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FORUM REVIEW ARTICLE

# Molecular Mechanisms Regulating Oxidative Activity of the Ero1 Family in the Endoplasmic Reticulum

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**Abstract**

Formation of disulfide bonds in the endoplasmic reticulum (ER) is catalyzed by the ER oxidoreductin (Ero1) family of sulfhydryl oxidases. Ero1 oxidizes protein disulfide isomerase (PDI), which, in turn, introduces disulfides into ER client proteins. To maintain an oxidized state, Ero1 couples disulfide transfer to PDI with reduction of molecular oxygen, forming hydrogen peroxide. Thus, Ero1 activity constitutes a potential source of ER-derived oxidative stress. Intricate feedback mechanisms have evolved to prevent Ero1 hyperactivity. Central to these mechanisms are noncatalytic cysteines, which form regulatory disulfides and influence catalytic activity of Ero1 in relation to local redox conditions. Here we focus on the distinct regulatory disulfides modulating Ero1 activities in the yeast and mammalian ER. In addition to considering effects on the Ero1 catalytic cycle, we consider the implications of these mechanisms with regard to function of Ero1 isoforms and the roles of Ero1 during responses to ER stress. *Antioxid. Redox Signal*. 13, 1177–1187.

## Introduction: Disulfide Formation Within Cells

FORMATION OF NATIVE DISULFIDE BONDS between cysteine residues is a crucial step in the folding and assembly of many proteins. Disulfides are commonly found within cell surface and secretory proteins and are often essential for acquisition of a functionally active molecule. In addition to conferring structural stability in the extracellular environment, disulfide formation is increasingly credited with important functions in eukaryotes. These roles include control of peptide loading onto major histocompatability complex class I molecules (10), the thiol mediated retention of proteins within the endoplasmic reticulum (ER) (2), and as functional switches controlling inflammation and thrombogenicity (1).

Most disulfides formed within cells are products of thiol-disulfide exchange reactions, the transfer of two electrons from a reduced cysteine pair to an existing disulfide bonded species. This exchange causes oxidation of the reduced thiols to form a new disulfide bond, with concurrent reduction of the existing disulfide to generate free thiols (Fig. 1). Enzymes facilitate these processes by catalyzing thiol-disulfide exchange between themselves and specific client proteins (15). A classic example of such an enzyme is protein disulfide isomerase (PDI), which is found within the ER of eukaryotes. PDI contains a pair of redox-active cysteine residues within each of its active sites (23, 42). These residues shuttle between thiol and disulfide states facilitating oxidation, reduction, or net rearrangement (isomerization) of disulfides (12). To continually introduce disulfides into client proteins, PDI must be maintained in an oxidized state. This function is primarily fulfilled by the ER oxidoreductin 1 (Ero1) family (17). Similar to PDI, Ero1 has active sites containing pairs of cysteine residues that alternate between thiol and disulfide status (18). However, Ero1 also has a conjoined flavin adenine dinucleotide (FAD) moiety to which it can transfer electrons and thus maintain its active sites in an oxidized form without requiring further thiol-disulfide exchange (49). In this way, Ero1 effectively creates disulfides *de novo* compensating for the loss of oxidizing equivalents caused by flux of proteins through the secretory pathway.

## Ero1 Is a Potential Source of Oxidative Stress

Mechanisms have arisen throughout evolution that utilize electron accepting cofactors such as FAD and ubiquinone to introduce disulfides into biological systems (Fig. 2). DsbB in *Escherichia coli* fulfils a function analogous to Ero1, maintaining periplasmic DsbA in a state permitting oxidation of client proteins (25, 27). DsbB transfers electrons to ubiquinone and, consequently, into the bacterial electron transport chain (ETC). Similarly, disulfide formation within the mitochondrial intermembrane space makes use of the flavoprotein Erv1. Erv1 transfers disulfides to the import receptor Mia40 and electrons *via* its FAD moiety to cytochrome C, again linking a disulfide relay to the local ETC (7). In both Dsb and Mia pathways, the terminal electron acceptor *via* the ETC is molecular oxygen, generating water as a product. In contrast to these systems, Ero1 in the ER lumen lacks a proximal ETC.

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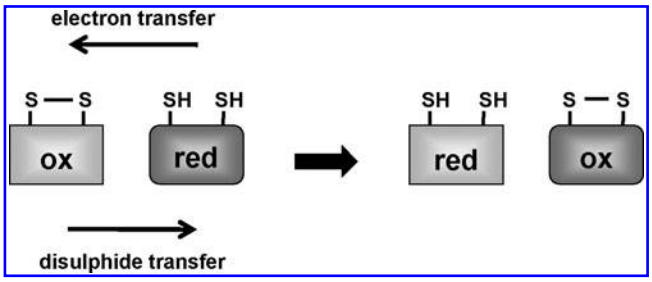


**FIG. 1. Thiol-disulfide exchange.** Cartoon illustrating redox exchange between oxidized (S–S, ox) and reduced (SH, red) pairs of cysteine residues. Reducing equivalents (two electrons) are transferred from the reduced to the oxidized species, whereas oxidizing equivalents (a disulfide) move in the other direction. As a result, the oxidized species are reduced from disulfide to free thiol state, whereas the free thiols of the reduced species are oxidized to form a disulfide.

Instead, electrons directly shuttle to molecular oxygen *via* the FAD moiety, generating hydrogen peroxide rather than water (21, 50); hence, Ero1 forms one molecule of hydrogen peroxide for every disulfide it introduces. Hydrogen peroxide is a reactive oxygen species (ROS) capable of bringing about oxidative stress and cell death. A burden is, therefore, attached to the process of disulfide bond formation within the ER, the cost of which is evident for cells with a heavy ER client–protein load (22, 24, 33).

Although putative mechanisms exist for hydrogen peroxide detoxification in the ER (44, 48), it is widely accepted that prevention is better than cure. Consequently, the prospect of Ero1-derived oxidative stress has raised significant questions with regard to how cells might moderate Ero1 activity. Given that its redox partners—PDI and oxygen —generally exist in abundance, how is flux through this pathway regulated? In recent years, elegant mechanisms for feedback regulation of Ero1 have been discovered (3, 4, 46). In this review, we focus on these regulatory processes and compare the yeast and mammalian Ero1 proteins.

## Ero1 Is Essential for an Oxidizing ER

Cells contain millimolar quantities of the tripeptide glutathione, which exists in a reduced thiol (reduced glutathione [GSH]) or an oxidized disulfide (oxidized glutathione [GSSG]) form. In the reducing environment of the mammalian cytosol, GSH exceeds GSSG by a ratio between 30:1 and 100:1 (26). For the mammalian ER, this ratio has been established as more like 5:1, reflective of a more oxidizing compartment (14). For many years, the elevated GSSG concentration was assumed to be the major source of oxidizing equivalents in the ER for transfer of disulfides into secretory proteins. This view was altered over a decade ago with parallel discoveries of the essential gene *ERO1* in *Saccharomyces cerevisiae* (16, 43).

