

Balanced Ero1 activation and inactivation establishes ER redox homeostasis

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The endoplasmic reticulum (ER) provides an environment optimized for oxidative protein folding through the action of Ero1p, which generates disulfide bonds, and Pdi1p, which receives disulfide bonds from Ero1p and transfers them to substrate proteins. Feedback regulation of Ero1p through reduction and oxidation of regulatory bonds within Ero1p is essential for maintaining the proper redox balance in the ER. In this paper, we show that Pdi1p is the key regulator of Ero1p activity. Reduced Pdi1p resulted in the activation of Ero1p by direct reduction of Ero1p

regulatory bonds. Conversely, upon depletion of thiol substrates and accumulation of oxidized Pdi1p, Ero1p was inactivated by both autonomous oxidation and Pdi1p-mediated oxidation of Ero1p regulatory bonds. Pdi1p responded to the availability of free thiols and the relative levels of reduced and oxidized glutathione in the ER to control Ero1p activity and ensure that cells generate the minimum number of disulfide bonds needed for efficient oxidative protein folding.

Introduction

A key step in the maturation of many secreted proteins and extracellular domains of membrane proteins is the formation of disulfide bonds in the ER. Disulfide bonds, which stabilize native and functional conformations of proteins, are formed by pairing and oxidation of cysteines during the initial folding process in the ER. In *Saccharomyces cerevisiae*, most biosynthetic disulfide bonds are formed by dithiol/disulfide transfer reactions with the oxidized form of protein disulfide isomerase (PDI), Pdi1p (Sevier and Kaiser, 2002; Wilkinson and Gilbert, 2004). Pdi1p, in turn, requires other oxidizing molecules to be recycled because Pdi1p cannot generate disulfide bonds by itself. We and other colleagues identified the major disulfide-generating flavoenzyme Ero1p in *S. cerevisiae* (Frand and Kaiser, 1998; Pollard et al., 1998). In both yeast and mammalian cells, Ero1 directly transfers disulfide bonds to PDI (Frand and Kaiser, 2000; Mezghrani et al., 2001). The oxidative capacity of the yeast ER depends primarily on the activity of Ero1p: a temperature-sensitive *ero1-1* mutation decreases the resistance of yeast to the reducing agent DTT and induces the unfolded protein response with accumulation of secretory proteins, and, after prolonged incubation

at the restrictive temperature, a strain with this mutation loses viability (Frand and Kaiser, 1998, 1999; Cuozzo and Kaiser, 1999; Tu and Weissman, 2002), and overexpression of *ERO1* from a multicopy plasmid increases the oxidizing capacity of yeast cells, as shown by their increased resistance to DTT (Frand and Kaiser, 1998).

The catalytic cycle of Ero1p depends on a relay of disulfide bonds from the conserved active-site cysteine pair, C352-C355, which is proximal to bound flavin adenine dinucleotide (FAD) to the second shuttle cysteine pair, C100-C105, which is responsible for direct disulfide transfer to Pdi1p (Frand and Kaiser, 2000; Gross et al., 2004; Sevier and Kaiser, 2006b). In addition to these two catalytic cysteine pairs, Ero1p contains three cysteine pairs (C90-C349, C143-C166, and C150-C295) that form regulatory bonds (Sevier et al., 2007). Several lines of evidence indicate that Ero1p is inactive with regulatory bonds formed, whereas it is active with regulatory cysteines in the reduced state. Ero1p is converted from an oxidized (inactive) state to the reduced (active) state in the presence of reduced thioredoxin-1 (Trx1) as a substrate; once Trx1 is fully oxidized through the action of active Ero1p, Ero1p returns to the oxidized (inactive) form (Sevier et al., 2007). A double mutant, Ero1p-C150A-C295A, which cannot form the crucial C150-C295 regulatory bond,

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Abbreviations used in this paper: BSO, buthionine sulfoximine; CPY, carboxypeptidase Y; FAD, flavin adenine dinucleotide; MBP, maltose binding protein; PDI, protein disulfide isomerase; SMM, supplemented minimal medium; TEV, Tobacco etch virus.

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exhibits increased Ero1p activity in vitro and in vivo (Sevier et al., 2007).

A byproduct of Ero1p activity is the generation of hydrogen peroxide as a result of a two-electron reduction of oxygen per disulfide generated. Thus, although Ero1p activity is essential for biosynthetic disulfide bond formation, uncontrolled Ero1p activity could produce too much reactive oxygen species that would be detrimental to the cell (Gross et al., 2006). Indeed, overexpression of the hyperactive Ero1p-C150A-C295A mutant inhibits cell growth, highlighting the physiological importance of the regulatory bonds in keeping Ero1p activity in check. Mammalian Ero1- α has been shown to modulate its activity through a similar mechanism involving regulatory bonds, showing that autoregulation in response to the redox environment is a general property of Ero1 (Appenzeller-Herzog et al., 2008; Baker et al., 2008). An additional layer of protection against reactive oxygen accumulation in the ER of mammalian cells is provided by an ER resident, peroxiredoxin, which reduces hydrogen peroxide generated by Ero1 to limit peroxide accumulation (Tavender et al., 2008, 2010; Zito et al., 2010). An obvious sequence homolog to peroxiredoxin is absent in yeast; whether additional pathways functionally similar to the peroxiredoxin system exist in the ER of fungi remains to be explored.

The precise mechanism by which the regulatory bonds of Ero1 are reduced and then reoxidized will be crucial to understand how Ero1p activity is controlled and which features of the redox environment of the ER are sensed by Ero1p. Pdi1p is the most abundant oxidoreductase of the yeast ER and is the preferred physiological substrate for oxidation by Ero1p (Nørgaard et al., 2001; Vitu et al., 2010); therefore, Pdi1p is likely to play an important part in regulating Ero1p. In the mammalian ER, the disulfide relay between Ero1- α and PDI is regulated at least in part by the availability of PDI (Appenzeller-Herzog et al., 2010; Inaba et al., 2010). Interaction between Ero1- α and PDI has been shown to be facilitated by a protruding β -hairpin in Ero1- α and the b' domain of PDI, which facilitates the interconversion between the Ox1 and Ox2 redox-active forms of Ero1- α (Masui et al., 2011). Differences between Ero1- α and Ero1p, including the lack of a β -hairpin in Ero1p and the preference of Ero1p for the a domain of Pdi1p (versus the preference of Ero1- α for the a' domain), suggest that the yeast and mammalian Ero1-PDI systems may operate differently.

