated knockdowns or overnight treatment with 5 µg/mL tunicamycin, total RNA was isolated from HepG2 cells and analyzed for Xbp-1 splicing by reverse-transcription PCR. Xbp-1\textsuperscript{U}, unspliced Xbp-1 product; Xbp-1\textsuperscript{S}, spliced Xbp-1 product. (C) HepG2 cells were subjected to PDI knockdown for 10 d. On day 9, cells were treated overnight with the indicated concentrations of tunicamycin (Tm). The following day, cell lysates were immunoblotted to detect PDI, BiP, and actin. Lanes shown were assembled from a single gel. Total RNA was also isolated and analyzed for Xbp-1 splicing.
imposed ER stress. HepG2 cells were subjected to knockdown for an extended period of ten days including an additional siRNA transfection on day 6 to maintain cellular siRNA concentration. As shown in Fig. 3.1C, greater than 95% knockdown of PDI was maintained during this period, yet remarkably there was no evidence of UPR induction as assessed either by Xbp-1 splicing or by increased BiP expression (Fig. 3.1C, compare lanes 1 and 4). These cells were subsequently tested for their sensitivity to ER stress induced by tunicamycin. Control cells exhibited Xbp-1 splicing and a 1.4-fold increase in BiP level only at 5 µg/mL tunicamycin, but in 10-day PDI-depleted cells Xbp-1 splicing and increased BiP expression (1.5-fold) were apparent at a 10-fold lower concentration of tunicamycin. Furthermore, at 5 µg/mL tunicamycin, the increase in BiP level (1.9-fold) and extent of Xbp-1 splicing in PDI-depleted cells exceeded those observed in control cells (Fig. 3.1C). These findings indicate that HepG2 cells depleted of PDI, although not exhibiting an overt UPR themselves, are more sensitive to ER stress imposed through tunicamycin treatment. Since the degree of Xbp-1 splicing and BiP upregulation in control HepG2 cells was remarkably modest following tunicamycin treatment (Fig. 3.1C), it may be that these professional secretory cells have higher basal levels of folding and ER-associated degradation (ERAD) machineries, and so they do not respond as readily as other cell types to stress conditions. Indeed, it has been reported that nearly 75% of endogenous apolipoprotein B-100 in HepG2 cells is constitutively degraded cotranslationally (Adeli et al., 1997), suggesting that misfolded substrates may be disposed of without the need for an additional UPR.

The preceding data indicate that the 6-day period chosen for PDI family member depletion is suitable to assess the functions of PDI family members without confounding factors such as compensatory upregulation of other family members or induction of the UPR.

### 3.3 Identification of PDI substrates

Human HepG2 cells were selected for these experiments because they express many well characterized secretory proteins that possess disulfide bonds. Albumin (Alb), α-fetoprotein (αFP), and transferrin (TF) are attractive study subjects since they are monomeric, contain multiple disulfides and have molecular weights sufficiently low for oxidative folding to be monitored by non-reducing SDS-PAGE. Furthermore, αFP and TF are glycosylated whereas Alb is not, which permits an assessment of glycoprotein preference by PDI family members during catalysis of oxidative folding. To first confirm that these secretory proteins are indeed substrates
of PDI, HepG2 cells were prepared that constitutively express an HA-tagged substrate-trapping mutant of PDI (PDI^{CXXA-HA}) (Fig. 3.2A). Mutation of the second Cys in both PDI active sites stabilizes mixed-disulfide reaction intermediates with substrates on which it is acting as a reductase or isomerase (Walker and Gilbert, 1997). Although our focus is on oxidative folding, it is likely that in the case of PDI it selects substrates largely through hydrophobic interactions regardless of the reaction catalyzed (Tian et al., 2006). Radiolabeled cells expressing PDI^{CXXA-HA} were subjected to a first round of immunoisolation with anti-HA mAb followed by disruption of immune complexes and a second round of immunoisolation with Ab directed against various secretory proteins. As shown in Fig. 2B, Alb, αFP, and TF could all be recovered as complexes with the PDI trap mutant, confirming them as PDI substrates. Other bands observed in complex with the PDI trap mutant were not identified, but presumably correspond to additional PDI substrates (Fig. 3.2B, lane 2). In addition to Alb, αFP, and TF, α_2-HS-glycoprotein (α_2HS) was selected for study based on high disulfide content and available reagents. As a negative control to detect any pleiotropic effects of PDI family member knockdown, α_1-antitrypsin (α_1AT) was selected since it is a glycoprotein that lacks disulfide bonds.

3.4 Individual knockdown of PDI family members

To determine the contributions of PDI, ERp57, ERp72, and P5 to the oxidative folding of secretory proteins, pulse-chase experiments were carried out under control and knockdown conditions. Free thiols were alkylated with membrane-permeable NEM prior to cell lysis to prevent air oxidation and disulfide isomerization. As shown for PDI knockdown in Fig. 3.3A, non-reducing SDS-PAGE was used to monitor the progression of substrate proteins from the fully reduced (R) form of slow mobility to partially oxidized (PO) and fully oxidized forms (O) of higher mobilities. For αFP and α_2HS, the fully oxidized form was further converted to one or two slower-mobility species (G, G_1), reflecting processing of their oligosaccharide chains to larger complex structures in the Golgi. A reduction in signal was also apparent at late chase times due to secretion and fully oxidized species could be recovered from the culture medium (Fig. 3.3B). These were included when calculating percentages of proteins oxidized or secreted (Fig. 3.3B, C). In addition, the region near the top of the gel as well as the stacking gel were monitored for disulfide-linked aggregates, since previous PDI and ERp57 depletion studies
Figure 3.2. Identification of PDI substrates. (A) Lysates of HepG2 cells stably expressing either the pQCXIH expression vector (vector) or HA-tagged PDI carrying C56A and C400A mutations (PDI\textsuperscript{CXXA-HA}) were immunoblotted with anti-HA antibody. (B) HepG2 cells expressing either vector or PDI\textsuperscript{CXXA-HA} were radiolabeled with \[^{35}\text{S}]\text{Met}\) for 15 min, and treated with 20 mM NEM in PBS. Cell lysates were immunoisolated with anti-HA antibody, immune complexes were disrupted and then immunoisolated a second time with the indicated antibodies. Proteins were separated by reducing SDS-PAGE and visualized by fluorography. Alb, albumin; \(\alpha\text{FP}, \alpha\)-fetoprotein; TF, transferrin.
Figure 3.3. Effect of PDI knockdown on oxidative folding of secretory proteins. HepG2 cells were depleted of PDI or treated with control siRNA for 6 d, radiolabeled with $^{35}$SMet for 3 min then chased with unlabeled Met for the indicated times. The medium was removed and then cells were treated on ice with 20 mM NEM in PBS and lysed in RIPA buffer containing 20 mM NEM. Both cell lysates and media were immunopurified with antibodies directed against the indicated proteins. (A) Kinetics of disulfide formation. Immune complexes from cell lysates were subjected to SDS-PAGE under reducing (1st lane) or non-reducing conditions and proteins were visualized and quantified by phosphorimaging. Shown are representative gels of three independent experiments for substrates albumin (Alb), α-fetoprotein (αFP), transferrin (TF), and α2-HS-glycoprotein (α2HS). Reduced (R), partially oxidized (PO), oxidized (O), and Golgi (G, G1) forms of each protein are indicated. (B) Secretion kinetics. Immune complexes recovered from media samples at the indicated chase times were analyzed by SDS-PAGE and visualized and quantified by phosphorimaging. Histograms indicate the amount of protein observed in the medium as a percentage of the combined intra- and extra-cellular signal. Black and grey bars represent control (C) and knockdown (KD) conditions, respectively. (C) Quantification of gels shown in panels A and B was carried out by expressing the amount of fully oxidized protein in lysate and media samples as a percentage of the total combined amount of reduced, partially oxidized, and oxidized forms at a given time point. Solid lines represent control conditions; dashed lines represent knockdown conditions. (D) ER to Golgi transport kinetics. The indicated proteins were immunopurified and either digested or not with endo H as indicated and analyzed by reducing SDS-PAGE. Endo H$^R$ and Endo H$^S$ represent endo H-sensitive and -resistant species, respectively. G and ER represent Golgi-processed and ER forms which could be resolved without endo H digestion. (E) Quantification of the gels in panel D. Histograms represent the amount of protein remaining in the ER as a percentage of the total protein recovered from cells and medium at each time point. Black bars, control; grey bars, knockdown. Error bars represent the average of three independent experiments +/- one standard deviation, except in the case of TF which was examined in a single experiment.
involving overexpressed exogenous substrates had reported significant quantities of such aggregates (Solda et al., 2006; Lee et al., 2010). However, we did not observe disulfide-linked aggregates upon PDI family member depletion, either individually or in combination, for any of our endogenous substrates (shown only for Alb in Figs. 3.3-3.6).

During the oxidative folding of Alb and αFP, formation of disulfide bonds was very rapid and a substantial shift in gel mobility was seen even in the 3 min pulse period (Figure 3.3A, control; compare lane P -/+ DTT). Only one distinct partially oxidized species was observed under control conditions that slowly progressed to fully oxidized forms (species O and G) over the course of 30-60 min. Upon depletion of PDI, the impact on oxidative folding was remarkably modest for both proteins. The initial rapid formation of the partially oxidized species during the 3 min pulse period was unchanged, but progression to the fully oxidized species during the subsequent 10 min of chase was delayed (Fig. 3.3A, PDI lanes and Fig. 3.3C, dashed lines). No other partially oxidized forms were observed. By 20-30 min of chase, the proportion of fully oxidized Alb and αFP (O + G species) in PDI-depleted cells became similar to that in control cells, although it was noted that in the PDI-depleted cells the conversion of the αFP O species to the Golgi-processed G species was delayed. Since non-native protein conformers are normally subject to quality control and retained in the ER, we also monitored secretion rates and, in the case of glycoproteins, ER to Golgi transport rates as an indirect measure of folding efficiency. This method has been used previously to detect incorrectly disulfide-bonded conformers in proteins that exhibit an otherwise normal extent of oxidation (Chakravarthi and Bulleid, 2004; Solda et al., 2006). Both Alb and αFP appeared in the culture medium at similar rates in the absence or presence of PDI (Fig. 3B). ER to Golgi transport of αFP was measured by the rate at which its oligosaccharides were processed from an endo H-sensitive ER form to a complex, endo H-resistant form in the medial-Golgi (Fig. 3D, quantified in Fig. 3E). In the absence of PDI, ER to Golgi transport of αFP was notably delayed at the 30 min chase time, consistent with the delay noted above in the conversion of the αFP O species to the G species at 30 min in Fig. 3A. Since control experiments revealed no delay in the ER to Golgi transport of non-disulfide-bonded α₁-antitrypsin (α₁AT), the delay observed for αFP was specific to this protein. Given that αFP exhibited a similar degree of disulfide formation at the 30 min chase point in the absence or presence of PDI (Fig. 3.3C), these data are consistent with the presence of non-native
folding intermediates that may require a disulfide isomerization event, delayed in the absence of PDI, in order to be released from the ER quality control machinery.

The oxidative folding of TF occurred at a slower pace than Alb or αFP and the fully oxidized form was not detectable until 10 min of chase under control conditions (Fig. 3.3A, C). Multiple partially oxidized folding intermediates were visible as a smear between the fully reduced and fully oxidized forms. Upon depletion of PDI, the formation of the various partially oxidized species appeared unaffected but the rate of their subsequent conversion to the fully oxidized species was delayed. However, by 60 min of chase, TF was maximally oxidized in both control and PDI-depleted cells. PDI-depletion also resulted in slower ER to Golgi transport of TF, most apparent by 60 min of chase (Fig. 3.3D, E), although no difference in its rate of secretion was detected (Fig. 3.3B). The final secretory protein tested, α2HS, yielded clearly distinguishable fully reduced, partially oxidized and fully oxidized forms, but only a slight delay in oxidative folding was observed at the 10 and 20 min chase times in PDI-depleted cells (Fig. 3.3A, C). By 30 min of chase, α2HS was fully oxidized (species O, G, G1) in both control and PDI-depleted cells. A modest delay in ER to Golgi transport was also observed in PDI-depleted cells at 20 and 30 min of chase (Fig. 3.3D and E). It is noteworthy that slower ER to Golgi transport was manifested for both TF and α2HS at time points when they were maximally oxidized, again suggestive of non-native disulfides in the absence of PDI.

Overall, PDI appears to be a versatile and prominent oxidoreductase in that its depletion affected all substrates tested and other PDI family members could not fully complement its loss. However, the phenotypes observed were more modest than expected given the essential role of PDI in yeast, raising the question of whether other PDI family members are able to substitute to some degree for the functions of PDI.