*ERO1* encodes Ero1p, a membrane-associated glycoprotein whose expression helps to protect against fatal effects of the thiol-reducing agent, dithiothreitol (DTT) (16, 43). *ERO1* deletion proves lethal, whereas temperature-sensitive mutants display an unfolded protein response (UPR) caused by an accumulation of nonnative proteins within the ER. The phenotype is attributable to a lack of disulfide formation which induces protein misfolding, evident by the fact that maturation of disulfide-free ER client proteins is unaffected (43). Consistent with a lack of disulfide formation, defects associated with Ero1p functional mutants are complemented by addition of chemical oxidants (16). *ERO1* expression is itself directly upregulated as part of the UPR, presumably to fulfill

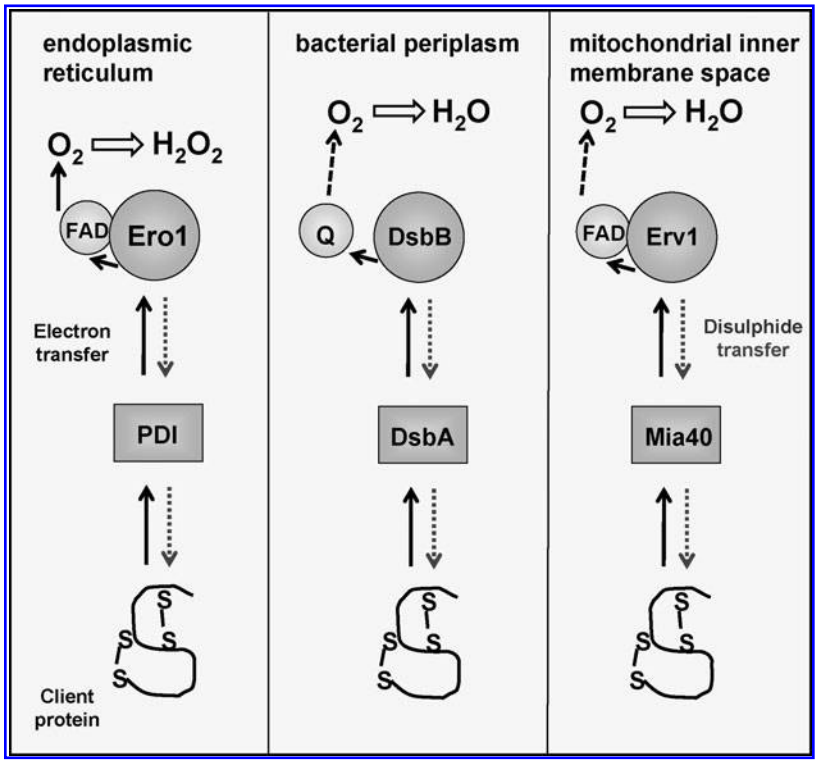


**FIG. 2. Electron flow in disulfide relays.** A schematic representation of pathways for disulfide formation in the endoplasmic reticulum (*left*), bacterial periplasm (*middle*), and mitochondrial intermembrane space (*right*). In each case, oxidation of client protein is ultimately linked to reduction of molecular oxygen. *Dotted, light gray arrows* indicate direction of disulfide transfer, *solid black arrows* show direct electron transport, and *dashed black arrows* represent electron flow *via* an electron transport chain.

a requirement for disulfide formation during refolding of nonnative ER client proteins (16, 43).

Strikingly, glutathione has since been shown to be dispensable for the oxidative branch of disulfide formation (9, 16). Rather, the role of glutathione lies in GSH providing a source of reducing equivalents for isomerization of nonnative disulfides (9, 37). In catalyzing *de novo* disulfide synthesis, Ero1p, therefore, provides the principle source of oxidizing equivalents within the yeast ER.

### Ero1α and Ero1β Are Distinct Mammalian Oxidases

Characterization of Ero1p paved the way for identification of mammalian counterparts (8, 40). Humans possess two isoforms encoded by distinct genes and designated Ero1α (Ero1-like α [Ero1-Lα]) and Ero1β. Both are functional homologs of Ero1p able to complement temperature-sensitive yeast mutants (8, 40), whereas overexpression of each increases the oxidizing power of the mammalian ER (36). Regions of significant conservation are seen between yeast and human Ero1 sequences, and the active site cysteine residues are invariant (Fig. 3). A major distinction, however, is the presence of a hydrophobic C-terminal tail in Ero1p that facilitates membrane association and is essential for functions. Human Ero1 proteins lack this domain and, as such, can only complement Ero1p loss-of-function mutants, not deletion strains entirely lacking the protein (41). Human Ero1α can, however, complement a deletion strain if the yeast hydrophobic tail is appended to the human sequence (41). This result demonstrates the importance of membrane association for Ero1 activity in yeast. A clear need for membrane association of human Ero1 has yet to be established, but both isoforms depend on binding partners to maintain their localization within the ER (39). Ero1α and Ero1β each lack a conventional Lys-Asp-Glu-Leu (KDEL)-type motif for Golgi-to-ER retrieval and instead rely on ER retention by thiol-dependent association with either PDI (KDEL) or ERp44 (RDEL).

Human Ero1 proteins differ from each other in distribution profile (40). *ERO1-Lα* is constitutively expressed with relatively little fluctuation across a range of cells and tissues, a notable exception being elevated transcript accumulation in the oesophagus. *ERO1-Lβ* expression is less ubiquitous and varies from low level in most tissues to high transcript abundance in certain secretory tissues, especially pancreas, stomach, and testis. Regulation of expression is also a dividing factor. *ERO1-Lα* is upregulated in response to hypoxia and hypoglycemia under control of the hypoxia-inducible factor (HIF)-1 transcription factor (19, 34) and is also directly activated by the C/EBP homologous protein (CHOP) (33). CHOP is itself positively regulated by ATF4 as part of an integrated stress response initiated by the action of PKR-like eukaryotic initiation factor 2α kinase (PERK) (during the UPR), Gcn2 (during amino acid starvation), and others (22). Ero1α responds to a diverse range of insults, whereas *ERO1-Lβ* expression is only known to be induced during the UPR (40), though not *via* the CHOP-dependent pathway that regulates *ERO1-Lα* (33). Further, even in stressed mouse cells levels of mRNA, encoding Ero1β are just a tenth those of Ero1α (33). Given such a prevalent role for Ero1α, the precise function of Ero1β remains unclear, though we will see hereafter that subtle differences in regulation of Ero1α and Ero1β activity may have profound implications regarding their action within the ER.