In vitro assays showing activation of Ero1p have typically used the nonphysiological substrate Trx1, which has a single CxxC-containing domain with a highly reducing reduction potential rather than the physiological substrate Pdi1p because reduced Trx1 is more effective than Pdi1p at activating Ero1p by reduction of regulatory bonds (Sevier et al., 2007; Baker et al., 2008; Vitu et al., 2010). Here, we examine the role of Pdi1p in the reduction and reoxidation of the Ero1p regulatory bonds to reveal how the activity of Ero1p is set in a living cell. We have also sought to understand the role of glutathione in this process as the most abundant redox buffer of the ER. Although glutathione itself does not activate Ero1p nor is glutathione efficiently used as a substrate for Ero1p (Tu et al., 2000; Sevier and Kaiser, 2006b), reduced glutathione can stimulate Ero1p activity when used in conjunction with Pdi1p as a substrate (Sevier et al., 2007; Baker

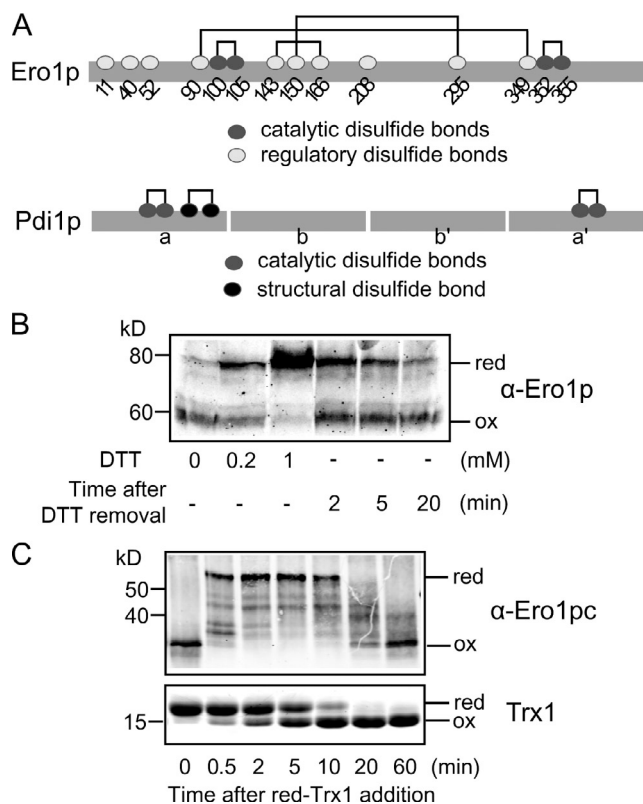


Figure 1. Ero1p regulation by reduction or oxidation of its regulatory bonds. (A) Disulfide connectivity of Ero1p and Pdi1p. In Ero1p, dark gray and light gray circles indicate catalytic and regulatory cysteines, respectively. Pdi1p has four thioredoxin domains, a, b, b', and a'. In Pdi1p, dark gray and black circles indicate catalytic and structural cysteines, respectively. (B) Ero1p cycles between oxidized (ox) and reduced (red) regulatory states in vivo according to the redox environment. Cells were incubated with 0, 0.2, or 1 mM DTT for 30 min. DTT was removed by placing cells in fresh medium. (C) Ero1p regulation in vitro. 1 μ M Ero1pc was incubated with 50 μ M reduced Trx1.

et al., 2008). We have reconstituted this coupled reaction in vitro and show that it recapitulates properties of the ER in vivo; as long as an excess of reduced glutathione is available, Pdi1p is maintained in a state enabled to activate Ero1p, which in turn provides oxidizing equivalents to oxidize glutathione.

Results

Ero1p is capable of autonomous inactivation

Because the regulatory bonds link distant sites of the Ero1p polypeptide chain, the presence or absence of these bonds can be readily discerned by mobility differences on nonreducing SDS-PAGE (Fig. 1 A). Activity of Ero1p can be inferred from the redox state of the regulatory bonds. In live yeast cells, Ero1p is converted to the reduced (active) form in cells treated with the reducing agent DTT and then returns to the oxidized (inactive) form after DTT removal (Fig. 1 B). Activation of Ero1p by reduction of the regulatory bonds and subsequent inactivation by reformation of the regulatory bonds could also be readily observed in an in vitro assay containing recombinant Ero1p (Ero1pc, 56–424, with an N-terminal (1–55) and a C-terminal (425–563) fragment removed) and an excess of reduced Trx1,