We next knocked down ERp57 and monitored the same substrates for changes in oxidative folding and intracellular transport. For Alb there was no observed difference in oxidative folding in cells with or without ERp57 and no changes in secretion efficiency (Figure 3.4A-C). In contrast, αFP and α2HS, exhibited modest delays in oxidative folding similar to those observed upon depletion of PDI. However, unlike PDI depletion, there was little impact on rates of ER to Golgi transport, suggesting that native disulfides were being formed (Fig. 3.4D, E). For TF, ERp57 depletion resulted in a marked delay in oxidative folding that was
Figure 3.4. ERp57 depletion delays oxidative folding of some glycosylated substrates. HepG2 cells were depleted of ERp57 or treated with control siRNA and pulse-chase experiments were conducted and analyzed as described in Figure 3.3. (A) Kinetics of disulfide formation. (B) Secretion kinetics. Black and grey bars represent control and knockdown conditions, respectively. (C) Quantification of oxidative folding. Solid lines, control conditions; dashed lines, knockdown conditions. (D) ER to Golgi transport kinetics. (E) Quantification of the gels in panel D. Error bars represent the average of three independent experiments +/- one standard deviation, except in the case of TF which was examined in a single experiment.
considerably more pronounced than that observed with PDI depletion (Fig. 3.4A-C). This occurred through 60 min of chase, at which time only 45% of transferrin had reached a fully oxidized state in the absence of ERp57 compared to 85% fully oxidized transferrin in PDI-depleted cells (compare Fig. 3.3C and 3.4C). By 2h, almost all of TF in the cells and medium was fully oxidized (Figure 3.4A-C, TF panel). Although secretion of TF was not significantly delayed upon ERp57 depletion, ER to Golgi transport was substantially impaired (Fig. 3.4D, E). This was observed throughout 30-90 min of chase, reflecting the slower formation of disulfide bonds. These data indicate that TF is a substrate highly specific to ERp57 and that other family members, such as PDI, are acting on this substrate with reduced efficacy. Even the modest phenotypes observed upon ERp57 depletion for αFP and α2HS suggest that PDI, despite being able to act on these substrates, is not able to fully complement the loss of ERp57 for these client proteins. The lack of impact of ERp57 depletion on the folding of Alb stands in contrast to the other substrates. Alb is the only protein in this set that is not glycosylated and hence is less likely to be targeted via Cnx or Crt to ERp57 (Jessop et al., 2009a). This is in agreement with previous work suggesting that the action of ERp57 is largely specific for glycoproteins (Oliver et al., 1999; Solda et al., 2006; Jessop et al., 2009a).

Individual knockdowns of ERp72 and P5 were also carried out. However, there was no change in the oxidative folding of any of the substrates tested and no changes were observed in their rates of ER to Golgi transport or secretion (data summarized in Table 3.1). Since the preceding data indicated that the secretory proteins under study are substrates for PDI and/or ERp57, it is possible that any contribution of ERp72 or P5 to the oxidative folding of these proteins was simply masked by the action of PDI or ERp57. Alternatively, ERp72 and P5 may not act on these substrates, but are specific for other client proteins. In order to distinguish between these possibilities as well as to characterize the degree of functional overlap between PDI and ERp57, PDI family members were depleted in combination.

3.5 Combinatorial knockdown of PDI family members

Initially, PDI and ERp57 were knocked down with high efficiency (Fig. 3.1A). As shown in Figure 3.5, A and B and quantified in Figure 3.6A, we observed a marked decrease in the total radioactive signal recovered from the combined cell lysates and medium in double-knockdown cells beyond 30 min of chase, consistent with degradation of substrate proteins.
(explored further below). Because this degradation complicated the determination of the percentage of fully oxidized or ER-localized forms of the substrates at later time points, quantifications were carried out up to the 30-min time point but not beyond (indicated by the asterisks in Figure 3.5, C and E). Additionally, the amount of each substrate secreted was quantified as a percentage of the total radioactive signal recovered at the 10-min chase time rather than as a percentage of the total signal recovered at each chase time. As shown in Fig. 3.5A and C, the oxidative folding of Alb was delayed to the same extent as when PDI alone was depleted, supporting our finding that ERp57 is not acting on this non-glycosylated substrate (Fig. 3.4). For the glycoprotein substrates, a range of impairments in oxidative folding was observed. For αFP, there was little change relative to PDI or ERp57 depletion alone. For α2HS, there was a greater delay in oxidative folding compared with that observed with either of the single knockdowns and, in the case of TF, oxidative folding was profoundly impaired. By 30 min of chase, only 15% of TF was fully oxidized compared with 25 and 50% when either PDI or ERp57 were depleted, respectively (compare Fig. 3.3C, 3.4C, and Fig. 3.5 C). Indeed, after 2 h of chase, only trace amounts of fully oxidized molecules could be observed (Fig. 3.5A). Furthermore, both secretion and ER to Golgi transport were strongly impaired for all proteins tested (Fig. 3.5B, D, E). Upon comparison to the α1AT control, it was further observed that even a secretory protein lacking disulfide bonds was delayed both in its trafficking from ER to Golgi and its secretion. This suggests that combined depletion of ERp57 and PDI leads to a generalized impairment of ER function.

The observed degradation of substrate proteins (Fig. 3.6A) was investigated further in the case of TF by treating control and PDI/ERp57-depleted cells with the proteasome inhibitor lactacystin and examining both the subcellular distribution of TF by immunofluorescence microscopy and its steady state levels by immunoblotting. As shown in Fig. 3.6B, ER morphology as assessed by Cnx staining was not grossly altered upon combined PDI and ERp57 depletion either alone or following treatment with lactacystin. TF exhibited a reticular ER staining pattern in both control and PDI/ERp57-depleted cells. However, staining was more intense in the depleted cells consistent with ER accumulation and the intensity increased substantially upon inhibition of proteasomal degradation. This was confirmed by immunoblotting wherein cellular levels of TF increased 2.2-fold upon combined PDI and ERp57 depletion relative to control cells and 3-fold following lactacystin treatment (Fig. 3.6C). These
Figure 3.5. Combined knockdown of PDI and ERp57 substantially impairs protein folding. HepG2 cells constitutively depleting ERp57 by shRNAAmir were subjected to knockdown of PDI by siRNA for 6 days. HepG2 cells transfected with control siRNA were prepared in parallel. Pulse-chase experiments were conducted and analyzed as described in Figure 3. (A) Kinetics of disulfide formation. (B) Secretion kinetics. Percent secreted quantified as a function of the total signal recovered at the 10 min chase time point. Black and grey bars represent control and knockdown conditions, respectively. (C) Quantification of oxidative folding. Solid lines, control conditions; dashed lines, knockdown conditions. (D) ER to Golgi transport kinetics. (E) Quantification of the gels in panel D. Error bars represent the average of three independent experiments +/- one standard deviation, except in the case of TF which was examined in a single experiment. Asterisks (*) indicate quantifications were not included beyond 30 min of chase due to substrate degradation.
Figure 3.6. Misfolded transferrin is degraded by the proteasome in PDI/ERp57-depleted cells. (A) Pulse-chase data from Figures 3.3-3.5 was analyzed for changes in the total amount of radiolabeled Alb, αFP, TF, α2HS and α1AT recovered from the combined intracellular and medium fractions. Total protein, as measured by densitometry, was defined as 100% at the 10 min chase time. Error bars represent the average of three independent experiments +/- one standard deviation. (B, C) HepG2 cells were treated with control siRNA or depleted of PDI and ERp57 for 6 d and then incubated in the absence or presence of 5 µg/mL lactacystin for 5 h. Cells were either plated on Cell-Tak-treated coverslips (BD Biosciences), fixed in paraformaldehyde, and permeabilized for confocal microscopy (B) or lysed in RIPA buffer for Western blot analysis (C). Immunoblot or immunofluorescence was carried out using the indicated antibodies (Scale bar = 10 µm).
data are consistent with a substantial degree of protein misfolding accompanying the combined loss of PDI and ERp57 and the subsequent disposal of misfolded proteins by ERAD. Such efficient disposal of misfolded proteins by HepG2 cells may explain the absence of an observable UPR upon depletion of PDI and ERp57 (Fig. 3.1A, B).

Collectively, the stronger phenotypes observed in the double versus single knockdown studies support the view that both PDI and ERp57 are involved in the oxidative folding of the glycoprotein substrates and that neither can fully replace the functions of the other. Furthermore, in the context of the mild phenotypes observed with the other combinatorial knockdowns (see below and Table 3.1), these experiments underscore the prominent role that these thiol oxidoreductases play among the four PDI family members examined.

We next tested whether depletion of ERp72 or P5 in combination with PDI would yield enhanced defects in folding. The combination of PDI + P5 showed no differences for any of the four substrates tested compared to data obtained for PDI knockdown alone (Table 3.1). For the PDI + ERp72 knockdown combination, oxidative folding profiles were comparable to PDI-depleted cells for the three glycoprotein substrates. In contrast, there was a more pronounced delay in the oxidative folding of Alb compared to PDI-depletion alone suggesting that both enzymes can act on this substrate (compare Fig. 3.3A, C and Fig. 3.7). Unlike the PDI + ERp57 combination, these cells did not display a generalized delay in ER to Golgi trafficking or in secretion rate; indeed, the rate of Alb secretion was the same in both control and PDI + ERp72 knockdown cells (data not shown). Therefore, ERp72 likely recognizes a more limited set of substrates compared to PDI or ERp57. Its effect on Alb was likely masked in the individual ERp72 knockdown by the presence of PDI.

To assess whether an effect of ERp72 or P5 knockdown could be assigned in the absence of ERp57, combination knockdowns of ERp57 + ERp72 as well as ERp57 + P5 were carried out. There was no change observed in the oxidative folding of the four substrates beyond what had been noted for ERp57 knockdown alone (Table 3.1). Since the presence of PDI in these cells may have yet been influencing our ability to observe the impact of these family members on glycoprotein folding, a triple knockdown was carried out, depleting cells of PDI, ERp57, and ERp72. Remarkably, the cells grew and remained viable and behaved as previously observed for the PDI + ERp57 double knockdown. Alb oxidation was delayed in a manner similar to what
Figure 3.7. Effect of combined PDI and ERp72 knockdown on oxidative folding of albumin. HepG2 cells were depleted of PDI and ERp72 by siRNA or treated with control siRNA for 6 d and then subjected to pulse-chase radiolabeling and immunoisolation of Alb as described for Fig 3. (A) Representative gel of Alb oxidation from three independent knockdown experiments. (B) Quantification of gel shown in panel A. Solid lines, control conditions; dashed lines, knockdown conditions. Error bars represent the average of three independent experiments +/- one standard deviation.
Table 3.1 Summary of oxidative folding defects accompanying single and combined PDI family member knockdown.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>MW (kDa)</th>
<th>Glycosylation sites (N-linked)</th>
<th>PDI</th>
<th>ERp57</th>
<th>ERp72</th>
<th>P5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>66</td>
<td>0</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>α-Fetoprotein</td>
<td>66</td>
<td>1</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Transferrin</td>
<td>77</td>
<td>2</td>
<td>++</td>
<td>+++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>α₂-HS glycoprotein</td>
<td>44</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>α₁-Antitrypsin</td>
<td>52</td>
<td>3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

The degree of delay in formation of the fully oxidized protein substrate and/or its ER to Golgi trafficking rate in response to the indicated individual or combined PDI family member knockdowns is scored from –, signifying no effect, to ++++, signifying a profound delay.
had been observed in the PDI + ERp72 combination, but there was no additional change in the oxidative folding of the glycoproteins due to ERp72 depletion (Table 3.1). This again suggests that ERp72 does not act on these substrates, but is specific for others such as Alb. The inability to detect any effect of P5 depletion may indicate specificity for a narrow range of substrates, none of which were included in this study, or that it may be involved in other processes such as substrate reduction.

3.6 Discussion

Our current understanding of the relationships among mammalian PDI family members comes mainly from the use of substrate trap mutants to examine the spectrum of client proteins acted on by individual family members (Jessop et al., 2007; Jessop et al., 2009b). However, this approach does not detect substrates undergoing oxidative folding nor does it permit an assessment of the functional impact of each family member. Some functional studies on select substrates have been undertaken by depletion of individual family members but comparative assessment of the impact of depleting different PDI members on oxidative folding has been very limited (Solda et al., 2006; Kang et al., 2009; Zhang et al., 2009). The present study represents the first systematic comparison of the functions of mammalian PDI, ERp57, ERp72 and P5 in the oxidative folding of multiple endogenous substrates.