### Ero1 Flexibility Maintains Disulfide Transfer to PDI

Ero1 directly introduces disulfides to reduced PDI, evident *in vitro* from the ability of recombinant Ero1p and Ero1α to oxidize PDI (4, 49) and suggested *in vivo* by the detection of mixed disulfides containing the two proteins in yeast and mammalian cells (5, 17). Disulfide transfer is thought to be facilitated by contact between the CGHC active site motifs of PDI and the "outer" active site (also referred to as the "shuttle disulfide") of Ero1. After disulfide transfer to PDI, the Ero1 outer active site is re-oxidized by disulfide exchange with its C-terminal "inner" active site (depicted in Fig. 4). Current models predict direct exchange between the inner and outer active sites (18).

Resolution of the Ero1p structure by X-ray crystallography established several features important to the cycle of Ero1

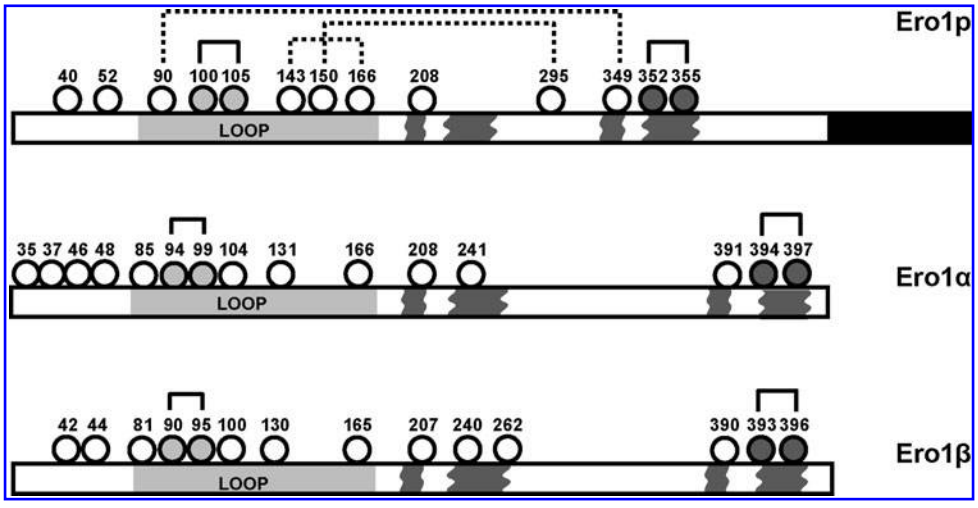


**FIG. 3. Disulfide arrangement in *Saccharomyces cerevisiae* Ero1p.** A simple representation of key features in the Ero1p primary structure. *Circles* represent cysteine residues present in the mature protein. Disulfide connectivity is illustrated using *solid lines* to show catalytic disulfides and *dashed lines* for noncatalytic disulfides. Cysteine residues of the shuttle disulfide are shaded light gray, with the inner active site filled in dark gray. Relative positions of the flexible loop region and the four core helices are also indicated by gray shading, whereas the hydrophobic C-terminal tail is shown in black. Human Ero1α and Ero1β are also presented for comparison. Catalytic disulfides are indicated as are the flexible loop and helical core regions. Noncatalytic disulfides are omitted (see Fig. 6 for details). Ero1, endoplasmic reticulum oxidoreductin 1.

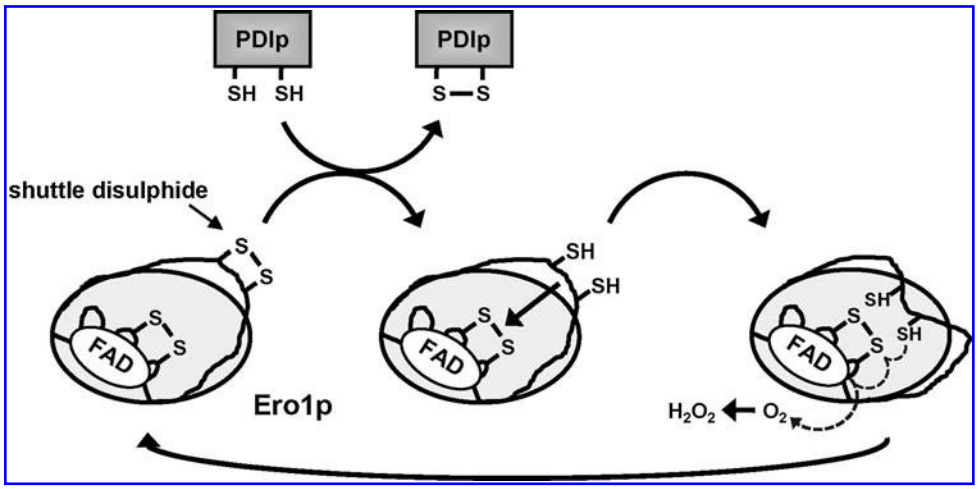


**FIG. 4. Disulfide transfer and electron flow within Ero1p.** A cartoon depicting the catalytic cycle of Ero1p. The shuttle disulfide of the outer active site oxidizes free thiols within the CGHC motif of PDI and is, in turn, reduced to a thiol state. Flexibility of the loop region (indicated by an *arrow*) allows interaction of the reduced outer active site with the oxidized inner active site, resulting in thiol-disulfide exchange and restoration of the shuttle disulfide. The inner active site is re-oxidized by electron transfer (*gray dashed line*) to the associated FAD moiety and on to molecular oxygen. Ero1, endoplasmic reticulum oxidoreductin 1; FAD, flavin adenine dinucleotide; PDI, protein disulfide isomerase.

activity (20). The position of the inner active site places it adjacent to the redox-active isoalloxazine moiety of the FAD cofactor. The FAD nestles among four antiparallel helices (whose relative positions within the primary structure are depicted in Fig. 3), creating a rigid core central to enzyme activity. In contrast, the shuttle disulfide of the outer active site is situated on a flexible loop resting close to the surface of the protein while also within proximity to the inner active site. The loop region contains little secondary structures and crystallizes in two distinct conformations differing in positions by up to 17 Å. The mobile range of the shuttle disulfide itself is around 5.5 Å, flexible enough to allow the outer active site to physically migrate between a position of accessibility for the PDI substrate and then back to the inner active site for re-oxidation (illustrated in Fig. 4). In addition to conferring flexibility, the unstructured nature of the Ero1 loop has been suggested to recruit PDI before disulfide exchange (20). As a molecular chaperone, PDI can bind unfolded peptides, a feature that may promote direct binding of Ero1 in the vicinity of the shuttle disulfide. In keeping with this, PDI lacking any active site cysteine residues still interacts with Ero1 (39).