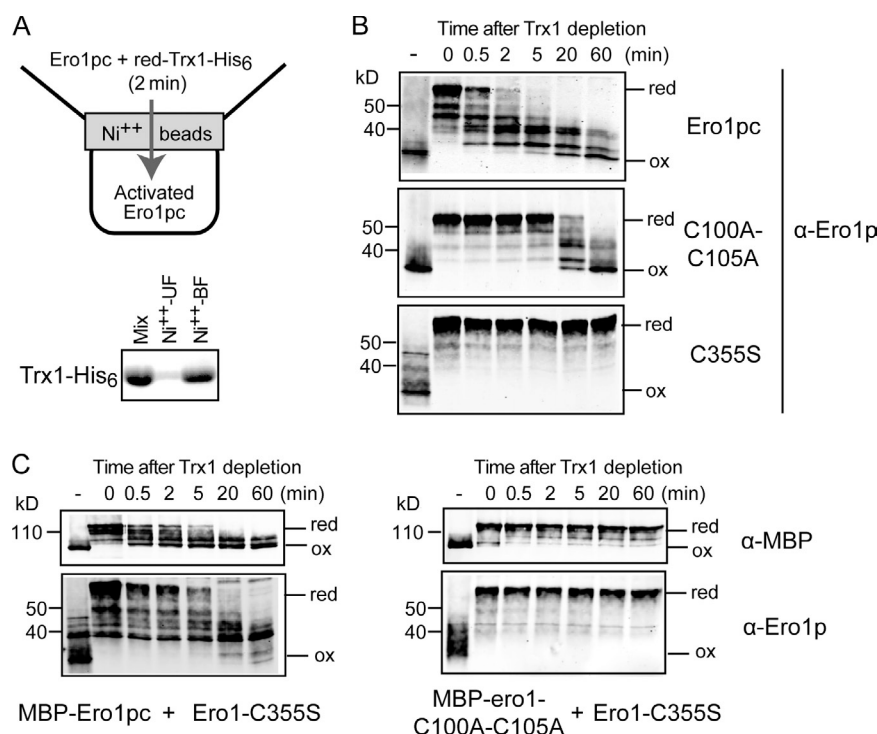


Figure 2. Pathways for Ero1p inactivation. (A) To test the ability of Ero1p to oxidize itself autonomously, 1 μ M reduced (red) Ero1pc was prepared by incubation with 100 μ M reduced His₆-tagged Trx1 for 2 min and then passage through Ni²⁺ beads to remove Trx1. Trx1 depletion was confirmed by SDS-PAGE (Mix; initial mixture: Ni²⁺-BF [beads bound fraction] and Ni²⁺-UF [unbound fraction]). (B) Autonomous reoxidation (ox) of Ero1pc after removal of Trx1 occurs rapidly and efficiently but depends on a functional active site (C355), and efficient reoxidation depends on the shuttle disulfide (C100-C105). The first lane in each time course (–) shows the oxidation state of Ero1pc before reduction by Trx1. (C) Ero1pc, expressed as an MBP fusion (MBP-Ero1pc), can reoxidize Ero1pc-C355S in trans. An equal mixture of MBP-Ero1pc (or MBP-ero1-C100A-C105A; right) and Ero1pc-C355S was reduced by incubation with reduced Trx1 followed by Trx1 depletion. The time course of reoxidation of MBP-Ero1pc and Ero1pc-C355S was followed independently by separating MBP-Ero1pc and Ero1pc-C355S by size in SDS-PAGE and immunoblotting for MBP or Ero1.

serving as an effective substrate for Ero1p (Fig. 1 C). First, Ero1pc is inactive with the regulatory bonds in a fully oxidized state. In ~ 0.5 min after addition of reduced Trx1, the regulatory bonds of Ero1pc become reduced, and Ero1pc is thus activated for the oxidation of Trx1. As a consequence of Ero1pc, after ~ 60 min, all of the Trx1 is oxidized, and Ero1pc returns to the oxidized inactive form (Fig. 1 C).

Activation of Ero1pc by reduction of the regulatory bonds initiates on addition of reduced Trx1, indicating that the regulatory bonds can be reduced by direct dithiol/disulfide exchange with reduced Trx1. For the end phase of the reaction in which most of the Trx1 is oxidized and the regulatory bonds of Ero1pc become reoxidized, we wished to know whether reoxidation of the regulatory bonds occurred by direct oxidation by oxidized Trx1 or instead by autonomous reoxidation by Ero1p when all of the reduced Trx1 had been consumed. To distinguish between these possibilities, we first tested the ability of active Ero1pc to oxidize regulatory cysteines in the absence of Trx1. For this experiment, 1 μ M Ero1pc was incubated with 100 μ M His₆-tagged reduced Trx1 for 2 min, a time sufficient for complete activation of Ero1pc by reduction of the regulatory bonds. Subsequently, His₆-tagged Trx1 was removed by affinity to Ni²⁺ beads (Fig. 2 A). Trx1 depletion was confirmed by the significant absence of Trx1 in the Ni²⁺ unbound fraction and corresponding presence of Trx1 bound to the Ni²⁺ beads (unbound fraction; Fig. 2 A, bottom). A small amount of residual Trx1 was observed in the unbound fraction (Fig. 2 A, bottom), but it was anticipated that this limited fraction of Trx1 would be rapidly oxidized by Ero1pc and not contribute to our Ero1pc inactivation assay. After depletion of reduced Trx1, Ero1pc was converted from a fully reduced to an oxidized form over a period of 60 min with a half time for completion of ~ 10 min (Fig. 2 B, top). The bands migrating between the fully reduced

and oxidized forms likely correspond to partially oxidized states of Ero1pc. Parallel reactions using Ero1pc mutants lacking the shuttle disulfide bond (Ero1pc-C100A-C105A) or the catalytic disulfide bond (Ero1pc-C355S) in the absence of Trx1 exhibited greatly slowed or no autonomous reoxidation of Ero1pc, respectively (Fig. 2 B, middle and bottom). In addition, when the reoxidation reaction was performed in the absence of O₂ as an electron acceptor (Fig. S1 A, right), Ero1pc exhibited slowed autonomous reoxidation. These observations show that Ero1pc is capable of autonomous oxidation to form regulatory bonds and that this oxidation has catalytic requirements similar to biosynthetic disulfide bond formation.

We considered the possibility that autonomous oxidation of the Ero1p regulatory bonds was promoted by the hydrogen peroxide generated by Ero1pc before removal of Trx1. Therefore, we examined the effects of hydrogen peroxide. Addition of catalase to the reaction, which should convert any hydrogen peroxide into water and oxygen (Fig. S1 B), did not significantly slow autonomous oxidation of the Ero1p regulatory bonds (Fig. S1 A, middle). Conversely, addition of 100 μ M hydrogen peroxide, the maximum concentration that could be obtained from complete oxidation of 100 μ M Trx1, did not enhance the oxidation of Ero1pc-C100A-C105A (Fig. S1 C).

Autonomous oxidation of Ero1p occurs via an intermolecular reaction

Autonomous oxidation of Ero1p could conceivably occur by intramolecular (in cis) or intermolecular (in trans) disulfide bond transfer. In the crystal structure of Ero1pc (Gross et al., 2004), one of the regulatory bonds, C90-C349, is close enough to the catalytic site to be oxidized in cis, whereas the other two regulatory bonds, C150-C295 and C143-C166, are located >20 Å away from the catalytic site, suggesting that the oxidation of these

bonds would require involvement of additional dithiol/disulfide exchanges with other cysteines. To determine whether one Ero1p protein can oxidize the regulatory bonds of another Ero1p, we asked whether Ero1pc-C355S, which is catalytically inactive and is not capable of autonomous oxidation, could nevertheless be oxidized in trans by active Ero1pc. A maltose binding protein (MBP) fusion to Ero1pc was used in this experiment so that MBP-Ero1pc and Ero1pc-C355S could be distinguished by their distinct mobilities on SDS-PAGE. An autonomous oxidation assay was established by combining MBP-Ero1pc and Ero1pc-C355S with reduced Trx1 to reduce the regulatory bonds on both forms of Ero1p. After Trx1 was removed by Ni^{2+} affinity, both Ero1pc-C355S and MBP-Ero1pc were reoxidized at nearly equal rates, showing that efficient trans oxidation can occur (Fig. 2 C). Consistent with the oxidation of Ero1pc-C355S being a product of MBP-Ero1pc catalytic function, disruption of the MBP-Ero1pc catalytic cycle by mutating the C100-C105 pair (MBP-ero1-C100A-C105A) prevented Ero1pc-C355S oxidation (Fig. 2 C). We also tested the effect of dilution of Ero1pc in our autonomous oxidation assay and found that a fourfold dilution of Ero1pc slowed autonomous oxidation of Ero1pc (unpublished data). Together, the trans oxidation experiment and the dilution experiment show that autonomous oxidation of the Ero1p regulatory disulfides occurs predominantly, if not exclusively, by one Ero1p protein oxidizing the regulatory bonds of another Ero1p. The simplest mechanism for such dithiol/disulfide exchange would be for successive direct transfer of the C100-C105 shuttle cysteine disulfide bond to each of the three regulatory cysteine pairs. However, we cannot rule out the possibility that more complicated dithiol/disulfide rearrangements occur. Slow oxidation of Ero1pc facilitated by the Ero1pc-C100A-C105A (but not the Ero1pc-C355S) mutant could be observed also at late time points of the autonomous oxidation assay (>20 min), suggesting that the C352-C355 active site may contribute directly to oxidation of Ero1pc.