One of our more surprising findings is that depletion of PDI in HepG2 cells had a relatively modest impact on the oxidative folding capacity of the ER. For all substrates tested, oxidative folding was delayed rather than arrested, with the half-time for acquisition of the fully oxidized state being extended from less than 2-fold for the α2HS and TF substrates to about 8-fold for Alb and αFP. A trivial explanation is that low levels of PDI remaining after knockdown can still catalyze substantial disulfide formation. However, this possibility is unlikely given that estimates of PDI activity required to maintain yeast viability range from 10-20% (Hatahet and Ruddock, 2009), far higher than the trace levels detectable in our experiments. Rather, the modest phenotypes we observe are consistent with a substantial degree of redundancy among mammalian PDI family members, although they were not able to fully complement PDI deficiency. Interestingly, less redundancy is observed in yeast where PDI is absolutely required for efficient oxidative folding of carboxypeptidase Y; none of the remaining family members could support carboxypeptidase Y folding and maturation to even 50% of the efficiency
observed with PDI (Norgaard et al., 2001). We also noted that PDI depletion resulted in a delay in ER to Golgi transport of αFP, α2HS and TF. This occurred after the acquisition of an apparent fully oxidized state, suggesting the presence of non-native disulfides and hence a role for PDI in catalyzing disulfide isomerization. This is consistent with the finding that the isomerase activity of yeast PDI is required for efficient carboxypeptidase folding in vivo (Xiao et al., 2004).

Despite the relative subtlety of the folding defects observed upon loss of PDI, it is noteworthy that PDI silencing affected the oxidation of all substrates examined, irrespective of their glycosylation status. This observation is in agreement with the generally held view that PDI is the predominant ER thiol oxidoreductase (Hatahet and Ruddock, 2009), directly binding to most folding proteins via hydrophobic interactions mediated largely through its b’ domain (Pirneskoski et al., 2004; Tian et al., 2006).

The modest delay in oxidative folding upon PDI depletion is also surprising given that oxidizing equivalents within the ER are thought to pass from Ero1 through PDI to substrate protein (Sevier and Kaiser, 2008). Although controversial (Mezghrani et al., 2001), there is mounting evidence that other PDI family members such as ERp57, ERp46 and ERp18 form mixed disulfides with Ero1 and hence may accept oxidizing equivalents in a similar manner (Jessop et al., 2009b). Alternatively, since more than half of ER-localized glutathione has been shown to be in the form of mixed disulfides with protein (Bass et al., 2004), this may serve as an alternative source of oxidative equivalents in the absence of PDI.

Our studies suggest that ERp57 is a leading candidate among PDI family members for activities that partially complement PDI deficiency. ERp57 had the broadest substrate specificity of the remaining PDI family members examined in this study, contributing to the efficient oxidative folding of αFP, α2HS and TF but not of Alb. Furthermore, when ERp57 was depleted in combination with PDI, a greater impairment in oxidative folding rates was observed for both TF and α2HS compared to either single knockdown alone and slower export of all substrates from the ER was apparent along with evidence of misfolding and disposal by ERAD. Such extensive effects on protein folding and trafficking were not observed when either ERp72 or P5 was depleted in combination with PDI, although a greater delay in the oxidation of Alb was noted with combined PDI and ERp72 knockdown. These findings indicate overlapping substrate specificities for PDI and ERp57 as well as illustrating that both enzymes are required for the efficient folding of certain substrates, most notably TF. The generalized impairment of protein
export from the ER and disposal by ERAD upon depletion of PDI and ERp57, but not with either enzyme individually, suggests that both enzymes are required for the folding of many other substrates as well. Our findings indicate that, of the four PDI family members examined, PDI and ERp57 contribute most substantially to oxidative protein folding within the ER. It will be of considerable interest in the future to test the generality of this observation with an expanded set of glycosylated and non-glycosylated substrates as well as other PDI family members.

Why might an individual substrate require the activities of both PDI and ERp57 during oxidative folding? One possibility is that individual substrate thiols exhibit substantial variation in redox potential that require different PDI family members for thiol-disulfide exchange reactions. However, both PDI and ERp57 possess similar reduction potentials that are substantially more oxidizing than those of substrate thiols (reviewed in (Hatahet and Ruddock, 2009), rendering such a requirement unlikely. Another possibility is that one enzyme acts primarily to catalyze disulfide formation whereas the other is more involved in isomerization reactions. However, current evidence supports the ability of PDI and ERp57 to catalyze both types of reactions in vivo ((Xiao et al., 2004) and see below) and further work would be required to determine if this varies from one substrate to another. Perhaps a more likely possibility is that PDI and ERp57 recognize distinct substrate conformers during the folding process. This may occur either through direct interaction with hydrophobic patches on the substrate in the case of PDI or indirectly through association with different chaperones. As discussed below, ERp57 is thought to be recruited to folding glycoproteins primarily through its association with Cnx and Crt which recognize non-native folding intermediates by virtue of a lectin site specific for monoglucosylated oligosaccharides as well as through a hydrophobic polypeptide binding site (Williams, 2006). PDI has also been detected in a large complex that contains BiP and Grp94, both of which may influence PDI-substrate interactions (Meunier et al., 2002). Such distinct modes of substrate recognition may dictate the utilization of PDI or ERp57 at different stages of the substrate folding pathway.

Previous studies employing substrate-trapping mutants of ERp57 (Jessop et al., 2007) or that interfered with the formation of ERp57-Cnx/Crt (Jessop et al., 2009a) or Cnx/Crt-glycoprotein complexes (Oliver et al., 1997; Zapun et al., 1998; Solda et al., 2006) have concluded that ERp57 has glycoprotein specificity mediated through its association with these lectin-chaperones. Our finding that Alb, the only non-glycosylated substrate examined in this
study, was conspicuously excluded from those substrates affected by the loss of ERp57 is in
good agreement with this view. Interestingly, the impact of ERp57 depletion varied among
the glycoproteins examined. Whereas the effect was mild for αFP and α2HS, the impact on TF was
substantial. Molinari and co-workers recently examined the impact of ERp57 depletion on
different glycoproteins and found that the severity of the defect in oxidative folding correlated
with the dependency of the individual glycoprotein on the Cnx/Crt chaperone system for folding
(Solda et al., 2006). Although the dependence on Cnx and Crt of the various secretory
glycoproteins in the present study has not been rigorously examined, TF has been established as
an obligate client of these chaperones (Wada et al., 1997) consistent with the strong impairment
we observe upon ERp57 depletion. It is noteworthy that delays in oxidative folding were even
detectable for αFP, α2HS, and TF upon ERp57 depletion, given that all three glycoproteins were
demonstrated to be substrates for PDI and that PDI was fully present when ERp57 was depleted.
Recent studies from Jessop and co-workers have shed light on this issue (Jessop et al., 2009a).
They speculated that entry of glycoproteins into the Cnx/Crt cycle might impede access of other
PDI family members, even when ERp57 was depleted. Consistent with this view, they showed
that the impaired oxidative folding of influenza HA observed upon ERp57 depletion could be
largely bypassed if HA was prevented from associating with these chaperones.

There is some controversy as to whether ERp57 functions primarily as an isomerase or
oxidase during protein folding. Previous studies by Jessop and co-workers, in which the disulfide
content of HA was assessed through chemical alkylation, revealed that the HA produced in
ERp57-deficient cells was fully oxidized but contained non-native disulfides (Jessop et al.,
2009a). This led to the conclusion that ERp57 functions primarily as an isomerase. Our current
findings, as well as previous work showing that ERp57 deficiency slows the formation of fully
oxidized class I heavy chains by as much as 10-fold (Zhang et al., 2006), point to a role for
ERp57 in catalyzing disulfide formation, but do not preclude the possibility of an additional role
in isomerization. Future experiments employing the chemical alkylation approach on secretory
proteins in ERp57-depleted HepG2 cells will help to clarify this issue.

ERp72 is expected to possess a different substrate specificity compared to PDI or ERp57.
The crystal structure of the ERp72 bb’ domain fragment revealed that it lacks the hydrophobic
face that is thought to mediate PDI interactions with non-native substrates and, on the opposite
face, lacks the positively charged patch that mediates ERp57 binding to Cnx and Crt (Kozlov et
Consistent with a limited substrate specificity, a substrate trap mutant of ERp72 failed to stabilize a significant level of mixed disulfide complexes with substrates (Jessop et al., 2009b). Thus, our inability to detect any impact of ERp72 depletion is perhaps not surprising. Only when PDI was depleted in combination with ERp72 was a role for ERp72 revealed in the oxidative folding of Alb, a role previously masked by PDI in the single knockdown experiments. The lack of effect of this double knockdown on the three glycoprotein substrates further underscores the narrow substrate specificity of ERp72. It has been noted that when ERp57 is depleted (Solda et al., 2006) or the binding of Cnx or Crt to substrates is impeded through treatment with castanospermine (Jessop et al., 2009b) several glycoprotein substrates of ERp57 can shift to an association with ERp72, suggesting overlapping substrate specificities. However, we were unable to detect any involvement of ERp72 in the oxidative folding of αFP, α2HS, or TF when ERp57 was depleted along with ERp72 or even in triple knockdowns lacking PDI, ERp57 and ERp72. It is possible that the engagement of these glycoproteins within the Cnx/Crt cycle precludes interactions with ERp72 or that ERp72 simply has a narrow substrate range not sampled in the present study.

Finally, we did not observe any impact on oxidative folding of depleting P5 alone or in combination with other PDI family members. These data suggest either a minor role for P5 or one specific for substrates not included in this study. P5 has previously been reported to be part of a multi-enzyme complex containing several ER chaperones including BiP (Meunier et al., 2002). In the substrate-trapping study by (Jessop et al., 2009b), the authors confirmed a non-covalent interaction between P5 and BiP, and reported mixed disulfide complexes between P5 and a broad range of substrates tested, although such complexes could only be observed when substrates were selectively translated in a semi-permeabilized cell system. The authors speculated that in a manner analogous to ERp57 and Cnx/Crt, P5 is targeted to substrates via its interaction with BiP. Given that BiP interacts with many newly synthesized proteins in the ER including those in hepatoma cells (Molinari and Helenius, 2000; Schmidt and Perlmutter, 2005), the discrepancy between our respective findings may reflect differences in the methods used or may indicate that P5, although reported to have oxidase activity in vitro (Lappi et al., 2004), may act predominantly as a reductase to remove disulfide bonds in misfolded proteins targeted to BiP and degraded by ERAD (Jessop et al., 2009b).
The existence of a multi-enzyme complex containing PDI, ERp72, P5, BiP and Grp94, but lacking Cnx, Crt, and ERp57, has led to the suggestion that the folding of various proteins may be segregated either spatially or temporally between different chaperone networks (Meunier et al., 2002) and that this may partially explain differences in PDI family member substrate specificity (Hatahet and Ruddock, 2009; Jessop et al., 2009b). Our data highlighting the apparent functional overlap of PDI and ERp72 in Alb oxidation as well as the lack of substrate sharing for ERp57 and ERp72 are in good agreement with such physical separation of function. However, our observation that both PDI and ERp57 are involved in the oxidative folding of most substrates tested is consistent with a more homogenous distribution of PDI family members in the ER. We also do not observe any impact of combined depletion of PDI and P5 which might have been expected if substrate selection was based on physical proximity of the oxidoreductases within a chaperone complex.

In summary, using a functional approach we have begun to define the relative roles of different PDI family members in oxidative protein folding within the ER. Although revealing in terms of delineating the predominant roles of PDI and ERp57 and the lesser impact of ERp72 and P5, it is noteworthy that disulfide formation still occurred even in the context of triple depletion conditions. An extension of this approach to other members of the PDI family, or to potential ER oxidant enzymes, should prove informative in revealing the sources of additional oxidase activities.
Chapter 4

Vitamin K epoxide reductase contributes to protein disulfide formation and redox homeostasis within the endoplasmic reticulum

This chapter was originally published as:

4.1 Introduction

When PDI family members carry out net oxidation of a substrate, the active site CXXC shuttles from an oxidized disulfide state to a dithiol state as the disulfide is donated to the substrate protein (Hatahet and Ruddock, 2009). In order for the PDI family member to catalyze another oxidation reaction, the active site must be re-oxidized to the disulfide state. In yeast, the specific oxidation of PDIp (Tu et al., 2000; Gross et al., 2006; Sevier and Kaiser, 2006), is catalyzed by Ero1p, using molecular oxygen as the ultimate source of oxidizing equivalents (Tu and Weissman, 2002; Gross et al., 2004). The mammalian homologues, Ero1α (Cabibbo et al., 2000) and Ero1β (Pagani et al., 2000), have also been shown to carry out this function. Since Ero1p function in yeast is essential, it was surprising that combined Ero1α- and Ero1β-deficient mice were not only viable but that disulfide bond formation was only partially impaired for certain proteins, e.g., during insulin biogenesis (Zito et al., 2010a). These findings suggest the existence of Ero1-independent pathways for the formation of disulfide bonds and several candidates have been proposed that are defined by their central enzymes: peroxiredoxin IV (PRDX4), quiescin-sulfhydryl oxidase (QSOX1), and vitamin K epoxide reductase (VKOR) (reviewed in (Bulleid and Ellgaard, 2011).