### Noncatalytic Disulfides Form Molecular Shackles for Ero1p

Outside its active sites, Ero1 contains additional cysteine residues that also form intramolecular disulfides. Based on the crystal structure, disulfide arrangement for Ero1p is as depicted in Figure 3. Two long-range disulfides (C90–C349 and C150–C295) connect the flexible loop containing the shuttle disulfide to regions within the core of the protein. Once established, these connections have the effect of pinning the flexible loop in place and perhaps restraining migration of the shuttle disulfide toward the inner active site, thereby attenuating oxidation of PDI by Ero1p. These long-range "regulatory" disulfides, therefore, act as a redox switch controlling Ero1p, with their reduction crucial to allowing efficient Ero1p activity (Fig. 5) (46).

Redox state of regulatory disulfides also influences the physical properties of Ero1p. Formation of the bonds is accompanied by a decrease in hydrodynamic volume of the denatured protein, enhancing electrophoretic mobility under nonreducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis conditions. This property provides a means to assess the oxidation state of Ero1p in cells and was used to demonstrate that the regulatory disulfides inactivate Ero1p in the yeast ER at steady state (46). The cellular pool of Ero1, therefore, exists in a state of catalytic "standby," requiring reduction of its restraining bonds for transition to an activated form and subsequent oxidation of PDI.

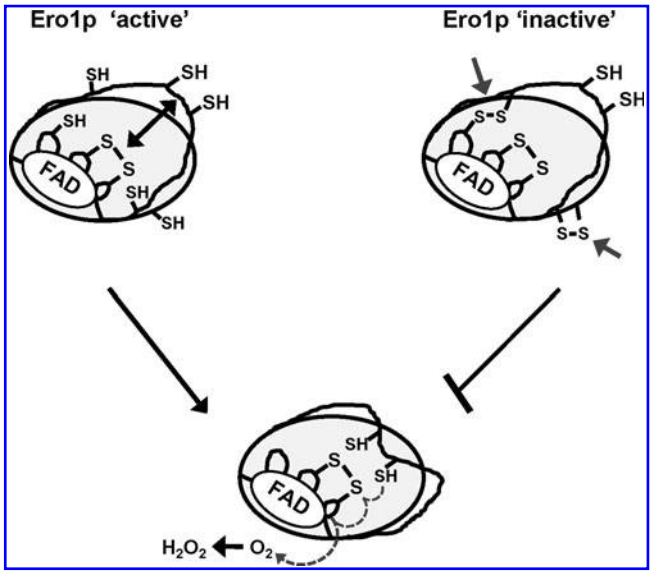


**FIG. 5. Inhibition of Ero1p activity by regulatory disulfides.** An illustration of the principle of Ero1p regulation. When Ero1p is active, the reduced outer active site can shuttle back and forth (indicated by the *double-headed arrow*) for continuous catalytic activity, as depicted in Figure 4. In the inactive state, formation of regulatory disulfide bonds (indicated by *solid gray arrows*) prevents migration of the outer active site toward the inner active site for re-oxidation. Continued oxidation of substrates and electron transport to molecular oxygen (*dashed gray line*) are, therefore, limited. Ero1, endoplasmic reticulum oxidoreductin 1.

The mechanism of transition from resting to active Ero1 emerged after experiments with recombinant protein *in vitro*. Purified Ero1p possesses a full complement of regulatory disulfides that disappear on addition of substrate (reduced PDI or thioredoxin), evident from corresponding changes in gel mobility (46). This remodeling is necessary for substrate oxidation and for transfer of electrons to oxygen and manifests as a characteristic lag preceding reaction of PDI with Ero1p *in vitro*. The initial lag can be significantly shortened if the requirement for regulatory disulfide reduction is removed, that is, by mutation of cysteine residues to prevent oxidation in the first place. Substrates such as PDI can, therefore, be defined not only by their capacity to be oxidized by Ero1 but (at least *in vitro*) also by their ability to first remove its regulatory shackles (46).

This prerequisite for removal of regulatory disulfides introduces a point of constraint whereby Ero1p activity can be modulated over and above the level of substrate availability. As described later for Ero1α, redox balance within the ER affects the propensity for activation of Ero1 and vice versa, providing a mechanism for regulation of Ero1 activity by environmental feedback.

### A Negative Feedback Loop Shuts Down Ero1 Activity

After reduction, the long-range disulfides in Ero1p can reform, though this is a relatively slow process during which multiple rounds of PDI oxidation can proceed (46). Once switched on, it is, therefore, unnecessary for Ero1p to be continually activated. Moreover, restoration of the regulatory disulfides is only favorable in the absence of sufficient reduced PDI for efficient Ero1p turnover. Thus, by oxidizing PDI and removing reducing equivalents, Ero1p alters the redox balance of its local environment and favors re-oxidation of the regulatory cysteine residues, ultimately curtailing its own activity. Ero1p activity is, therefore, self-regulated by a process of negative feedback.

A regulatory mechanism emerges in which electron transfer through Ero1p can occur as long as there is a client protein substrate that is (1) capable of reducing regulatory disulfides of Ero1p; and (2) present in sufficiently reducing balance to maintain Ero1p turnover and prevent re-formation of the regulatory disulfides. The fact that these criteria are disfavored after Ero1p activity has prompted the idea that Ero1p is central to the maintenance of ER thiol-disulfide balance (45, 46). In a reducing environment, Ero1 may become activated, promoting thiol oxidation, which, in turn, diminishes further Ero1 activity. This is an attractive idea when considered from a thermodynamic point of view, and it is clear that Ero1p influences ER redox conditions. However, the kinetics of regulatory disulfide reduction and oxidation questions whether Ero1p activity constitutes a homeostatic mechanism in its own right.

### Ero1p Regulatory Disulfides: The Hierarchy Within

While simple in principle, Ero1p feedback regulation undoubtedly involves intricate complexities that have yet to become clear. For example, a function has yet to be established for the C143–C166 disulfide. This pair is reduced while Ero1p is active, re-oxidized when inactive, and has been speculated to influence mobility of the loop region (46). Unfortunately, structural instability of C143–C166 mutants has made this contribution difficult to assess (46).

Though individually characterized, the relationship between the long-range disulfides is itself not fully understood. They are not functionally equivalent, as C150–C295 exerts a defining influence on redox properties of the C90–C349 pair. Cysteine-to-alanine mutants preventing formation of C150–C295 give rise to Ero1p, which also lacks the C90–C349 bond when expressed in yeast (46). The ability of C90–C349 to resist reduction in the ER, therefore, seems dependent on the stabilizing C150–C295 connection. The reverse is not true, as C90–C349 mutants still retain the C150–C295 disulfide. Moreover, expression of the C150–C295 mutant generates a protein with dramatically elevated activity *in vitro*, whereas the C90–C349 mutant is only moderately enhanced. C150–C295 can, therefore, be considered as the critical disulfide required for regulation of Ero1p activity. Redox status of these residues effectively determines whether Ero1p is switched on or off in the ER. Questions persist as to the exact purpose of the C90–C349 disulfide; whether this bond fulfils a specific structural requirement or a role that is simply imperceptible *in vitro* remains to be seen.