Ero1p is activated slowly but inactivated rapidly by Pdi1p

As we have shown, reduced Trx1 is a potent activator of Ero1p, and the use of Trx1 as an activator and substrate for Ero1p in *in vitro* assays made it possible to document the dramatic effect that reduction and oxidation of regulatory cysteines have on Ero1p activity (Fig. 1 C; Sevier et al., 2007). However, Pdi1p is the major physiological substrate of Ero1p (Frand and Kaiser, 1999), and we were interested to use the *in vitro* assay for Ero1p regulation to make a comparable assessment of the ability of Pdi1p to either activate or inactivate Ero1p activity through reduction or oxidation of regulatory bonds.

First, we tested the ability of oxidized Pdi1p to inactivate Ero1p. We prepared activated Ero1pc-C100A-C105A by treatment with reduced Trx1 followed by removal of Trx1. The use of the Ero1pc-C100A-C105A mutant prevented efficient autonomous reoxidation of Ero1p, allowing us to test the ability of exogenous agents to reoxidize Ero1p. Oxidized Pdi1p and Trx1 were prepared by air oxidation during purification from *Escherichia coli*. The endpoint of air oxidation yielded Pdi1p that had the a domain almost fully oxidized and the a' domain ~50%

oxidized (Fig. S2) and Trx1 that was almost fully in the oxidized form (not depicted). We found that addition of a 20-fold molar excess of oxidized Trx1 had no effect on the rate of reoxidation of Ero1pc-C100A-C105A (Fig. 3 A, top), showing that oxidized Trx1 has little or no capability to oxidize the regulatory bonds of Ero1p. In contrast, oxidized Pdi1p dramatically increased the rate of Ero1pc-C100A-C105A reoxidation (Fig. 3 A, top right). The ability of oxidized Pdi1p to efficiently oxidize the regulatory bonds of Ero1p was not a peculiarity of the Ero1pc-C100A-C105A mutant, as we could also show that oxidized Pdi1p accelerated the oxidation of wild-type Ero1pc as well as Ero1pc-C355S, which is unable to oxidize its regulatory bonds (Fig. S3). These results show that Ero1p can be inactivated by direct feedback from Pdi1p.

Next, we tested the converse process, the ability of reduced Pdi1p to reduce and thus activate Ero1p. To assess the activation process selectively, we again used Ero1pc-C100A-C105A because of this mutant's greatly diminished capacity for autonomous oxidation of regulatory cysteines once reduced with an exogenous agent (Fig. 2 B, middle). Reduced Pdi1p requires 60 min to reduce the Ero1p regulatory bonds completely, whereas reduced Trx1 requires <5 min, indicating that Pdi1p is a relatively poor activator of Ero1p (Fig. 3 B, top). In summary, Trx1 is a potent activator but poor inactivator of Ero1p, whereas Pdi1p is a weak activator but potent inhibitor of Ero1p. These contrasting abilities to reduce or oxidize the regulatory bonds of Ero1p can be accounted for by the much greater reducing potential of Trx1 as compared with Pdi1p. By assaying the rate of activation or inactivation of Ero1p in separate reactions, we could show that reduced Pdi1p activates Ero1p more slowly than oxidized Pdi1p can inactivate Ero1p. This explains why in an *in vitro* Ero1p oxidation reaction that contains reduced Pdi1p as a substrate, Ero1p rarely, if ever, becomes fully activated, and the reaction proceeds relatively slowly.

The a and a' domains of Pdi1p differ in their ability to regulate Ero1p

Yeast Pdi1p has two redox-active thioredoxin-like domains with different reduction potentials: the a domain (−195 mV) and a' domain (−164 mV; Fig. 1 A; Tian et al., 2006; Vitu et al., 2010). We recently showed that Ero1p preferentially oxidizes the a domain both *in vivo* and *in vitro*, suggesting Ero1p provides oxidizing equivalents to the ER mainly via the a domain (Vitu et al., 2010). Thus, the a' domain may play a lesser role in the oxidation of ER substrates despite its higher reduction potential than the a domain. To fully understand the interactions between Pdi1p and Ero1p, we wished to evaluate the contribution of each of the active Pdi1p domains to reduction and oxidation of Ero1p regulatory bonds.

Recombinant Pdi1p purified from *E. coli* had a fully oxidized a domain and a partially oxidized a' domain (Fig. S2), consistent with the different reduction potentials of the two domains. Moreover, naturally oxidized purified domain mutants Pdi1p-CCSS (a domain cysteines intact) and Pdi1p-SSCC (a' domain cysteines intact) had similar oxidation states to the corresponding domains in wild-type Pdi1p (unpublished data). The same assay for Pdi1p oxidation of the Ero1p regulatory bonds was performed