PRDX4 is well characterized in its ability to scavenge hydrogen peroxide to generate disulfide bonds (Tavender and Bulleid, 2010b). When depleted by RNAi in Ero1α+β null mouse embryo fibroblasts, PRDX4-deficient cells exhibited decreased cell growth, hypersensitivity to reducing agents, a more reduced ER redox balance and impaired collagen secretion (Zito et al., 2010b). In contrast, in vivo evidence for QSOX1 activity is limited to its ability to complement the Ero1 deletion when overexpressed in yeast (Chakravarthi et al., 2007), but physiological substrates for QSOX1 have not yet been identified in mammals. Human VKOR is an ER membrane protein that catalyzes the reduction of vitamin K epoxide, and in this process, a CXXC motif within VKOR is oxidized (Wajih et al., 2005). Subsequently, a complex containing VKOR and PDI was detected (Wajih et al., 2007) and substrate trapping experiments suggested that other members of the PDI family also interact with VKOR (Schulman et al., 2010). However, a direct impact of VKOR on oxidative folding or on ER redox status in cells has not yet been demonstrated.
As shown in Chapter 3, it was noted that rapid oxidation of several newly synthesized plasma proteins continued even in the combined absence of PDI family members such as PDI itself, ERp57 and ERp72, enzymes that would be expected to accept oxidizing equivalents from Ero1 (Rutkevich et al., 2010). This suggested either that Ero1 oxidizes many other PDI family members as well, or that oxidative pathways other than that involving Ero1 are participating, the latter being consistent with the modest phenotypes described for combined Ero1α+β knockout cells (Zito et al., 2010a). Accordingly, I assessed the contributions of candidate ER oxidant systems to oxidative protein folding in human hepatoma cells by systematically knocking down or inhibiting Ero1α+β, PRDX4, VKOR, and QSOX1, either alone or in combination.

4.2 Both PRDX4 and VKOR support cell growth and viability in the face of Ero1 depletion

To assess the contributions of various oxidative pathways to disulfide formation within the ER, I employed an RNAi-based approach in the human hepatoma cell line, HepG2. Ero1α has been reported to be widely expressed throughout mammalian tissues whereas Ero1β is enriched in pancreas and immunoglobulin-secreting cells (Pagani et al., 2000; Zito et al., 2010a). We detected both isoforms in HepG2 cells (Fig. 4.1A). Likewise, both QSOX1a and QSOX1b were detected, with the predominant species being the smaller QSOX1b isoform. By transfecting cells twice with siRNA over 6-7 days, Ero1α, Ero1β, PRDX4, QSOX1a/1b, or VKOR could be routinely depleted by 93-98% as determined by Western Blot analysis (Fig. 4.1A). This level of knockdown was also achieved when up to three targets were depleted simultaneously. In some experiments, 50 µM warfarin was used to inhibit VKOR function (Fasco et al., 1983; Rost et al., 2005), since it was determined that overnight treatment with the drug produced similar phenotypes to 7 days of siRNA-mediated VKOR depletion (data not shown). Furthermore, knockdown of one to three targets resulted in no detectable increase in expression of the ER Hsp70 chaperone BiP nor evidence of Xbp-1 splicing when measured on the final day of knockdown, indicating that an unfolded protein response was not being induced (data not shown).

Previously, a role for PRDX4 in ER redox homeostasis was revealed upon elimination of the Ero1 pathway in Ero1α+β combined knockout fibroblasts (Zito et al., 2010b). Cells lacking Ero1α+β and depleted of PRDX4 grew more slowly than Ero1α+β-deficient cells, exhibited a
Figure 4.1. Effect of Ero1, PRDX4, QSOX1 and VKOR depletion on cell growth and viability.

(A) Candidate ER oxidant enzymes were depleted for 6-7 days in human HepG2 hepatoma cells using siRNA either singly or in combination. Note that a single siRNA targeted both the QSOX1a and QSOX1b isoforms. Equivalent amounts of cell lysates were immunoblotted for the indicated ER enzymes as well as GAPDH which served as an internal loading control. Where two blots are shown, a single blot was stripped and re-probed for the second target. Results are representative of the level of knockdown achieved in both single- and multi-target siRNA treatment regimes. Numbers refer to molecular weight markers in kilodaltons.

(B) HepG2 cell growth and viability were monitored (using trypan blue exclusion) following depletion of a single candidate ER oxidant enzyme (both Ero1α+Ero1β in the case of Ero1). Day 0 is defined as the day of the second transfection with targeting siRNA (day 4 as described in Methods). Targeted enzymes are shown to the right and average percent viability on day 4 is indicated in the brackets. Plotted values are averages of three experiments and error bars represent one standard deviation.

(C) Combined depletion of candidate ER oxidant enzymes. Cell growth and viability were monitored daily as described in (B). On day 3, cells were treated with 50 µM warfarin to inhibit VKOR activity or DMSO carrier, as indicated by the arrow and “+ W”. Cell growth and viability were monitored for another two days (except for the PRDX4 and Ero1/QSOX1 knockdowns that were followed until 4 days only). Bracketed numbers indicate the average viability of recovered cells on day 5.
more reducing ER and were more severely impaired in collagen secretion. Consequently, we anticipated that any involvement of PRDX4, VKOR or QSOX1 in ER redox homeostasis in HepG2 cells would become apparent only upon Ero1α+β depletion. We first examined the impact of individual or combined depletion of oxidative pathways on cell growth and viability. These parameters were monitored for a period of 4-5 days following the last of the two siRNA transfections, as target protein levels declined from 10-17% to trace levels. As shown in Fig. 4.1B, individual knockdown of PRDX4, QSOX1 or VKOR had no impact on cell growth or viability whereas depletion of Ero1α+β resulted in a significant slowing of growth, but no effect on viability (% viabilities in brackets). This is in keeping with the substantive role that Ero1 plays in ER oxidative processes (Frand and Kaiser, 1998; Tien et al., 2008). Subsequent combined pathway depletion revealed the relative abilities of the PRDX4, QSOX1 and VKOR pathways to maintain cell function in the absence of Ero1. Cell growth was essentially arrested when PRDX4 was depleted in combination with Ero1α+β, although viability was only modestly affected (Fig. 4.1C). Interestingly, a marked growth defect was also observed when VKOR was depleted in combination with Ero1α+β, but no growth phenotype was observed upon combined QSOX1 and Ero1α+β depletion beyond that of Ero1α+β depletion alone (Fig. 4.1C). These findings are consistent with the previous demonstration that PRDX4 contributes to oxidative folding within the ER (Zito et al., 2010b) and, for the first time, implies a similar role for VKOR but not QSOX1.

I also inhibited VKOR function by treating cells on Day 3 with warfarin, either alone or in combination with siRNA-mediated depletion of other oxidative pathways, and then monitored growth and viability for the next two days (denoted by W in Fig. 4.1C). Control cells treated with warfarin exhibited no evidence of toxicity when monitored for two days (Fig. 4.1C, Control + W), or even for periods of up to 7 days (data not shown). In addition, warfarin did not induce a UPR response as monitored by Xbp-1 splicing and BiP expression levels (data not shown). Inhibition of VKOR function with warfarin resulted in a growth phenotype only in the context of Ero1α+β depletion (Fig. 4.1C, Ero1+W); combined impairment of the PRDX4 and VKOR pathways resulted in normal cell growth (Fig. 4.1C, PRDX4+W). Furthermore, warfarin treatment permitted assessment of the effects of combined impairment of the Ero1, PRDX4 and VKOR pathways. A gradual loss of cell viability ensued, consistent with the concept that all three pathways contribute to the essential process of ER oxidation (Fig. 4.1C, Ero1/PRDX4+W).
The strong growth phenotypes and loss of cell viability observed in these experiments also provide assurance that the trace levels of target proteins remaining following knockdown are not sufficient to maintain substantial function.

4.3 VKOR contributes to an oxidizing ER environment under conditions of Ero1 and PRDX4 deficiency

To extend these findings more directly to oxidative processes within the ER, I first monitored the secretion of the endogenous HepG2 protein albumin (Alb), since I had previously demonstrated Alb secretion defects when its oxidative folding was impaired through PDI member depletion (Rutkevich et al., 2010). Cells were radiolabeled with $[^{35}\text{S}]$Met, chased for 40 min, Alb was immunoisolated from cell lysate and medium samples, and then the percentage secreted was determined. Figure 4.2A depicts the results of Alb secretion under various knockdown conditions on Days 3 and 4 following the second siRNA transfection, and under the same knockdown conditions on Day 4 but with the inclusion of warfarin to inhibit VKOR function. Individual pathway depletion through knockdown of Ero1α+β, knockdown of PRDX4 or inhibition of VKOR (control + warfarin) had no effect on Alb secretion. Combinatorial pathway depletions revealed that loss of Ero1α+β plus PRDX4, Ero1α+β plus QSOX1 or PRDX4 plus VKOR did not impact Alb secretion. However, the combined loss of Ero1α+β plus VKOR did result in a modest reduction in secretion. Furthermore, additional depletion of the PRDX4 pathway in combination with Ero1α+β plus VKOR resulted in an even greater defect in Alb secretion. This contrasts with the lack of any additional defect when QSOX1 was depleted in combination Ero1α+β and VKOR. Collectively, these findings suggest that Ero1, PRDX4 and VKOR, but not QSOX, are involved in supporting the oxidative folding and secretion of Alb. Particularly noteworthy was the finding that Alb secretion was normal upon simultaneous depletion of the Ero1 plus PRDX4 pathways but became markedly impaired upon additional inhibition of VKOR with warfarin (Fig. 4.2A, compare Ero1αβ + PRDX4 with Ero1αβ + PRDX4 + warfarin). This suggests that VKOR is an important contributor to the maintenance of ER redox homeostasis upon combined depletion of the Ero1 and PRDX4 pathways.

As an additional means to monitor the consequences of oxidative pathway depletion/inhibition on ER redox homeostasis, I used a colorimetric assay to determine the ratio of oxidized glutathione (GSSG), which originates mainly from the ER.
Figure 4.2. Effects of Ero1, PRDX4, QSOX1 and VKOR depletion on the oxidative folding and secretion of albumin and on ER redox environment. HepG2 cells depleted of the indicated candidate ER oxidant enzymes were assessed on days 3 and 4 following the second transfection of siRNAs to monitor changes in (A) albumin secretion and (B) GSSG:GS\textsubscript{tot} levels. Cells on day 4 were also analyzed following overnight treatment with 50 µM warfarin to inhibit VKOR activity. (A) Albumin secretion was monitored by radiolabeling cells with [\textsuperscript{35}S]Met for 10 min followed by a chase with excess unlabeled Met for 40 min. Albumin was immunoisolated from cell lysates and chase medium, detected by SDS-PAGE and fluorography and band intensity determined by densitometry. Secreted albumin was calculated as a percentage of the total albumin in cell and medium fractions. Results are the mean of two independent experiments and error bars represent one standard deviation.

(B) GSSG and GS\textsubscript{tot} levels were monitored as described in Chapter 2. Results depict the ratio of GSSG to total GSSG+GSH (GStot), normalized to the Control Day 3 ratio, and are the mean of two independent experiments. Error bars represent one standard deviation. (C and D) Kinetics of disulfide formation in albumin. HepG2 cells were treated with control siRNA or the indicated targeting siRNAs, then radiolabeled with [\textsuperscript{35}S]Met for 3 min and chased for the indicated times. Following treatment with 20 mM NEM, cells were lysed and immunoisolated albumin was subjected to non-reducing SDS-PAGE. Shown are fluorograms representative of three independent experiments. The mobilities of reduced (R), partially oxidized (PO), and fully oxidized (O) albumin are indicated. (E) Quantification of gels shown in C and D was carried out using densitometry and by expressing the amount of fully oxidized protein in lysates and media samples as a percentage of the total combined amount of reduced, partially oxidized, and oxidized forms recovered at a given time point. Solid black line, Control; Dotted black line, Ero1α+β knockdown; Dashed black line, Ero1α+β &PRDX4 knockdown. Error bars represent the average of three independent experiments +/- one standard deviation.
(Hwang et al., 1992; Banhegyi et al., 1999), to total cellular glutathione (GS$_{tot}$, comprised of GSH and GSSG). As shown in Fig. 4.2B, Day 3 and Day 4, depletion of the Ero1α+β pathway resulted in a higher GSSG:GS$_{tot}$ ratio when compared to control cells, reflecting a more oxidizing environment. This seemingly paradoxical finding has been noted before (Appenzeller-Herzog et al., 2010) and presumably reflects the compensatory action of other oxidative pathways to increase the pool of GSSG when the Ero1 pathway is impaired. The increased GSSG pool could potentially re-oxidize PDI family members in the absence of Ero1 activity (Lyles and Gilbert, 1991b; Karala et al., 2009; Appenzeller-Herzog, 2011). No further alteration in GSSG:GS$_{tot}$ ratio was observed when QSOX1 was depleted in combination with Ero1α+β but an even greater increase in ratio was observed upon combined PRDX4 and Ero1 depletion. Of particular interest was the finding that warfarin treatment abolished the observed increases in GSSG:GS$_{tot}$ ratio, thereby implicating VKOR as the source of the compensatory oxidative activity (Fig. 4.2B, Day 4 + warfarin).