As the critical disulfide for control of Ero1p, the importance of the C150–C295 disulfide *in vivo* is most apparent in its absence. Overexpression of C150–C295 mutants drastically reduces yeast viability and increases the cellular ratio of GSSG:GSH, indicative of hyperoxidizing conditions within the ER (46). These findings provide compelling evidence that deregulated Ero1 activity causes unwarranted thiol oxidation and elevated ROS production, highlighting how crucial it is that C150–C295 can be stably oxidized in the ER. The fact that Ero1p is not indiscriminately reduced by free thiols (as evident by its poor activity toward GSH *in vitro*) is likely to be reflective of a low reduction potential for this cysteine pair, supported by the necessity of a highly reducing partner (*e.g.*, thioredoxin) or an abundance of more moderately reducing thiols (PDI) for efficient activation of Ero1p (46).

### Mammalian Ero1α Shares Catalytic Principles with Ero1p

Structural analyses of mammalian Ero1α and Ero1β have not met with the successes of those performed using Ero1p. Based on predictive modeling and sequence homology, however, the flexible loop model for Ero1p activity is likely to be conserved throughout the Ero1 family (11). Consistent with this, human Ero1α replicates many biochemical properties of Ero1p *in vitro* and *in vivo*.

Ero1α exhibits two major redox forms in the ER, termed OX1 and OX2 (5). Both contain disulfide bonds, evident by their increased electrophoretic mobility compared with reduced Ero1α. OX2 is the predominant species in human cells, although detectable levels of OX1 can vary significantly between experiments highlighting a delicate equilibrium between the two forms (3). OX2 is also the most mobile species in-gel prompting the idea that, as in yeast, Ero1α is predominantly inactivated *in vivo* through formation of multiple noncatalytic disulfides. Parallel studies confirmed this theory, identifying several regulatory disulfides for Ero1α (3, 4). Similar to Ero1p, *in vitro* studies show that the presence of reduced PDI or thioredoxin stimulates initial reduction of these bonds, igniting oxidative activity and substrate depletion, followed by re-oxidation of Ero1α to the OX2 form (4). Although this suggests a broadly similar regulatory mechanism to that found in yeast, critical disulfides within Ero1α appear

evolutionarily distinct, indicating subtle differences underlying Ero1 regulation in the mammalian ER.

Much of the disulfide connectivity within Ero1α was established by Ellgaard and coworkers using a mass spectrometry (MS) approach (3). Ero1α was first alkylated in mammalian cells to prevent disulfide exchange. The resulting alkylated protein was immunoisolated from cells, and the disulfide connectivity determined from peptides was derived after proteolytic digestion. At this time, our group employed a strategy built around systematic mutation of cysteine residues in Ero1α (4). By evaluating biochemical, biophysical, and enzymatic effects of the recombinant mutants *in vitro*, we were also able to identify and characterize regulatory disulfide pairs. The combined findings of the two studies determined disulfide patterning in the OX2 or "inactive" Ero1α species to be as illustrated in Figure 6.

### Ero1α Regulatory Disulfides Connect Catalytic with Noncatalytic Cysteine Residues

Ero1α disulfide arrangement differs from Ero1p in several aspects. Notably, there is no ortholog for the critical C150–C295 disulfide, as equivalent cysteine residues do not exist within Ero1α. Instead, C150–C295 is supplanted by alternative disulfides, one of which connects a cysteine residue in the outer active site (C94) with a noncatalytic cysteine further along in the flexible loop (C131). C94–C131, therefore, introduces a novel concept for Ero1α regulation, the use of regulatory disulfides to impound active site thiols and physically inhibit shuttle disulfide formation. Such direct interference with the disulfide exchange relay is a defining feature distinguishing the regulatory mechanism of Ero1α from that of Ero1p.

The C94–C131 connection is the only disulfide in Ero1α that is essential for OX2 formation. Reduction of this bond triggers transition from OX2 to OX1 (4, 6). As such, C94–C131 is analogous in influence, if not mechanism, to the Ero1p C150–C295 disulfide. Consistent with this, mutation of C131 to alanine prevents C94–C131 formation, causing hyperactivity of Ero1α toward PDI *in vitro* (4). Such deregulation of Ero1α is more pronounced in the absence of C94–C131 than for any other disulfide mutant. Unlike deregulation of Ero1 in yeast, expression of Ero1α C131A does not impinge on cell viability. That is not to say, redox balance is unaffected as significant increases in GSSG relative to GSH accompany overexpression of hyperactive Ero1 in human cells. Additional effects include a shift in the balance of predominantly reduced ER proteins, such as ERp57, to a more oxidized state (3). ERp57 is important for isomerization of nonnative disulfides in complex glycoproteins (29) and requires GSH to directly maintain its active thiols in a reduced form (28). The increased pool of oxidized ERp57 arising during Ero1α deregulation is most likely induced by perturbed glutathione balance and is, therefore, an indirect symptom of Ero1 hyperactivity. The ability of Ero1α to exert such knockon effects highlights the importance of its tight control in promoting ER redox homeostasis.

Since the noncatalytic C131 residue sequesters C94 to inhibit catalytic disulfide exchange, so C104 is likely to collaborate to contain the other cysteine of the outer active site, C99 (4). Thus, formation of the C94–C99 shuttle disulfide may well be prevented by thiol-occupation on two fronts. Removal of the C99–C104 bond does not activate Ero1α catalysis (removal of the C94–C131 bond is critical for this), but logic dictates that it must be reduced in OX1 for the shuttle disulfide to form. Moreover, C99–C104 does contribute to shutdown of Ero1α activity, as is evident from activity assays *in vitro* (4). Ero1α mutants lacking both the C104 and C131 cysteine residues display rapid oxidation of reduced PDI. Mutation of C131 alone, while still hyperactive, does not induce quite such heightened activity. From this, we can conclude that the C104