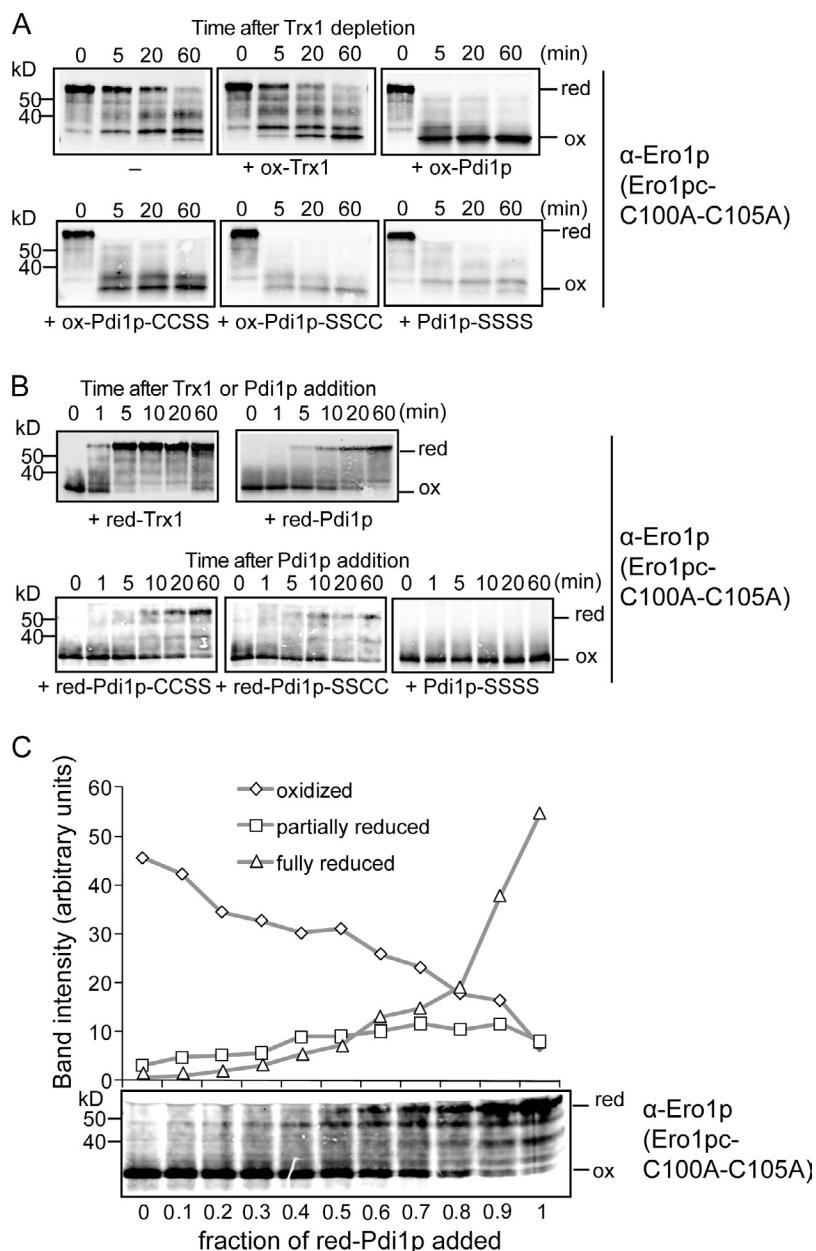


Figure 3. Regulation of Ero1p by Pdi1p. (A) Reoxidation (ox) of Ero1pc-C100A-C105A, which is impaired for autonomous oxidation, can be stimulated by oxidized Pdi1p but not by oxidized Trx1. Ero1pc-C100A-C105A was reduced (red) by incubation with reduced Trx1 followed by Trx1 depletion. Reoxidation of Ero1pc-C100A-C105A was followed after addition of a 20-fold molar excess of either oxidized Trx1 (ox-Trx1), oxidized Pdi1p (ox-PDI), or oxidized Pdi1p variants (α' mutant-ox-CCSS, a mutant-ox-SSCC, and both the α and α' mutant-SSSS). (B) Reduced Pdi1p is capable of reducing Ero1pc-C100A-C105A albeit at a slower rate than reduction by reduced Trx1. Ero1pc-C100A-C105A was incubated with a 20-fold excess of reduced Trx1 (red-Trx1), reduced Pdi1p (red-Pdi1p), or reduced Pdi1p variants (the red-Pdi1p panel is presented again in Fig. 4 C). (C) Oxidized Ero1pc-C100A-C105A was mixed with different admixtures of reduced and oxidized Pdi1p for 1 h (the time required for 100% reduced Pdi1p to reduce Ero1pc-C100A-C105A). The relative amounts of reduced, partially reduced, and oxidized Ero1pc-C100A-C105A were quantified by band intensity.

with Pdi1p-CCSS or Pdi1p-SSCC mutants. Both Pdi1p mutants accelerated oxidation of the Ero1p regulatory bonds. Despite the less-active disulfides, Pdi1p-SSCC reproducibly accelerated Ero1p oxidation more rapidly than Pdi1p-CCSS (Fig. 3 A), consistent with the higher reduction potential of the α' CxxC. A Pdi1p-SSSS that lacked both redox-active sites did not accelerate oxidation of Ero1p regulatory bonds to the extent of the other Pdi1p variants, but a modest acceleration of Ero1p oxidation was observed (Fig. 3 A, bottom right). It is possible that the structural (CX6C) disulfide in Pdi1p (Fig. 1 A) has some effect or that Pdi1p may stabilize the oxidized form of Ero1p through a mechanism not involving disulfide exchange with the redox-active motifs.

Next, we compared the ability of reduced Pdi1p-CCSS or Pdi1p-SSCC to reduce the regulatory bonds of Ero1pc-C100A-C105A. Although reduction potentials measured against a glutathione standard suggest that the α domain is a better reductant,

both reduced Pdi1p-CCSS and reduced Pdi1p-SSCC reduced Ero1p regulatory bonds at similar rates (Fig. 3 B, bottom). As expected, the single-domain mutants reduced the Ero1p regulatory bonds somewhat less efficiently than did wild-type Pdi1p, which has both active sites intact, and the double domain mutant Pdi1p-SSSS did not reduce the Ero1p regulatory bonds (Fig. 3 B, bottom right).

The redox state of Ero1p can be set by a mix of reduced and oxidized Pdi1p

Reduced and oxidized Pdi1p promote activation and inactivation of Ero1p, respectively, suggesting that the activity of Ero1p in steady state may be set by the ratio of reduced to oxidized Pdi1p. By taking advantage of Ero1pc-C100A-C105A, which lacks the ability to oxidize Pdi1p efficiently, we could test admixtures of oxidized and reduced Pdi1p for their effect on the steady-state redox balance of the regulatory bonds of Ero1pc-C100A-C105A.