To assess the contributions of ER oxidant enzymes directly on substrate-level oxidation, I monitored the kinetics of Alb disulfide formation. Cells were subjected to pulse-chase radiolabeling with $[^{35}\text{S}]$ Met, treated with N-ethylmaleimide (NEM) to alkylate free thiols and then immunoisolated Alb was analyzed by non-reducing SDS-PAGE. In control cells (Fig. 4.2C, left side), Alb was rapidly converted during the 3 min pulse from its reduced (R) form to more rapidly migrating, partially oxidized (PO) species as well as fully oxidized Alb (O). During the subsequent chase, the various partially oxidized species were gradually converted to the fully oxidized form. Upon depletion of Ero1α+β (Fig. 4.2C, right side), very rapid oxidation of Alb was also observed during the 3 min pulse period and, in fact, there was no observable defect in the overall kinetics of Alb oxidation (quantified in Fig. 4.2E). This is consistent with previous observations using combined Ero1α+β-knockout cells (Zito et al., 2010a; Zito et al., 2010b) and also with the mild to absent phenotypes observed in the cell growth and Alb secretion assays, respectively (Fig 4.1C and 4.2A).

To test the involvement of additional oxidative pathways, I next depleted PRDX4 in combination with Ero1α+β (Fig. 4.2D, right side). Remarkably, there was no evidence of impaired albumin oxidation as a consequence of this triple depletion. This was surprising given the growth arrest that accompanies these conditions (Fig. 4.1B) and the strong perturbations in redox balance and secretion observed by others when PRDX4 is depleted in Ero1α+β-knockout
mouse fibroblasts (Zito et al., 2010b). However, my findings are consistent with the lack of effect of combined Ero1α+β and PRDX4 depletion on Alb secretion (Fig. 4.2A) and may reflect the compensatory increase in GSSG:GS\textsubscript{tot} ratio that occurs under these conditions (Fig. 4.2B). Efforts to evaluate the participation of the VKOR pathway in maintaining Alb oxidative folding under conditions of combined Ero1α+β and PRDX4 depletion were hampered by the toxicity observed when warfarin was added to the cells (Fig. 4.1C), limiting the number of cells available for extensive pulse-chase analysis. Consequently, I sought a more sensitive assay to report on substrate level oxidative folding that would permit a clearer assessment of the contributions of each pathway.

4.4 The Ero1, PRDX4, and VKOR pathways contribute to recovery after reductive challenge

The technique of treating cells with DTT for a brief period and then washing away the reductant (Braakman et al., 1992) has proven to be a useful tool to monitor the involvement of Ero1 and PDI in the restoration of ER redox homeostasis (Cuozzo and Kaiser, 1999; Mezghrani et al., 2001; Appenzeller-Herzog et al., 2010). Specifically, Appenzeller-Herzog and co-workers demonstrated that, upon DTT washout, restoration of the pre-treatment ratios of oxidized:reduced PDI family members and GSSG:GS\textsubscript{tot} was rapid and these rapid kinetics depended on both Ero1 and PDI (Appenzeller-Herzog et al., 2010). However, recovery of homeostasis still occurred in the absence of Ero1 or PDI, indicative of the involvement of other oxidant systems. Live cell imaging studies using a redox-sensitive ER-localized GFP confirmed the importance of Ero1α, but not PRDX4, in oxidative recovery following reductive challenge (van Lith et al., 2011).

I extended these analyses by examining the involvement of QSOX1, PRDX4, and VKOR and by monitoring the oxidative folding of Alb as a readout in addition to the GSSG:GS\textsubscript{tot} ratio. The 8 min treatment with 5 mM DTT used in my study permitted full reduction of the ER, but was not long enough to induce a UPR (data not shown). As shown in Fig. 4.3A (upper panel, Control), fully reduced Alb was observed at 0 min chase and then progressed gradually through partially oxidized forms until it became fully oxidized by 15-30 min. Substantial oxidation was apparent even 5 min following DTT washout, reflecting the rapidity of ER re-oxidation. Upon depletion of Ero1α+β, the oxidative folding of Alb was substantially delayed, in keeping with the
Figure 4.3. Ero1 is the predominant activity responsible for oxidative recovery following DTT treatment. (A) HepG2 cells depleted of Ero1α+β (or control) were treated with 5 mM DTT for 5 min plus an additional 3 min during pulse radiolabeling with [35S] Met. Chase was carried out in the absence of DTT and constituted the “washout” period. NEM treatment, albumin immunosolubilation, and analysis were carried out as described in Figure 2 C and D. Upper panel, non-reducing gel; lower panel, reducing gel. Right panel depicts the quantification of the non-reducing gel. % oxidized albumin (black traces) was determined as described in Figure 2E (solid line, control; dashed line, knockdown). To determine the % reduced values (grey traces), the amount of albumin with mobility matching that of the fully reduced control was expressed as a percentage of the total signal recovered at a given time point (solid line, control; dashed line, knockdown). (B) Single knockdowns of the indicated targets were carried out and radiolabeling with reductive challenge was carried out as described in A. Left panel, non-reducing gel representative of three independent experiments. Right panel, quantifications as outlined in A were performed. Error bars show the average of three independent experiments +/- one standard deviation.
demonstrated role of Ero1 in restoration of redox homeostasis (Fig 4.3A, upper panel, Ero1α+β). A discrete band corresponding to fully reduced Alb persisted to 15 min of chase with the fully oxidized species being detectable only by 30 min with full oxidation at 60 min. Fig. 4.3A, right panel, depicts the slower kinetics of disappearance of fully reduced Alb (dashed gray trace) and the slower formation of the fully oxidized species (dashed black trace) under conditions of Ero1α+β depletion. I also noted some high molecular weight aggregates under both control and knockdown conditions, but much of this material was not resolved to the reduced form upon analysis by reducing SDS-PAGE and its level was not reproducible between experiments (Fig. 4.3A, lower panel). Consequently, it was not included in the quantitative analysis.

I next assayed the oxidation of Alb in cells depleted of PDI, QSOX1, PRDX4, or VKOR (Fig 4.3B). PDI knockdown generated a delayed Alb oxidation profile that was similar to that observed with Ero1α+β depletion. This is consistent with the role of PDI as a recipient of oxidizing equivalents from Ero1 (Frand and Kaiser, 1999) (Benham et al., 2000; Inaba et al., 2010) and with the previous demonstration that both Ero1 and PDI are required for rapid recovery following reductive challenge (Appenzeller-Herzog et al., 2010). However, it was noted that, unlike the situation with Ero1α+β depletion where Alb remained for 15 min as a discrete fully reduced species (Fig. 4.2A), PDI depletion was associated with significant production of partially oxidized Alb over the same period. This is consistent with the idea that PDI is not an essential conduit to Ero1 and that family members other than PDI are able to receive oxidizing equivalents from Ero1 for catalysis of Alb oxidative folding. In contrast to Ero1α+β and PDI, individual depletion of QSOX1 or VKOR showed no differences in the rates of Alb oxidation compared to control cells (Fig. 4.3B, 2nd and 4th panels). However, depletion of PRDX4 resulted in a slight slowing of Alb oxidation (Fig 4.3B, 3rd panel). This was particularly obvious at the 15 min chase time where a clear decrease in the proportion of fully oxidized Alb was apparent. Thus, even in the presence of the Ero1 to PDI pathway, the most efficient oxidation was observed only when PRDX4 was also present. Since Ero1 is highly active following reductive challenge (Sevier et al., 2007; Appenzeller-Herzog et al., 2008), the hydrogen peroxide it produces may be used by PRDX4 to contribute significantly to the re-oxidation of PDI family members (Tavender and Bulleid, 2010b; Zito et al., 2010b).

The individual knockdown experiments revealed that Ero1 plays the predominant role in recovery from reductive challenge. Since Ero1 activity may mask the involvement of other
oxidative pathways, we next assessed the participation of the PRDX4, QSOX1 and VKOR pathways through combinatorial depletions with Ero1α+β (Fig. 4.4A). In the case of Ero1α+β and QSOX1 combined depletion, no enhanced phenotype beyond that of Ero1α+β depletion was observed, suggesting that QSOX1 is not a significant participant in recovering from reductive challenge (compare Fig. 4.3A and 4.4A, top panels). By contrast, combined depletion of Ero1α+β &PRDX4 resulted in a greater delay in Alb re-oxidation than the depletion of Ero1α+β alone (Fig. 4.4A, second panel). Of particular interest was the finding that co-depletion of Ero1α+β &VKOR also delayed Alb oxidation to the same or even greater degree than that observed with combined Ero1α+β &PRDX4 depletion (Fig. 4.4A, third panel). This confirms a role for both PRDX4 and VKOR in ER oxidation. Further support for this conclusion comes from combined depletion/inhibition of the PRDX4 and VKOR pathways (Fig. 4.4A, fourth panel). Despite the continued presence of the Ero1/PDI pathway, Alb re-oxidation was notably slowed, and to a greater extent than that observed upon PRDX4 depletion alone (compare with Fig. 4.3B, 3rd panel).

Based on the preceding findings, one would predict that simultaneous loss of the Ero1, PRDX4 and VKOR pathways would have a profound impact on the oxidative recovery of the ER. Unfortunately, due to toxicity issues, I was unable to assess the consequences of inhibiting VKOR in the context of high efficiency knockdowns of Ero1α+β &PRDX4. As an alternative approach, Ero1α+β &PRDX4 were depleted to approximately 30% of normal levels and warfarin was added overnight to inhibit VKOR. With this modification, cell death was minimized and pulse-chase analysis following reductive challenge was performed (Fig. 4.4A, bottom panel). For comparison, the final lane shows the oxidation pattern of Alb after 15 min in the partially depleted cells without warfarin treatment. There is a much greater degree of oxidation of Alb in this final lane when compared to the 15 min time point in the high efficiency knockdown of Ero1α+β &PRDX4 (Fig. 4.4A, second panel). However, when VKOR was inhibited in the context of the partial Ero1α+β &PRDX4 knockdown, a marked slowing of Alb oxidation was apparent (Fig. 4.4A bottom panel, compare 15 min time points -/+ warfarin). Once again, this illustrates the significant contribution of VKOR to ER oxidation.

In addition to monitoring substrate level oxidation, we examined the impact of combined pathway depletion on the kinetics of recovery of the GSSG:GS\textsubscript{tot} ratio to its steady state level
Figure 4.4. Combinatorial knockdowns reveal that PRDX4 and VKOR contribute to recovery following reductive challenge. Co-depletions of the indicated enzymes were carried out over 7 days and then cells were subjected to DTT challenge followed by quantification of albumin oxidative folding kinetics (A) or GSSG:GS\text{tot} ratios (B). For the Ero1\(\alpha\)+β & PRDX4 + warfarin experiment (A, bottom panel), combined depletion was carried out to day 5 post-transfection to obtain approximately 30% expression of these targets. On day 4, cells were treated with warfarin and reductive challenge and radiolabeling were performed on day 5. A DMSO-treated sample of knockdown cells at 15 min of chase (last lane) is included for comparison with the 15 min time point of knockdown + warfarin-treated cells. (B) Following 8 min treatment with 5 mM DTT, cells were allowed to recover for the indicated times in DTT-free media then washed twice with ice cold PBS, and assayed for GSSG and GS\text{tot} as described in Methods. The GSSG:GS\text{tot} ratio following reductive challenge is quantified as a percentage of the steady state ratio (independently determined). Averages of three independent experiments (performed in triplicate) are shown +/- one standard deviation.
following reductive challenge. For technical reasons, a more rigorous DTT washout protocol was required to obtain reproducible results in this assay. This resulted in more rapid recovery of the ER redox state, which prevented a direct comparison with the kinetics of Alb oxidative folding following DTT washout. Nevertheless, the qualitative effects of pathway depletion can still be compared using both assays.

As shown in Fig. 4.4B, under control conditions, recovery of the steady state GSSG:GS_{tot} ratio occurred by 5 min of chase, followed by an overshoot that was corrected by 30 min of chase. In Ero1α+β depleted cells, recovery of the steady state GSSG:GS_{tot} ratio was delayed by about 5 min, followed by a similar overshoot and correction by 30 min. This is consistent with previous observations by others (Appenzeller-Herzog et al., 2010). However, we were further able to demonstrate that co-depletion of either PRDX4 or VKOR with Ero1α+β results in an even greater impairment in the recovery of steady state GSSG:GS_{tot} levels, particularly at times exceeding 5 min post-wash-out of the reductant. Thus, like PRDX4, VKOR plays a significant role in oxidative protein folding and the maintenance of ER redox homeostasis.