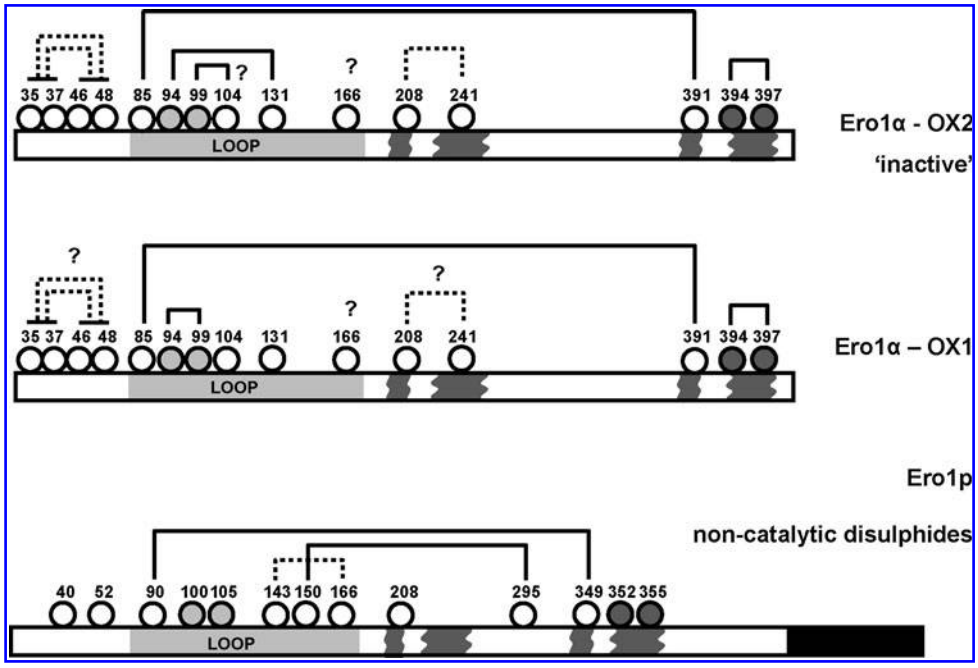


**FIG. 6. Disulfide patterning in Ero1α.** A simple depiction of disulfide bond arrangement as experimentally determined for Ero1α in its resting state (OX2) alongside predicted disulfide patterning after reduction of critical regulatory disulfides (OX1, see main text for details). Included for comparison is a representation of noncatalytic disulfides within Ero1p. In each case, *solid lines* illustrate potential disulfides that have been demonstrated to exert effects on Ero1 activity. *Dashed lines* show connections whose influence remains undetermined, and question marks indicate where redox state is uncertain. The flexible loop and cysteine residues of the outer active site are shaded light gray, whereas the core helices and cysteines of the inner active site are shaded dark gray. Ero1, endoplasmic reticulum oxidoreductin 1.

residue provides an additional element of regulation in the system, presumably helping to modulate Ero1 activity by hampering formation of the shuttle disulfide. Control of enzyme activity is, therefore, much more direct for regulatory cysteines of Ero1α than in Ero1p. A real contest exists in the human protein whereby noncatalytic thiols of C104 and C131 compete against reduced substrates for redox exchange with the C94–C99 shuttle disulfide (3). Intriguingly, these residues raise the possibility for positive as well as negative regulation of Ero1α. Stabilization of a heightened state of Ero1 activity could conceivably be achieved by formation of stable disulfides connecting C104 and C131 with other noncatalytic cysteines.

Theoretically, the C94–C131 and C99–C104 disulfides serve an equal purpose, yet the former clearly wields greater influence over Ero1α catalysis. C131 may simply be more potent in competing against C94–C99 oxidation. Alternatively, C94–C131 redox exchange may exhibit more widespread effects on Ero1α activity than mere hijacking of a catalytic cysteine. Favoring the latter idea, we have shown that reduction of Ero1α with DTT alters inherent tyrosine and tryptophan fluorescence, indicative of altered solvent accessibility to regions containing these residues (4). This behavior is fully attributable to reduction of the C94–C131 disulfide and cannot be replicated in a C131A mutant. Hence, changes in C94–C131 redox state exert minor conformational alterations in Ero1α, and it is probable that this disulfide modulates Ero1α activity by influencing both structural mobility and availability of catalytic thiols. Conformational changes associated with C94–C131 oxidation state may explain why reduction of this disulfide is so crucial in switching on Ero1α.

### Ero1α Is Rich in Additional Intramolecular Disulfides

In addition to its novel regulatory links, Ero1α shares some familiar aspects of Ero1p disulfide arrangement, most notably the long-range C85–C391 disulfide. This is equivalent to the C90–C349 bond of Ero1p and, as such, displays several similar characteristics, in particular the connection of a cysteine in the flexible loop region with the N-terminal residue of the CXXCXXC motif housing the inner active site. Mutation of C85 to alanine eliminates the disulfide and enhances the already elevated levels of oxidase activity displayed by C94–C131 mutants *in vitro* (4). In contrast to Ero1p, however, the C85–C391 disulfide of Ero1α is more resistant to reduction *in vivo* and appears stably oxidized in the OX1 form despite the absence of other regulatory disulfides (3). Unfortunately, the phenotypes of C85 and C391 mutants have not been probed further, as the residues appear important for solubility and stability of the recombinant protein (4). Moreover, expression of C85–C391 mutants in human cells gives rise to a protein that displays aberrant gel migration and forms atypical high molecular weight associations with PDI (6). The ability to form a C85–C391 disulfide during parts of the catalytic cycle may, therefore, be important to structural integrity of Ero1α.

Ero1α contains several other disulfides with undefined purpose. As illustrated in Figure 6, a cluster of four cysteine residues at the N-terminus of the protein are involved in a pair of disulfides. Each of C35 and C37 link one of C46 and C48, though the exact pairs could not be matched by MS (3). Whether these have any influence on catalysis, are structurally important, or serve some other function is unknown. No inspiration can be drawn from Ero1p, as its own N-terminal pair C40 and C52 is absent from the crystal structure (20). Another disulfide, C208–C241, covalently links two of the helical motifs predicted to encase the catalytic center of Ero1α, whereas the only seemingly unpaired cysteine within the protein, C166, is itself of dubious status. Disulfide mapping of Ero1α determined that C166 within the OX2 species could not be alkylated, indicative of the cysteine being either oxidized or solvent inaccessible. No partner could be allocated to the residue for disulfide formation, which leads to the conclusion that the residue is disulfide bonded or inaccessible to modification (3).

It is interesting to note that of the 15 cysteine residues within Ero1α, all but the first three (C35, C37, and C46) are strictly conserved in Ero1 orthologs throughout vertebrates, including Ero1β (Fig. 3). The likelihood that these residues have important roles, either structural or regulatory, is, therefore, fairly high.

### Low Reduction Potential of Ero1α Tightly Regulates Disulfide Transfer to PDI

Reduction of regulatory disulfides initiates oxidizing activity of both Ero1p and Ero1α in the ER. Current models predict the reductant to be PDI as a prelude to its oxidation by Ero1. In support of this view is the fact that the oxidation state of Ero1α in mammalian cells depends on the level of reduced PDI (3). However, turnover of PDI in both yeast and human systems *in vitro* is unimpressive with each Ero1 molecule introducing around three disulfides per minute (4, 46). This may be a consequence of the delicate redox poise that exists between PDI and Ero1. Provision of a powerful reducing agent such as thioredoxin greatly favors Ero1 catalytic activity, unbalancing the competition between substrate oxidation and regulatory disulfide formation within Ero1. As a more conservative activator, PDI may help prevent Ero1 hyperactivity by ensuring that oxidative transfer is constantly on the verge of shutdown and is, therefore, integral to the concept of Ero1 feedback control.