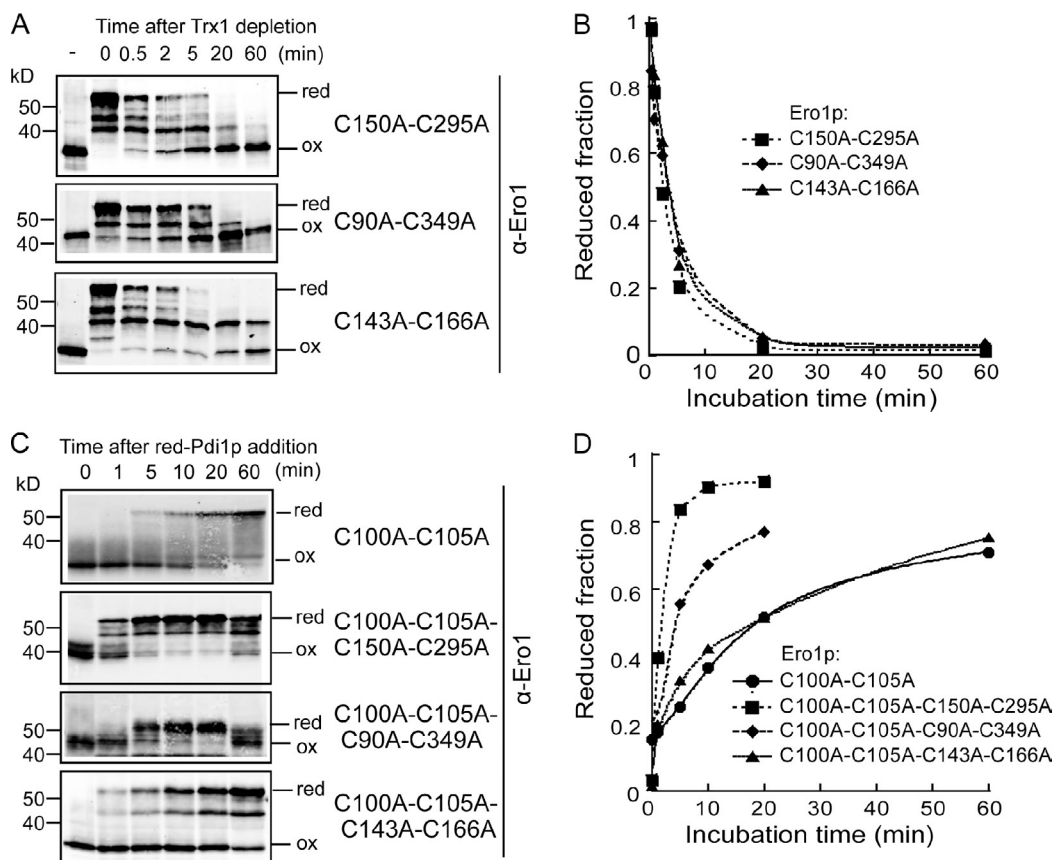


Figure 4. Mutational analysis of the roles of individual regulatory bonds in autonomous reoxidation of Ero1p. (A) Ero1pc-C150A-C295A, Ero1pc-C90A-C349A, or Ero1pc-C143A-C166A was reduced (red) by incubation with reduced Trx1 followed by Trx1 depletion and the oxidation (ox) state analysis. (B) The fraction of reduced Ero1p was calculated by the ratio of the band intensity of reduced Ero1p species to the sum of fully reduced and fully oxidized band intensities. (C) Mutational analysis of the roles of individual regulatory bonds in reduction by reduced Pdi1p of Ero1pc-C100A-C105A. The respective mutants were incubated with a 20-fold excess of reduced Pdi1p followed by the oxidation state analysis (the result of Ero1pc-C100A-C105A was reproduced from Fig. 3 B). (D) The fraction of reduced Ero1p determined as in B reveals that the greatest rate enhancement occurs as a consequence of elimination by mutation of the C150-C295 disulfide bond.

Although accurate quantitative amounts cannot be obtained as a result of the complications that oxidized Pdi1p contains a partially reduced domain and also nonlinear detection by immunoblot analysis, we found that when the fraction of reduced Pdi1p was >0.8 , most Ero1pc-C100A-C105A was fully reduced, whereas when the fraction of reduced Pdi1p was <0.3 , most Ero1pc-C100A-C105A was oxidized (Fig. 3 C). In a steady state, *in vivo* Pdi1p is partially reduced (Vitu et al., 2010) like air-oxidized recombinant Pdi1p, and almost all Ero1p is in a fully oxidized form (see 0 time point of Figs. 1 B and 5 E). Therefore, we could conclude that partially reduced Ero1p is at least partially active and will continue to oxidize Pdi1p until a ratio of oxidized-to-reduced Pdi1p is achieved that no longer facilitates reduction and activation of Ero1p.

An Ero1p regulatory mutant is rapidly activated by Pdi1p

Previously, we showed that an Ero1p mutant that lacks only one of the three regulatory bonds, Ero1p(c)-C150A-C295A, has high enzymatic activity both *in vivo* and *in vitro* (Sevier et al., 2007; Vitu et al., 2010). This suggests that in the absence of the C150-C295 regulatory bond, Ero1p is either more readily activated by reduced Pdi1p or less easily inactivated by reaction with oxidized Pdi1p or by autonomous oxidation.

First, the autonomous oxidation of Ero1p regulatory bond mutants Ero1pc-C150A-C295A, Ero1pc-C90A-C349A, and Ero1pc-C143A-C166A was evaluated for their ability to undergo autonomous reoxidation after being reduced by Trx1. This comparison is complicated by differing band mobility for the oxidized and partially oxidized states of each of the mutants (Fig. 4 A). As a measure of the oxidation rate that could be consistently applied to each of the mutants, we represented the progression of the oxidation process as the decline in the fraction of Ero1p that was in a fully reduced state. All regulatory mutants showed similar overall autonomous oxidation rates (Fig. 4 B), indicating that formation of the C150-C295 bond does not significantly affect oxidation of other regulatory bonds. Because the pattern of partially oxidized states was more complex for wild type (Fig. 2 B) than for the mutants, it was not possible to make a precise quantitative comparison of rates.

Next, we tested each of the three regulatory bond mutants for their ability to be activated by reduced Pdi1p. This test required addition of the shuttle cysteine mutant to each of the three regulatory bond mutants. Reduction of Ero1pc-C100A-C105A-C150A-C295A by reduced Pdi1p was significantly faster than for Ero1pc-C100A-C105A or for the other regulatory bond mutants (Fig. 4, C and D). Ero1pc-C100A-C105A-C90A-C349A also