### 4.5 Discussion

In this investigation, I used a systematic knockdown approach to examine the relative contributions of known and candidate oxidant enzymes to ER redox homeostasis and disulfide bond formation. I confirm that both Ero1 and PRDX4 are important contributors and demonstrate a novel role for VKOR in these processes. However, no phenotypes associated with depletion of QSOX1 could be detected, either in single knockdowns or in combinatorial depletions with other ER oxidants.

Ero1 is essential in both yeast and worms (Frand and Kaiser, 1998; Pollard et al., 1998), but deletion of both Ero1 paralogues in mice generated only mild phenotypes (Zito et al., 2010a). In my studies involving individual depletion of candidate oxidative pathways, I also noted that knockdown of Ero1α+β produced no defects in the oxidative folding or secretion of Alb. However, it was the only pathway that, when depleted individually, resulted in slow cell growth and a substantial delay in recovery of ER redox balance and oxidative folding of Alb following reductive challenge. Furthermore, in combinatorial depletions of candidate pathways, those involving Ero1 were associated with the strongest phenotypes. Collectively, these findings suggest that Ero1 represents the predominant pathway for the de novo generation of disulfide bonds.
bonds. Although this distinction could be observed, the mild Ero1-depletion phenotype supports the view that other oxidase pathways are acting in parallel to maintain ER redox homeostasis.

Ero1 utilizes molecular oxygen, producing one molecule of \( \text{H}_2\text{O}_2 \) upon transfer of oxidizing equivalents to PDI (Gross et al., 2006), and the role of PRDX4 in scavenging \( \text{H}_2\text{O}_2 \) to generate disulfide bonds has been well characterized (Tavender and Bulleid, 2010b, a). Zito and co-workers illuminated a key role for PRDX4 in supporting cell growth, secretion and ER redox homeostasis in mouse embryo fibroblasts lacking Ero1\( \alpha+\beta \) (Zito et al., 2010b). I confirmed the importance of PRDX4 in human hepatoma cells since its depletion resulted in a delay in recovery from reductive challenge, even in the presence of Ero1. Furthermore, combined depletion with Ero1 resulted in growth arrest and an even greater delay in recovery from reductive challenge as measured by restoration of oxidized:total glutathione ratio and oxidative folding of Alb. Strikingly, however, under unchallenged conditions, the kinetics of Alb disulfide formation and secretion were normal in the combined absence of Ero1 and PRDX4. This contrasts with the severe secretion defect associated with PRDX4 depletion in Ero1-deficient mouse embryo fibroblasts (Zito et al., 2010b). Furthermore, in the same fibroblasts there was clear evidence of a more reducing ER, yet in HepG2 cells, we observed a more oxidizing ER upon depletion of both pathways. Cumulatively, these data suggest that in HepG2 cells an additional oxidative pathway is functioning that is absent or inefficient in mouse embryo fibroblasts.

Since QSOX1 utilizes molecular oxygen to generate disulfide bonds, its activity could be sufficient to maintain oxidation in the absence of Ero1 and PRDX4. In my study, depletion of both isoforms of QSOX1 either alone or in combination with Ero1 had no impact on cell growth, on oxidized glutathione levels, on the kinetics of Alb oxidative folding or secretion, or on recovery from reductive challenge. I conclude that at least in human hepatoma cells, QSOX1 does not contribute significantly to ER oxidation. Rather, I discovered that VKOR activity provides the oxidizing power required to maintain ER redox homeostasis in the absence of Ero1 and PRDX4.

In vertebrates, VKOR has a broad tissue distribution that features greatest expression in the liver (Rost et al., 2005; Wang et al., 2005b). In contrast, mouse embryos do not express VKOR mRNA at day 7.5 (Ko et al., 1998) and there was only weak expression, sporadically
distributed, of VKOR mRNA at day 14.5 (Diez-Roux et al., 2011). These differences in VKOR expression may explain the less severe phenotype we observed in human hepatoma cells upon combined Ero1 and PRDX4 depletion compared with that reported in mouse embryo fibroblasts (Zito et al., 2010b). Homologues of VKOR have also been reported in some plants, archaea, and bacteria (Goodstadt and Ponting, 2004). One key feature shared among VKOR homologues is the CXXC motif common in thiol-disulfide exchange reactions. In the case of bacterial VKORs, some homologues cooperate with a periplasmic thioredoxin partner for electron transfer, whereas in others, such as in *Synechococcus* sp., a thioredoxin domain is found on the same polypeptide chain. The crystal structure of this fusion VKOR homologue recently revealed the pathway of electron flow to VKOR from its thioredoxin partner and, importantly, confirmed that topologically this exchange would occur in the ER lumen for mammalian VKOR counterparts (Li et al., 2010).

Wajih and co-workers first proposed the coupling of the vitamin K cycle to disulfide bond formation via an interaction between VKOR and PDI (Wajih et al., 2007). These authors reported a decrease in reduced RNaseA-triggered VKOR activity upon PDI knockdown in HEK293 cells or upon inhibition of PDI with bacitracin in rat liver microsomes. Recent work, however, has shown that Cys43 in the luminal loop of human VKOR preferentially forms mixed disulfides with the membrane anchored PDI family member, TMX, and to a lesser extent to TMX4 and ERp18 (Schulman et al., 2010). Although there is debate concerning its PDI family partners, the present work provides the first evidence in intact cells that VKOR contributes to disulfide bond formation and redox homeostasis within the ER. Specifically, the phenotype of combined Ero1 and VKOR depletion was similar to that observed when Ero1 and PRDX4 were depleted, i.e., slow growth rate and a prolonged delay in recovering from reductive challenge. Importantly, when both Ero1 and PRDX4 pathways were depleted, VKOR oxidative activity was sufficient to maintain normal kinetics of Alb oxidative folding and secretion and, although delayed, the recovery of redox homeostasis following reductive challenge. Inhibition of VKOR activity in the context of such combined Ero1 + PRDX4 depletion was lethal, suggesting that no other ER oxidative pathway exists in hepatoma cells with sufficient activity to support essential cell functions. Thus, as depicted in Fig. 4.5, the use of VKOR to generate disulfide bonds *de novo* in the ER represents an important alternative to the Ero1 and PRDX4 pathways. In this pathway, VKOR accepts electrons during oxidation of PDI family members and in the process
Figure 4.5. Pathways contributing to oxidative protein folding. VKOR, as well as Ero1 and PRDX4, accept electrons from PDI family members resulting in the conversion of reduced PDIs (PDI$^{\text{red}}$) to their oxidized forms (PDI$^{\text{ox}}$). PDI$^{\text{ox}}$ can catalyze disulfide formation in folding proteins or oxidize glutathione to GSSG. Under conditions when GSSG is abundant, it may also provide oxidizing equivalents for the re-oxidation of PDIs.
reduces vitamin K epoxide (Vit.K1\(^{\text{ox}}\)) to the hydroquinone (Vit.K1\(^{\text{red}}\)). The vitamin K hydroquinone is utilized by the integral membrane ER protein \(\gamma\)-glutamyl carboxylase for the production of \(\gamma\)-carboxyglutamate (Gla) in vitamin K-dependent proteins (reviewed in (Presnell and Stafford, 2002)). During this reaction, the vitamin K epoxide is regenerated with molecular oxygen serving as the ultimate electron acceptor, producing a molecule of water. Of the 18 known Gla-containing proteins in humans, at least 10 are synthesized in the liver including 7 blood coagulation and anti-coagulation factors. This suggests that the \(\gamma\)-glutamyl carboxylase activity is high enough in HepG2 cells to support the observed oxidative functions of VKOR.

During the course of these studies, we observed a higher ratio of oxidized:total glutathione in cells depleted of Ero1\(\alpha+\beta\), in keeping with a previous report (Appenzeller-Herzog \textit{et al.}, 2010). This was further increased upon combined depletion of the Ero1 and PRDX4 pathways. I reasoned that parallel oxidant pathways could be generating oxidized glutathione as one means to support continued disulfide bond formation in compensation for the lack of Ero1 and PRDX4 activities. Notably, inhibition of VKOR activity eliminated the higher levels of oxidized glutathione, consistent with VKOR being a significant source of this oxidative activity. This process is summarized in Figure 4.5, where VKOR as well as Ero1 and PRDX4 participate in \textit{de novo} formation of disulfides via PDI or other PDI family members (PDI\(^{\text{ox}}\)). Oxidized PDI then passes on oxidizing equivalents to substrate or to reduced glutathione to form GSSG. In the absence of one or more oxidative inputs into PDI, such as loss of Ero1 activity, the remaining oxidative pathways may compensate by driving GSSG formation as detected by its elevated levels. By shifting the ER glutathione buffer to a higher proportion of GSSG, cells have a greater pool available for the GSSG-driven oxidation of PDIs and the maintenance of substrate oxidation as proposed by Appenzeller-Herzog (Appenzeller-Herzog, 2011). The existence of these parallel and compensatory pathways in complex eukaryotes highlights the remarkable complexity associated with maintaining ER redox balance in support of the essential process of disulfide bond formation.
Chapter 5

Discussion & Future Directions
5.1 Substrate Selection by PDI Family Members

In this thesis, an approach of systematic depletion allowed assessment of the roles of the many enzymes involved in delivery of oxidizing equivalents to the ER and to substrate proteins for disulfide bond formation. The initial hypothesis that the abundance of PDI family members in the ER was due to substrate specificity in their action was supported by the single and combinatorial knockdown studies discussed in Chapter 3. PDI was the only thioloxidoreductase shown to have universality in its action on substrates, while ERp57 was specific for glycoproteins, although with differing degrees of consequence. Since the substrate range observed for ERp72 was limited to a single protein of the four assayed, it was interpreted to have a much narrower substrate specificity. By this same interpretation then, the specificity of P5 towards oxidative folding of substrates was found to be even more constrained as its loss failed to impact the folding of any of the substrates studied.

Although PDI family members showed clear substrate preferences, there was also strong evidence for sharing of substrates as well. The relatively meager strength of the phenotypes observed upon single PDI-family member knockdown illustrated the likelihood of some functional redundancy among family members. Furthermore, combinatorial knockdown studies revealed the apparent use of more than one PDI family member by a single substrate. The collective contribution of the substrate selection motifs of PDI family members as well as the characteristics of substrates dictated which family members were utilized in oxidation of a given substrate. This has been most clearly defined in the literature by the specificity of ERp57 for glycoproteins (Maattanen et al., 2006); the modified b’ domain feature of ERp57 determines its interaction with Cnx and Crt and the glycosylation feature of substrates such as α-fetoprotein, α-2-HS glycoprotein, and transferrin direct their use of the Cnx/Crt cycle. It is a likely scenario that similar protein folding networks may also influence the use of PDI family members along the folding pathways of disulfide-containing folding intermediates. Some examples of these networks and the impact on substrate accessibility are discussed below.

The four PDI family members chosen for this study were all previously shown to possess the catalytic ability to catalyze disulfide bond formation in vitro. Therefore, their selection of one substrate over another would be anticipated to reside in other regions of the protein. Since PDI is the only family member with a clear site for direct binding to substrates via its
hydrophobic b’ domain, it is anticipated that, as observed for ERp57-Cnx/Crt, interaction with chaperones or folding factors may bring other PDI family members into contact with their client proteins. Recent studies have supported this possibility for both P5 and ERp72. In the case of P5, Jessop and coworkers illustrated a non-covalent interaction between P5 and BiP and proposed that substrates utilizing BiP are also acted upon by P5 due to this interaction (Jessop et al., 2009b). However, the molecular mechanism of this interaction is still unknown and is of great interest to uncover. The BiP-P5 interface may be unique and specific to these two proteins, but alternatively it may reveal an interaction mode utilized by other folding factors and this important chaperone. Indeed, a recent study of ER protein interactions mapped interactions between BiP and the PDI family members ERp29, ERp18, and PDIr, as well as P5 (Jansen et al., 2012) and it would be revealing should a similar interaction interface be used for some of these family members. By carrying out domain deletions on P5 and determining the relative ability of the resulting truncated proteins to bind to BiP, the general region of P5 that is responsible for BiP-binding could be deduced. If the identified region is the a or a’ domain, then sequence comparisons of PDIr and ERp18 could reveal similarity to P5 since these family members also possess these domains. However, if the identified region is in the b domain of P5, then similarity to PDIr and ERp29 may be found since these family members also contain a b domain, while ERp18 does not.