Several factors govern the efficiency with which PDI may activate Ero1, a component being the reduction potential of the Ero1 regulatory disulfides. This value provides a quantitative guide for the propensity of other redox couples to reduce the regulatory bonds. Lower values indicate a readiness to transit to an oxidized state (*i.e.,* disulfide), though this requires redox exchange with a partner of higher reduction potential (favoring a reduced state). To gauge the actual requirements for Ero1 activation, we determined the reduction potential of Ero1α regulatory disulfides (4). Using changes in electrophoretic mobility associated with their reduction, we found that both C94–C131 and C85–C391 are similarly susceptible to reductants. Mid-point reduction potential for these disulfides and, therefore, for activation of Ero1α was calculated as −275 mV. This is very low compared with values determined for the general ER environment and for PDI active sites (both around −180 mV) (26, 32). Although this disparity illustrates how the regulatory disulfides remain stable within the ER, it also suggests that their reduction by PDI is thermodynamically unfavorable, perhaps accounting for the slow kinetics of PDI oxidation by Ero1 *in vitro*.

The low reduction potential of Ero1α regulatory disulfides dictates that PDI must be both reduced and highly abundant

to activate Ero1 and initiate its own oxidation. Hence, catalysis *in vitro* demands inclusion of GSH to maintain a 50-fold molar excess of reduced PDI over Ero1 (4). GSH is neither an effective activator nor a substrate for oxidation by Ero1 but instead recycles PDI active sites back from disulfide to thiol status. In the absence of GSH, Ero1 activity shuts down after oxidation of just a fraction of available PDI. Intriguingly though, even in the presence of GSH, oxidative activity of Ero1 dies away at a point where regeneration of reduced PDI should not be a limiting factor. It is possible that this arises from accumulation of the other reaction products, hydrogen peroxide (from electron transfer to oxygen) and GSSG (from recycling of PDI).

Certain conditions *in vivo* may replicate the *in vitro* setup, as PDI is highly abundant in the human ER and exists in a predominantly reduced state (28). In contrast, up to 70% of PDI is oxidized in yeast (17). Reduction potential of the Ero1p C150–C295 disulfide has not been determined, but its stability in the ER again predicts that it will be lower than for PDI. It is, therefore, unclear as to how PDI can overcome the particular challenge of Ero1p activation. Individually determined reduction potentials are certainly not definitive in predicting redox exchange, and binding of PDI to Ero1 may itself alter their relative reduction potentials from those established using alternative redox couples. Alternatively, efficient catalysis of oxidative folding *in vivo* may involve accessory factors that smooth the transition of Ero1 from its resting state. Consistent with this, we have recently identified a number of PDI family members that can be trapped in mixed disulfides with Ero1$\alpha$ using a substrate-trapping approach (30). Formation of the mixed disulfide is a consequence of the PDI family member reducing a disulfide bond within Ero1. Although we have yet to confirm whether each of these interactions occurs *via* redox exchange with the shuttle disulfide or noncatalytic disulfides, we know that not all are predicted to be mediators of oxidative transfer from Ero1$\alpha$. It is, therefore, tempting to speculate that other thiol-dependent reductants within the ER can prime Ero1 for oxidation of PDI. A variety of activators differing in spatial distribution, temporal regulation, and inherent redox properties would provide an invaluable toolbox for fine tuning of Ero1 activity.

### Ero1$\beta$ Displays Hallmarks of Ero1$\alpha$ Deregulation

From a point of scientific interest, Ero1$\beta$ is the poor relation of the Ero1 family, conspicuous by the lack of knowledge relating to its function in the mammalian ER. Focus in humans generally favors Ero1$\alpha$ due to its widespread expression profile and apparent housekeeping function. Study of Ero1$\beta$ has meanwhile been hampered by the fact that expression is not only tissue specific but also cell-type specific within tissues (13). Moreover, distribution of Ero1$\beta$ within tissues erratically correlates with expression of predicted substrates such as PDI and its pancreatic sibling PDIp. Fortunately, recent data (3) have established some surprising features of Ero1$\beta$ behavior in human cells, explaining why Ero1$\beta$ may be more moderately expressed in the ER and how it may occupy a catalytic niche distinct from Ero1$\alpha$ even when present in the same space.

Unlike the OX1 and OX2 species of Ero1$\alpha$, Ero1$\beta$ exhibits a single oxidized species within the ER (13). This is surprising, given that it has the potential for similar disulfide patterning to Ero1$\alpha$ (Fig. 3) and suggests that equivalent regulatory disulfides are not formed in Ero1$\beta$. Consistent with this, Ero1$\beta$ overexpression in human cells elicits phenotypes characteristic of Ero1 hyperactivity; increased GSSG:GSH ratio; and altered redox poise of ER oxidoreductases (3). Ero1$\beta$, therefore, mirrors the behavior of the deregulated C131A mutant of Ero1$\alpha$, suggesting either that Ero1$\beta$ is not subject to control by regulatory disulfides or that Ero1$\beta$ regulatory disulfides only form under conditions established in the highly secretory cells in which it is expressed.

The idea that Ero1$\beta$ lacks regulatory disulfides seems counterintuitive, given the paradigms established in Ero1p and Ero1$\alpha$, and the extensive conservation of cysteine residues that exists between Ero1$\alpha$ and Ero1$\beta$. Moreover, Ero1$\beta$ contains an additional cysteine within one of its core helices (C262), providing further scope for unique disulfide bonding in this region. Another feature distinguishing between Ero1$\beta$ and Ero1$\alpha$ is the presence of extra motifs for addition of *N*-linked glycans. Ero1$\alpha$ possesses two glycosylation sites situated in the C-terminal half of its primary structure, whereas Ero1$\beta$ has four predicted glycans. Three of these sit in an area to the C-terminus of the shuttle disulfide, rich in noncatalytic cysteines whose equivalents are oxidized in Ero1$\alpha$. The capacity for glycosylation to interfere with disulfide formation in this region is unclear, but it is not a trivial point. A crucial factor in pioneering Ero1 research has been the ability to purify recombinant Ero1 isoforms from *E. coli*, yet protein generated in this way is not glycosylated as would be the case *in vivo*. This is all the more intriguing, given that the Ero1 family are relative anomalies in being both resident in the ER lumen and highly glycosylated.

An alternative to the prospect of Ero1$\beta$ lacking a regulatory mechanism is the idea that its disulfides are simply more sensitive to reduction and, therefore, do not form in the ER of HEK 293 cells. Such a property would render Ero1$\beta$ hyperactive under most circumstances, necessitating its expression be kept at a minimum. However, in pancreatic islets or enzyme-secreting chief cells, the environment could be more suited to disulfide formation by Ero1$\beta$ than Ero1$\alpha$. Highly secretory tissues and cells with a high ER client protein load are often subject to oxidizing conditions within the ER (22, 24, 33). This may be caused by peroxide generation during Ero1 activity, consumption of GSH, or ROS production by as-yet-undetermined processes resulting from UPR induction. In this relatively oxidizing milieu, the stability of Ero1$\alpha$ regulatory disulfides may stall its oxidizing activity, providing an obstacle to disulfide formation even in the presence of reduced PDI. In increasing the oxidative threshold at which it can efficiently operate, Ero1$\beta$ could effectively meet this challenge, replicating the catalytic control of Ero1$\alpha$ in a more oxidizing environment.