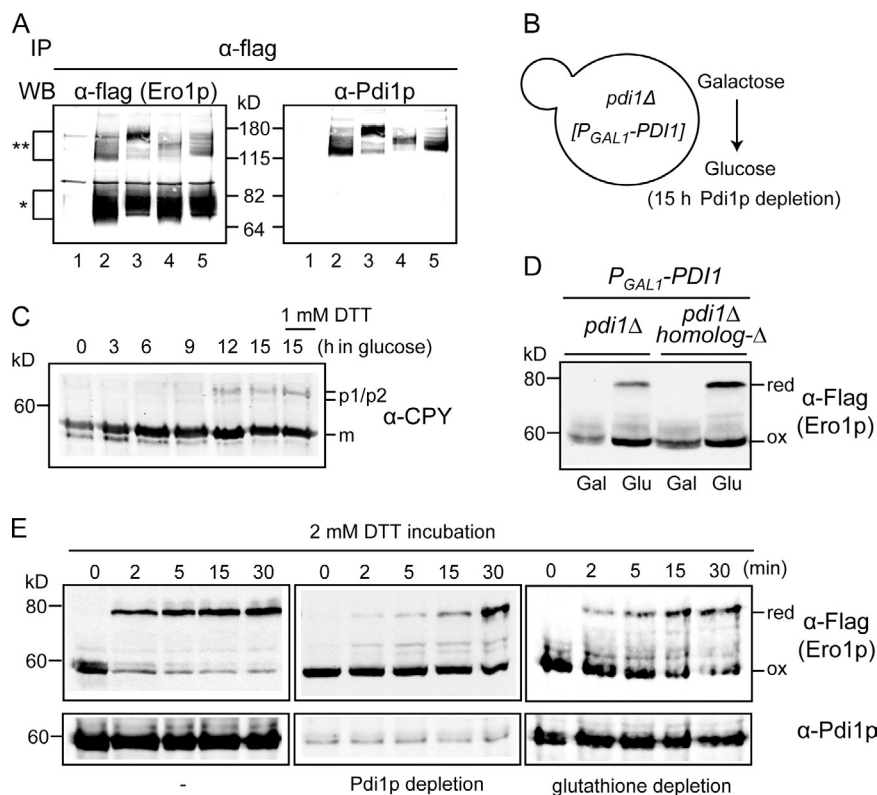


Figure 5. Pdi1p-mediated Ero1 regulation in vivo. (A) Pdi1p forms mixed disulfide with the Ero1p regulatory cysteines. Ero1p-C100A-C105A-Flag and its variants were immunoprecipitated (IP) by anti-flag antibody and analyzed by immunoblots against anti-flag antibody and anti-Pdi1p serum. Lane 1, Ero1p-C100A-C105A-Myc; lane 2, Ero1p-C100A-C105A-flag; lane 3, Ero1p-C100A-C105A-C90A-C349A-flag; lane 4, Ero1p-C100A-C105A-C143A-C166A-flag; lane 5, Ero1p-C100A-C105A-C150A-C295A-flag. The single asterisk indicates a monomer of Ero1p mutants, and the double asterisks indicate mixed disulfides between Ero1p mutants and Pdi1p. WB, Western blot. (B) Pdi1p was depleted by growing *pdi1Δ* covered with *P_{GAL1}-PDI1* in SMM (glucose medium) for 15 h. (C) Immature CPY accumulates as Pdi1p is depleted. A control for fully reduced environment was prepared by treating cells with 1 mM DTT for 1 h. m, mature CPY; p1/p2, nascent and glycosylated CPY, respectively. (D) Oxidation (ox) states of Ero1p after Pdi1p depletion (glucose medium) in a genetic background with *pdi1Δ* or with *pdi1Δ* with deletions of all PDI homologs (PDI homolog-Δ). red, reduced. (E) The roles of Pdi1p or glutathione on in vivo activation of Ero1p. Pdi1p-depleted cells (middle) and glutathione-depleted cells (right) were prepared by growing CKY1081 in glucose medium and in galactose medium with 5 mM BSO, respectively. Cells were treated with 2 mM DTT for the indicated time.

exhibited slightly faster reduction than Ero1p-C100A-C105A or Ero1p-C100A-C105A-C143A-C166A (Fig. 4, C and D). These results suggest that removal of the C150-C295 regulatory bond lowers the threshold for Ero1p activation. Interestingly, both the Ero1p-C100A-C105A-C150A-C295A and Ero1p-C100A-C105A-C90A-C349A mutants exhibited some reoxidation of Ero1p (Fig. 4 C) and oxidation of Pdi1p (not depicted) at the 60-min time point, suggesting that Ero1p may have alternative pathways to relay disulfide bonds to Pdi1p when the shuttle disulfide is unavailable (see Discussion).

Pdi1p is the primary in vivo regulator of Ero1p

If Pdi1p regulates Ero1p through reduction and oxidation of its regulatory bonds, it should be possible to trap mixed disulfide complexes between Pdi1p and the regulatory cysteines of Ero1p in vivo. To specifically isolate mixed disulfides with regulatory cysteines, we used FLAG epitope-tagged Ero1p-C100A-C105A (expressed ectopically in cells that carry wild-type *ERO1*). Ero1p-C100A-C105A is unable to support yeast viability as a result of the disruption of its ability to oxidize Pdi1p in what has been shown to be the primary pathway for electron exchange between Pdi1p and Ero1p in living cells (Frand and Kaiser, 2000). Therefore, any observable mixed disulfides trapped between Ero1p-C100A-C105A and Pdi1p in living cells would likely represent thiol/disulfide exchange between the Ero1p regulatory cysteines and Pdi1p.

Ero1p-C100A-C105A-FLAG, isolated from cysteine-alkylated cell extracts by immunoprecipitation with FLAG antibody, formed a predominant mixed disulfide complex that also reacted with Pdi1p antiserum (Fig. 5 A, lane 2). Analysis of

the crude immunoprecipitate by mass spectroscopy identified Pdi1p peptides but failed to identify additional PDI family members, including Eps1p, Eug1p, Mpd1p, or Mpd2p (unpublished data). This result suggests that Pdi1p is the primary oxidoreductase engaged in oxidation or reduction of the Ero1p regulatory bonds. Loss of any single regulatory cysteine pair in Ero1p-C100A-C105A did not prevent trapping of a mixed disulfide bond between Ero1p-C100A-C105A and Pdi1p, indicating that Pdi1p interaction with Ero1p-C100A-C105A is not limited to a single regulatory cysteine pair (Fig. 5 A). The mixed disulfides between Pdi1p and the various Ero1p-C100A-C105A regulatory bond mutants showed distinct mobilities on SDS-PAGE (Fig. 5 A), which likely corresponded to different variable combinations of cysteines in Ero1p and Pdi1p that engaged in oxidation and reduction of Ero1p regulatory bonds.