In the case of ERp72, its interaction with the peptidyl prolyl isomerase, cyclophilin B (CypB), has been recently characterized. An acidic N-terminus, unique to ERp72, was found to interact with a basic region on CypB and in vitro assays revealed that the combination of both folding factors synergistically accelerated the refolding of immunoglobulin G. Interestingly, further characterization revealed that the same interaction interface was utilized in the interaction between CypB and Grp94 (Jansen et al., 2012), supporting the possibility of elucidating common interaction motifs between foldases in ER protein folding networks. The consequences of these interactions in vivo have yet to be studied, but all three proteins, as well as BiP and P5 were crosslinked together in a large ER multichaperone complex (Meunier et al., 2002). In light of this interaction, it is notable that I observed that the folding of the substrate albumin was delayed in the absence of PDI, and that this delay was enhanced by depletion of both PDI and ERp72 (Fig. 3.7). Since Alb also contains cis-prolines (Sugio et al., 1999) potentially requiring the action of CypB, it may be that, in addition to PDI, ERp72 is routinely recruited to Alb via the
CypB. Thus, in the absence of PDI alone, the oxidation (albeit delayed) that took place in Alb occurred due to the action of ERp72. This hypothesis also suggests albumin as a potential candidate for studying the CypB-ERp72 interaction in cells. By knockdown of endogenous ERp72 and replacement with siRNA-resistant ERp72 with a mutated (acidic \(\rightarrow\) basic) N-terminal interaction site, the oxidation of Alb in control- and PDI-depleted cells could be assessed. If the oxidative folding is slower in the mutant-ERp72 cells, it would argue that ERp72 gains access to Alb via its CypB interaction and that the complex promotes accelerated folding of substrate. The coupling of CypB activity to ERp72 activity may have arisen due to a specific folding need of a particular substrate or class of substrates, or it may be of more general use. Since the trans-cis isomerization of a proline would change the structural constraints of the folding intermediate, it can be speculated that upon proline isomerization by CypB, the local environment of a substrate cysteine may change considerably. In the case of oxidation, an environmental alteration that stabilizes the thiolate anion on the substrate would allow more efficient nucleophilic attack on an ERp72 active site disulfide, or a substrate-ERp72 mixed disulfide intermediate for net transfer of the disulfide bond to the substrate.

Differences in folding conformers and access to folding networks could also explain the apparent use of both ERp57 and PDI by glycoprotein substrates like transferrin. With signal sequence, human TF is 698 residues in length and the first N-linked glycosylation site to send TF to the Cnx/Crt cycle does not occur until N432. Prior to this site, there are twenty-two cysteine residues (MacGillivray et al., 1982) cotranslationally translocated into the ER which are likely forming disulfide bonds via PDI before TF is partitioned into the Cnx/Crt protein network and ERp57 takes over for catalysis of the remaining sixteen cysteine residues. It is tempting to speculate that in the absence of PDI, disulfide formation prior to glycosylation may have occurred incorrectly, or not at all, and that subsequent oxidation (and isomerization) by ERp57 may have been less efficient due to substantial conformational differences in the TF intermediate, or that a significant number of isomerization events were needed to modify incorrect disulfides. Interestingly, it was upon PDI depletion that TF was retained in the ER, despite being nearly-fully oxidized (Figure 3.3), suggesting the need for isomerization of disulfides. Thus, one interpretation of the need for both PDI and ERp57 is a sequential hand-off from PDI acting in early oxidation events prior to N432, and presenting ERp57 with a folding-competent intermediate in the Cnx/Crt cycle. Notably, four out of transferrin’s five long-range
disulfides (separated by more than 100 residues) are located downstream of the glycosylation signal. Although these cysteine residues may be spatially close to one another in intermediate tertiary structures, their correct formation may be more complex. Since the loss of ERp57 perturbed TF folding more strongly than PDI depletion, it may be that ERp57 is ideally situated for this disulfide formation or isomerization due to a conformational constraint of the TF intermediate presented to ERp57 in the “on” phase of the Cnx cycle.

Since TF is an obligate client protein of the Cnx/Crt cycle, undergoing multiple rounds of binding and release, it may also be that both PDI and ERp57 participate in later folding events. ERp57 could effectively carry out thiol-disulfide exchanges while TF is still bound to Cnx/Crt or, if a mixed-disulfide between ERp57 and TF is maintained upon TF dissociation from Cnx, ERp57 could complete its action during the “off” phase as alternate new conformers are attempted by TF. Additionally, PDI could act on incorrect TF intermediates during the “off” phase of the Cnx cycle, supporting an alternative model in which both PDI and ERp57 are needed concurrently at late stages for most efficient TF oxidative folding.

In contrast, for the other two glycoproteins studied, α-fetoprotein and α2HS-glycoprotein, only a modest effect of ERp57 depletion was observed. The N-linked glycosylation signals for these substrates occur earlier in the sequence (at 251/609 residues and 156&176/367 residues, respectively (Aoyagi et al., 1977; Araki et al., 1989)) and there are no intrachain long-range disulfides. They may have more simple folding pathways, requiring fewer interaction cycles with Cnx/Crt, and therefore less dependence on ERp57.

Upon loss of both PDI and ERp57, defects in substrate trafficking became much more marked for all substrates and all were subject to degradation by ERAD (recall section 1.2.2). Although PDI has been shown to play a role in reducing disulfide bonds in proteins destined for ERAD (Sakoh-Nakatogawa et al., 2009), efficient degradation of client proteins was observed in its absence. This would suggest that other PDI family members are fully capable of catalyzing reduction for ERAD. The most likely candidate for fulfilling this role is ERdj5 (Dong et al., 2008), however P5 has also recently been implicated in the degradation of MHC Class I (D. Chapman, personal communication).

It is also notable that early oxidation steps still occurred in cells co-depleted of PDI, ERp57, and ERp72 indicating that other PDI family members, or potentially small molecules
like GSSG, are able to initiate disulfide bond formation. Due to the predominant roles of PDI and ERp57 however, identifying the functional importance of these family members will be challenging. Additionally, little previous work exists on target recognition of these PDI family members to inform which substrates may be of use to assay. Since the client proteins used in this study exhibit some disulfide formation in the absence of PDI and ERp57, they may provide a good starting point for substrate selection, but their use of alternate family members will likely only be observed in the combined absence PDI and ERp57, as well as the alternate family member. The use of CXXA and/or cis-proline-to-threonine trapping mutants for these alternate PDI family members may prove more informative for assaying substrate specificity and more technically feasible. Endogenous substrate could be trapped in cells expressing a PDI family member trap mutant and identified by mass spectroscopy (as in (Jessop et al., 2009b)) and by sequential immunoprecipitation for selected substrates (as in Figure 3.2). By expressing the trap mutants in cells depleted of PDI and/or ERp57, one could observe the shifting of substrates to the trap mutant PDI family member; interaction with endogenous substrates may become more stabilized without PDI present and interaction of substrates usually catalyzed by PDI and ERp57 will be shifted to the alternate PDI family member. The experimental system could be further pushed to determine whether the glutathione disulfide buffer plays a role in substrate oxidation by carrying out these experiments following overnight treatment of cells with buthionine sulfoximine (BSO), which inhibits the synthesis of glutathione. If glutathione disulfide is responsible for these early oxidation events, its loss might be expected to shift substrate to the PDI trap mutants for detection. The use of trap mutants of alternative PDI family members to identify new targets would have numerous benefits. Identified proteins could be added to the repertoire of substrates assayed for oxidative folding following knockdown of PDI family members (as carried out in Chapter 3) and allow expansion of knockdown studies to the alternate PDI family members. This will allow classification of the relative dependence of these substrates on that PDI family member. Furthermore, the identification of which PDI family members shuttle electrons to substrate in the absence of PDI and ERp57, will also inform our understanding of how ER oxidant pathways interact with PDI family members. Finally, since the remaining PDI family members do not have known substrate recognition motifs, promising strong substrates identified by trapping studies could potentially be used in crosslinking studies to identify the protein interaction network that mediates the substrate-oxidoreductase interaction.
In addition to identifying the roles of other PDI family members in oxidative folding, it would also be of interest to determine whether they have a role in disulfide reduction in the ER, and the potency of such a role. Since disulfide reduction is most obvious in ERAD of misfolded substrates, one approach would be to knockdown PDI family members, perhaps in combination with ERdj5, and look for stabilization of the ERAD substrate to assess reductase activity. Alternatively, some novel substrates identified by substrate trapping studies, as described in the previous section may not exhibit a defect in oxidation by pulse-chase and non-reducing SDS-PAGE analysis upon depletion of their trapped PDI family member partner. Such a substrate-oxidoreductase pair may be a good candidate in seeking reductase activity of the PDI family member.

5.2 ER Oxidant Enzymes

In the second half of this study, the observation that oxidation of substrates occurred after depletion of PDI, was more thoroughly investigated. Since PDI had been shown in yeast to be the primary acceptor of oxidizing equivalents from the ER oxidant Ero1, the mild phenotype observed upon loss of PDI was surprising. Subsequent evidence from the laboratories of Ron (Zito et al., 2010a; Zito et al., 2010b) and Bulleid (Tavender et al., 2010) revealed that other oxidant pathways were active in the mammalian ER and soon a number of proposed pathways were crowding for attention. My initial hypothesis that other oxidant activities could functionally replace Ero1α+β was tested by systematic knockdown of the enzymes central to three additional oxidant pathways, PRDX4, QSOX1, and VKOR. Indeed, I found that PRDX4 and VKOR, but not QSOX1, provided the oxidizing power needed to sustain disulfide bond formation. Further stressing of cells by addition of a reductive challenge by DTT washout also revealed that the active oxidant pathways were not equally potent; a ranking of Ero1 followed by PRDX4 and then VKOR was formed by monitoring their relative contributions to oxidative recovery. Additionally, it was observed that the loss of Ero1 drove up the GSSG:GS\textsubscript{tot} ratio in cells. This was interpreted to be a compensatory mechanism employed by cells when oxidation was solely dependent on alternative oxidant pathways.

A key result in the identification of VKOR as a significant provider of oxidizing equivalents to the ER was the observation of cytotoxicity in Ero1+PRDX4 depleted cells treated with warfarin. Since cells survived prior to addition of warfarin, it was proposed that VKOR
transferred sufficient oxidizing equivalents to PDIs and/or ER glutathione to support viability (recall Figure 4.5). Although the drug-mediated inhibition of VKOR is immediate, acute cell death in cells lacking Ero1+PRDX4 was not observed. Indeed, complete cell death was not seen until 2 days following warfarin treatment, arguing for a cellular pool of oxidizing equivalents that was gradually depleted and resulting in a loss of oxidative capacity in the ER triggering protein retention, UPR, and apoptosis. Glutathione disulfide is the prime candidate for this oxidizing pool as its levels were elevated in Ero1+PRDX4 depleted cells and then reduced upon overnight treatment with warfarin. To investigate this interpretation further, the ratio of GSSG:GS\text{tot} could be monitored over time following warfarin treatment to illuminate how quickly the excess GSSG pool is used up. Such experiments would clarify whether VKOR was responsible for generating the higher GSSG pool observed in Ero1+PRDX4 depleted cells. This interpretation could also be supported by treating Ero1+PRDX4 depleted cells with BSO to deplete glutathione. In pulse chase analysis, this combination could be anticipated to impede disulfide bond formation in substrate more prominently than in cells without BSO treatment and would indicate that the higher GSSG pool is used to support more rapid disulfide bond formation. Furthermore, in viability studies, Ero1+PRDX4 depleted cells first depleted of glutathione by BSO treatment and then subjected to warfarin treatment would be anticipated to exhibit cytotoxicity much more rapidly. Under these conditions, pulse chase analysis after just one to two hours of warfarin treatment may reveal a near-complete loss of substrate level disulfide bond formation thereby illustrating the link between VKOR and the GSSG pool in substrate oxidation.

The difference in the usage of VKOR by HepG2 cells versus mouse embryonic fibroblasts was attributed to differences in expression levels of VKOR by these two cell types (Section 4.4). However, there may be additional factors that limit VKOR activity in different tissues. Because its electron acceptor, vitamin K epoxide, is regenerated by the action of \(\gamma\)-glutamyl carboxylase, how potent VKOR is in generating oxidizing equivalents in the ER may be directly related to the activity of the carboxylase and the load of Gla-containing proteins produced by a given cell type. In cells with low \(\gamma\)-glutamyl carboxylase activity, a lower regeneration of vitamin K epoxide would be anticipated and VKOR’s impact on disulfide formation may be greatly diminished. One way to assess the involvement of Gla-containing substrates and \(\gamma\)-glutamyl carboxylase activity on VKOR function would be to inhibit VKOR in
different cell lines with varied VKOR or Gla-containing substrate expression levels and monitor the magnitude of the effect on the GSSG:GS_{tot} ratio and on cell viability. It would be of interest to compare the results obtained in Ero1+PRDX4 depleted HepG2 cells plus and minus warfarin treatment, with those from another high-expressor of VKOR such as cardiac fibroblasts and another cell line with low VKOR expression. Liver cells like HepG2 are able to generate the majority of known Gla-containing proteins, while the action of \(\gamma\)-glutamyl carboxylase in cardiac fibroblasts may be more limited, for example to the substrate Gas6 (Stenhoff et al., 2004). Such experiments could comment on the general usefulness of VKOR as an ER oxidant beyond liver cells and reveal how tightly its activity is coupled to that of the \(\gamma\)-glutamyl carboxylase.