### Regulation of Ero1: Effects on ER Stress

Ero1 isoforms in yeast, and humans are upregulated in response to various cellular insults but also have the capacity to exacerbate conditions of cellular stress. Moreover, Ero1 hyperactivity is detrimental *via* three distinct yet overlapping pathways. In oxidizing PDI, a commensurate increase in GSSG:GSH is brought about, leading to thiol-disulfide imbalance within the ER. Simultaneously, reduction of molecular oxygen liberates hydrogen peroxide,

providing a source of ROS. ROS may amplify preexisting redox imbalance by necessitating the consumption of free thiols by peroxiredoxins and glutathione peroxidases. In addition, hydrogen peroxide elicits widespread effects as a second messenger in cell-signaling cascades (51). Finally, a link has been recently established between Ero1α and the release of calcium from the ER during the UPR (31). In the absence of Ero1α, IP3-induced calcium release is attenuated, which leads to a protection from apoptosis after the UPR. Hence, the relationship between Ero1 activity and ER stress is, therefore, complex, with potential for Ero1 upregulation to do harm as well as good.

The distinct ways in which *ERO1-Lα* and *ERO1-Lβ* are expressed during UPR may provide insight into their function during recovery from stress. Ero1β was initially characterized as inducible during treatments that specifically brought about a UPR (40). The wide range of treatments initiating *ERO1-Lβ* expression combined with the rapid time frame in which this happens indicate that Ero1β may be produced as part of the initial wave of responses initiated in human cells. One possible mechanism for this involves transcriptional regulation by XBP1, a transcription factor whose activation is at the forefront of the UPR. Interestingly, *ERO1* in *S. cerevisiae* is regulated by the yeast XBP1 ortholog Hac-1 as well as by a heat shock transcription factor Hsf-1 (47). These response pathways are consistent with important roles for oxidative activity of Ero1β and Ero1p in alleviating early effects of ER stress.

In contrast, Ero1α was not originally considered to be influenced by the UPR, as its induction was not seen within a similar time frame to Ero1β. However, experiments by Ron and coworkers showed that *ERO1-Lα* expression in mice was upregulated during a much later stage of tunicamycin-induced stress response (33). Transcriptional activation of *ERO1-Lα* is not an initial effect of UPR induction, as it requires consecutive inductions of several response regulators, thus leading to production of CHOP. As a transcriptional regulator downstream of the PERK, eIF2α and ATF4 cascade, CHOP is important in determining cell fate during prolonged stress induction. In addition to increasing Ero1α levels, CHOP also activates expression of *GADD34*, which reverses the protein synthesis block installed by eIF2α phosphorylation during early UPR (33). Resumption of translation does not necessarily signal a return to normality, as the combined elevation of Ero1α with an increasing ER client protein load can have lethal consequences. The effect is heightened in $Perk^{-/-}$ mice that lack eIF2α phosphorylation during UPR and, therefore, have a consistently high (and misfolded) ER client protein load (22). In each case, high levels of intracellular peroxides are observed, a phenomenon that can be remedied in *Caenorhabditis elegans* by knockdown of *ero-1* (22, 33).

Taken together, these findings have prompted the postulation that CHOP induces cell death by creating a lethal cocktail of Ero1 and abundant client protein for the oxidative protein folding pathway (33). As detailed throughout this review, a mix of Ero1, PDI, and client protein does not necessarily lead to Ero1 activity unless redox conditions in the environment are favorable. Intriguingly, single cell monitoring of yeast has recently revealed a remarkable shift to a more reducing ER thiol-disulfide balance in response to treatment with tunicamycin (35). Such conditions would be predicted to favor Ero1 activity and in keeping with this, a chronic accumulation of misfolded protein in the yeast ER eventually results in excess peroxide production and attenuated viability (24). Further, increased thiol content has been described as a feature of diabetic rat liver (38). A conceivable pathway, therefore, exists by which Ero1 hyperactivity could bring about death in mammalian cells by linking oxidative stress with ER calcium release (31). Whether this constitutes a directed mechanism for apoptosis initiation or is simply a consequence of chronic UPR remains uncertain.

It is interesting to note that the effects of Ero1 hyperactivity appear progressively more severe in lower eukaryotes. The lethality of Ero1 deregulation in yeast has been discussed earlier, whereas Ero1 activity in *C. elegans* significantly contributes toward toxic effects of chronic UPR (22, 33). Knockdown of *ero-1* expression consequently enhances lifespan during treatment with tunicamycin, a treatment that otherwise leads to peroxide accumulation and GSH depletion (22). Expression of hyperactive Ero1α or Ero1β in human cells is less detrimental, and extensive manipulation of the mammalian system is required to attenuate viability in a way that is attributable to enhanced Ero1 activity (33). This is perhaps a reflection of the mammalian ER being buffered more effectively against redox imbalances due to its diverse family of catalysts for thiol-disulfide exchange and a complement of ER localized peroxidases. Potential ER peroxidases have yet to be isolated in yeast or *C. elegans*, potentially a consequence of lowered demand for secretion of complex disulfide-linked proteins from highly active secretory tissues.

## Concluding Remarks

Since the discovery of the Ero1 family, we have become increasingly aware of the need for tight regulation of their catalytic activities in the ER. Just as compromised Ero1 activity can be lethal, hyperactivity can be equally deadly. Ero1 has, therefore, evolved built-in mechanisms that trigger oxidative activity in the presence of appropriate reduced substrate and shut down activity as the redox environment becomes more oxidizing. This negative feedback loop regulates Ero1, moderates PDI oxidation, and is a cornerstone for redox homeostasis in the ER.

Despite our understanding of the critical regulatory disulfides in Ero1 molecules, there is much to be determined regarding other factors influencing Ero1 activity. Of specific interest is the possibility of alternative proteins that can act as activators and substrates for Ero1 disulfide transfer other than PDI. Alongside this, characterization of mechanisms for removal of hydrogen peroxide in the ER will help us understand how cells buffer against peroxide stress during normal oxidative protein folding.

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**Abbreviations Used**

CHOP = C/EBP homologous protein
DTT = dithiothreitol
ER = endoplasmic reticulum
Ero1 = endoplasmic reticulum oxidoreductin 1
ETC = electron transport chain
FAD = flavin adenine dinucleotide
GSH = reduced glutathione
GSSG = oxidized glutathione
MS = mass spectrometry
PDI = protein disulfide isomerase
ROS = reactive oxygen species
UPR = unfolded protein response

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