Notably, all of the ER oxidant pathways characterized to date have been shown to require molecular oxygen as the terminal electron acceptor, either directly (as for Ero1) or indirectly (PRDX4 and VKOR). This has implications for the ability of cells to generate disulfides under conditions of hypoxia, or more extremely, anoxia. It is known that under hypoxic conditions Ero1\(\alpha\) is upregulated by the activation of HIF-1, presumably to make use of more \(O_2\) by the ER’s most regulated oxidase (Gess et al., 2003). However, responses by the alternate oxidant pathways to hypoxia are unknown. Certainly PRDX4 can gain access to \(H_2O_2\) via pathways other than Ero1 (discussed in (Zito et al., 2010b)), but the reduction of molecular oxygen is still required. Interestingly, the case is slightly different for VKOR. Although recycling of vitamin K to the epoxide form requires molecular oxygen in the \(\gamma\)-carboxylation reaction, if a source of vitamin K epoxide is available, either from an (unknown) alternate mechanism or extracellularly, then VKOR mediated disulfide formation could continue, even under hypoxic conditions. It would be of interest to study disulfide formation in HepG2 cells under hypoxic conditions by incubation in a nitrogen incubator. If some level of substrate oxidation occurs under these conditions, systematic depletion of Ero1, PRDX4 and VKOR would reveal which pathway is responsible. Should VKOR be found to play a role, it would support a possible uncoupling of VKOR activity from oxygen consumption. Since various forms of vitamin K can be obtained through diet, it would be of interest to supplement cells with exogenous vitamin K in these experiments. Addition of vitamin K would be anticipated to enhance VKOR-mediated disulfide formation under hypoxic conditions and may represent a compensatory mechanism employed by cells under conditions of oxygen starvation. Alternatively, if VKOR-dependent disulfide bond formation occurs relatively efficiently without addition of exogenous vitamin K, or upon
knockdown of the \(\gamma\)-glutamyl carboxylase, it would suggest an oxygen-independent mechanism of recycling the vitamin K epoxide, although no such means is currently known. These experiments would be informative in the study of many aggressive cancers as hypoxic tumors are associated with poor response to therapies and poor patient prognosis (Keith et al., 2011).

I observed cell death upon loss of Ero1, PRDX4, and VKOR function in HepG2 cells, arguing for their predominance in these cells. However, other oxidant pathways also exist and may play a supplementary role in HepG2 cells, or a more significant role in cells types with low VKOR expression. Among these pathways are the glutathione peroxidases (GPX7 and GPX8), that scavenge \(\text{H}_2\text{O}_2\) and transfer oxidizing equivalents to PDIs (Nguyen et al., 2011).

Additionally, the pathway mediated by dehydroascorbate (DHA) following reaction of reactive oxygen species with the antioxidant ascorbate, is thought to introduce disulfide bonds directly to substrate proteins rather than via PDIs as DHA is recycled back to ascorbate (Saaranen et al., 2010). Since cytotoxicity was also observed upon loss of both Ero1 and PRDX4 in MEFs (Zito et al., 2010b) with negligible VKOR expression, it seems that neither of these alternative pathways can functionally replace Ero1 or PRDX4, even in cells without an active VKOR pathway, so the role of these pathways may be limited to specific cell types or developmental stages.

### 5.3 Consequences of redox tuning in the ER

The regulation of ER redox enzymes has been elegantly elucidated in the case of Ero1 in mammalian cells (Appenzeller-Herzog et al., 2010), but tuning of the activity of other oxidant pathways or thiol oxidoreductases is less clear. Additionally, there is mounting evidence that a number of ER folding factors, not directly involved in substrate-level disulfide formation, are redox sensitive and that their activity is modulated by the redox status of the ER. For example, the UPR sensor ATF6 has been recently revealed to possess disulfides that promote its activation upon their reduction (Nadanaka et al., 2007; Walter and Ron, 2011), although the specifics of this activation remain to be determined.

Another proposed redox sensitive complex in the ER is the OST complex for delivering preassembled glycans to N-linked glycosylation motifs. The yeast OST subunits Ost3p and Ost6p each possess a CXXC motif on predicted thioredoxin-like folded domains and their reduction has been shown to modulate OST function in yeast (Schulz and Aebi, 2009; Schulz et
Although homologous subunits are thought to exist in mammalian cells, no such redox regulation of OST activity has yet been assigned (Mohorko et al., 2011). Interestingly, however, when I used transferrin as an alternative to albumin to monitor oxidative recovery and substrate level folding upon Ero1α+β or PDI depletion and DTT washout (as carried out in Chapter 4; Figure 5.1), a new intermediate of transferrin was revealed at early time points. In control cells, a faster migrating band at the 0 min chase point was converted to a more slowly migrating band by 5 min of chase, but in Ero1α+β or PDI depleted cells, this lower band persisted to 15–30 min of chase (Fig. 5.1A and B). Digestion of samples from early chase times with PNGase revealed that the lower band represented an unglycosylated form of transferrin (Fig. 5.1C). These preliminary experiments indicate that OST activity may indeed be regulated by the redox status of the ER. Further experiments, such as systematic mutation of cysteines (e.g. CXXA) in the mammalian homologues of Ost3p and Ost6p to force a reduced form of the subunits, and potentially reveal a PDI-family member interaction, are required to determine if this effect is solely due to the complete reduction of the ER. However, the tuning of OST activity by redox sensing could be an important switch in partitioning folding intermediates to alternate ER chaperone networks, or even directly to ERAD, under varying ER folding capacity conditions.

The regulation of the alternate ER oxidant pathways and possibly PDI family members by ER folding factors is another important question to be addressed. The PDI family members focused upon in this study have all been linked to the peptidyl prolyl isomerase, cyclophilin B, either in a large multichaperone complex (PDI, ERp72, P5), by common association with Cnx/Crt (ERp57), or directly (ERp72). Work by others in the Williams lab revealed that another cyclophilin, CypC is present in the secretory pathway and that these PDI family members can be found in a more oxidized state (i.e. the CXXC motifs are oxidized) upon co-depletion of CypB/C by RNAi or inhibition of CypB/C activity by the drug cyclosporin A (Dr. P. Stocki, personal communication). These PDI family members all possess cis-proline residues spatially adjacent to their CXXC motifs, although available structural data indicates that these prolines are not solvent exposed. To initiate investigation into CypB/C-mediated effects on PDI oxidation state, the substrate trapping pattern of the PDI trap mutant (as used in Chapter 3) in control cells was qualitatively compared to that in CypB/C-depleted cells following reductive challenge. As shown in Figure 5.2A and B, efficient knockdown of ER cyclophilins resulted in a substantially
Figure 5.1. Unglycosylated transferrin persists upon Ero1α+β or PDI depletion. (A,B) Assessment of substrate level folding by pulse-chase following the indicated siRNA depletion and reductive challenge were carried out as described in Chapters 2 and 4, but immunoisolation of transferrin was carried out. Its oxidative folding, assessed by non-reducing SDS-PAGE (indicated by −DTT), revealed a novel band at about 66 kDa (*) that disappears by 5 min in control cells, persists as a discrete band in Ero1 depleted cells to 15 min, and persists and is subject to oxidation until 30 min in PDI depleted cells. R – reduced, PO – partially oxidized, O – oxidized. (C) Treatment of isolated transferrin at 5 min chase in control (C) or Ero1 knockdown (KD) cells with PNGase, reveals that the lower band has the same mobility as an unglycosylated intermediate of transferrin.
Figure 5.2. CypB+C impact on PDI substrate oxidation and ER redox balance. (A) Co-depletion of cyclophilin B and C was carried out, as monitored by Western blot. (B) HepG2 cells stably expressing the PDI\(^{\text{CXXA}}\)-HA construct described in Ch.3 were subjected to depletion of CypB+C by siRNA. DTT washout protocol was performed as in Ch.4 using NEM alkylation to halt disulfide exchange at the indicated time points. Whole lysates were subjected to non-reducing SDS-PAGE and Western blotting with both anti-HA and anti-GAPDH. The lower GAPDH band indicates equivalent loading of the lanes and the uncomplexed PDI-HA as well as PDI-HA in complex with disulfide bonded partners (DBP) are indicated. The bands demarked with the (*) indicate a non-specific HA antibody signal. (C) Steady state GSSG:GSH levels were assayed by the protocol used in Ch.4. with and without overnight treatment with 5 µg/µL cyclosporin A on the final day of the indicated knockdown (x-axis). Ratios were normalized to untreated control levels and are the averages of three independent experiments +/- one standard deviation. CsA – Cyclosporin A
different banding pattern for PDI-trapped substrates, with strong appearance of some novel bands. Future experiments using mass spectroscopy to identify and compare bands under both conditions may reveal a CypB/C-dependent modulator of PDI’s activity or preferred substrates in the absence of CypB/C.

To address the observation of more oxidized PDI family members more directly, the GSSG:GS\textsubscript{tot} ratios were assayed to determine if the total ER redox balance had shifted as well. Interestingly the ratio of glutathione disulfide: total glutathione dramatically increased upon treatment with the cyclophilin inhibitor cyclosporin A (CsA). Knockdown of CypB/C generated the same phenotype with no further GSSG increase upon CsA treatment of CypB/C depleted cells (Fig. 5.2C). This six-fold increase in GSSG:GS\textsubscript{tot} levels far exceeds what has been previously reported upon overexpression of an over-active mutant of Ero1α (Appenzeller-Herzog et al., 2008) and is consistent with the more oxidized state of PDI family members upon CypB/C co-depletion. Since previous work presented in this thesis had shown that GSSG ratio could be modulated by the activity of ER oxidant pathways, the knockdown of oxidant pathways was carried out and GSSG:GS\textsubscript{tot} ratios were determined with and without CsA treatment. These depletion studies revealed that knockdown of Ero1α+β failed to prevent the CsA-dependent ratio shift, but, surprisingly, knockdown of QSOX1 limited the over-oxidation of the ER upon CsA treatment by nearly 50%. Although not a complete inhibition, these observations suggest that QSOX1 may be responsible, at least in part, for the increased GSSG:GS\textsubscript{tot}. In context with the apparent lack of QSOX1 activity in HepG2 cells (Chapter 4), it may be that QSOX1 is a tightly regulated oxidase. In the presence of CypB/C in the ER, QSOX1 is perhaps inactive, either by direct action of CypB/C on either of QSOX1’s two exposed cis-prolines (Alon et al., 2010), or indirectly by CypB/C action on a (presently unknown) negative regulator of QSOX1. Upon depletion or inhibition of the cyclophilins, QSOX1 is active in the ER and, consistent with in vitro kinetic data, could promiscuously and rapidly generate disulfide bonds, shifting ER equilibrium to more oxidizing conditions. Such a model is also in agreement with the apparent lack of QSOX1 activity in the ER during knockdown studies and with proposed roles for QSOX1-mediated activity beyond the ER (Thorpe et al., 2002). Future experiments to distinguish these possibilities will make use of siRNA-insensitive QSOX1 additionally mutated at its cis-prolines. Should such a mutation lead to a de-regulated and active QSOX1, the over-oxidation of the ER would be expected. Mutation of cis-prolines may prove problematic due to
associated misfolding, so crosslinking and co-immunoprecipitation of QSOX1 may alternately reveal its regulator(s), particularly if compared with and without CsA treatment.

5.4 Conclusions

Disulfide bond formation in the ER is relatively simple in its chemistry, but strikingly complex in its execution. Although of differing functional importance, multiple PDI family members and oxidant pathways are all at work in the productive disulfide formation and folding of client proteins. The work presented in this thesis represents the first systematic characterization of the functional relationships among these PDI family members and ER oxidants and has revealed their relative importance in carrying out this essential posttranslational modification. Additionally, this work has supported increasing evidence of the partitioning of client proteins to chaperone-foldase networks, though it is likely that substrate proteins are able to move between folding networks as needed. The proposed role of glutathione disulfide as a productive participant in disulfide generation has also been upheld and GSSG:GS\text{tot} steady state ratio has been shown to be fluid, depending on the overall oxidative power of the ER. The regulation of these pathways, as well as their impact on redox sensing ER proteins will be topics of great interest in the future.
References


Allen, S., and Bulleid, N.J. (1997). Calnexin and calreticulin bind to enzymically active tissue-type plasminogen activator during biosynthesis and are not required for folding to the native conformation. Biochem J 328 (Pt 1), 113-119.