If reduced Pdi1p were essential for activating Ero1p, depletion of Pdi1p from the ER would be expected to decrease activation of Ero1p by reduction of regulatory bonds even under prevailing reducing conditions in the ER. We have established conditions to deplete a cell of Pdi1p, which is essential for viability, by glucose repression for 15 h of *P_{GAL1}-PDI1* in a *pdi1Δ* genetic background (Fig. 5 B; Tachibana and Stevens, 1992; Frand and Kaiser, 1999). As expected, Pdi1p depletion disrupts folding in the ER, as shown by the accumulation of the ER form of carboxypeptidase Y (CPY), similar to that in cells that had been treated with DTT (Fig. 5 C). Radiolabeling of cells to specifically follow CPY synthesized after Pdi1p depletion confirmed a complete block of biosynthetic disulfide bond formation in newly synthesized CPY (Fig. S4). However, under the same conditions of Pdi1p depletion, most Ero1p remains oxidized (Fig. 5 D), indicating that the signal for an insufficiently oxidized ER

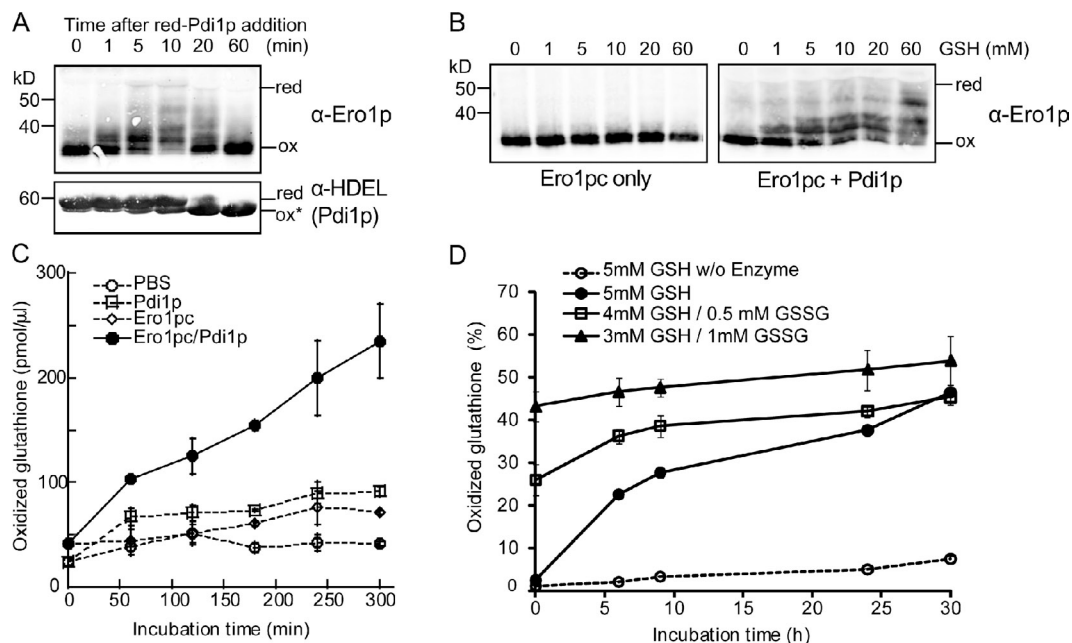


Figure 6. A coupled Pdi1p/GSH system recapitulates Ero1p homeostasis in vitro. (A) Ero1p is only partially activated when Pdi1p is used as a substrate. 1 μ M Ero1pc was incubated with 25 μ M reduced (red) Pdi1p. Ero1pc and Pdi1p were detected by immunoblotting with anti-Ero1p serum or anti-HDEL antibody. At the endpoint of in vitro oxidation (ox), Pdi1p is in a partially oxidized state, indicated by ox*. (B) Pdi1p is necessary for efficient reduction of Ero1pc by GSH. Ero1pc or a mixture of Ero1pc and oxidized Pdi1p was mixed with different concentrations of GSH for 1 h followed by TCA precipitation, NEM modification, and Western analysis. (C) Pdi1p is necessary for efficient oxidation of GSH by Ero1pc. 10 mM GSH was incubated with 1 μ M Ero1pc in the presence or absence of 10 μ M oxidized Pdi1p. The amount of GSSG produced was measured after alkylating GSH followed by reduction with glutathione reductase and assay of free thiols with Ellman's reagent. (D) The balance of GSH and GSSG can be established by the mixture of Ero1pc and Pdi1p. 10 μ M Pdi1p and 5 μ M Ero1pc were incubated with three different glutathione mixtures, 5 mM GSH, 4 mM GSH/0.5 mM GSSG, and 3 mM GSH/1 mM GSSG. The natural oxidation of 5 mM GSH without enzymes was also measured. The percentage of oxidized glutathione was calculated for the indicated time points. (C and D) Error bars represent the SEM generated by three independent experiments.

cannot be transduced to activate Ero1p in the absence of Pdi1p. Pdi1p-depleted cells showed very slow activation of Ero1p even when they were treated with the reducing agent DTT, a condition that leads to rapid activation of Ero1p in Pdi1p-expressing cells (Fig. 5 E).

In the Pdi1p-depleted cells, a small amount of activated (reduced) Ero1p was detected (Fig. 5 D), and catalytically inactive Ero1 mutants Ero1p-C355S and Ero1p-C100A-C105A exhibited significantly more of the reduced forms (Fig. S5), suggesting that under conditions of Pdi1p depletion, although Ero1p can be reduced to some extent, most of the Ero1p remains in an oxidized state if autonomous reoxidation is possible. We tested the possibility that the residual reduction of Ero1p in cells that had been depleted for Pdi1p was catalyzed by another PDI homolog. By conducting the depletion experiment in a strain depleted for the other homologs (*mpd1 Δ* , *mpd2 Δ* , *eug1 Δ* , *eps1 Δ* , *pdi1 Δ* , and *CEN-P_{GALI}-PDI1*), we found that reduced Ero1p formed to at least the same extent as in a *pdi1 Δ P_{GALI}-PDI1* strain (Figs. 5 D and S5), indicating that no other PDI family members contribute significantly to reduction of Ero1p regulatory bonds.

Glutathione acts synergistically with Pdi1p to activate Ero1p

The rapid Pdi1p-dependent reduction of Ero1p regulatory bonds that occurs in cells treated with DTT could be the result of a pool of reduced Pdi1p formed by direct reduction with DTT that had entered the ER. Alternatively, DTT treatment may produce

a pool of reduced glutathione (GSH) in the ER, which in turn could lead to reduction of Pdi1p. To test the possibility that DTT acts indirectly through glutathione, we tested the effect of depleting cells of glutathione by treating cells with 5 mM buthionine sulfoximine (BSO) for 15 h, which blocks glutathione synthesis by inhibiting γ -glutamylcysteine synthetase. Reduction of Ero1p regulatory bonds by DTT treatment occurred more slowly in glutathione-depleted cells than in untreated cells (Fig. 5 E). This result suggests that the presence of a reducible pool of glutathione may accelerate the overall process of reduction of Pdi1p by DTT. Alternatively, depletion of glutathione by BSO treatment may subtly affect Pdi1p expression or have some other indirect effect on the redox state of the ER.

To address the role of glutathione in Ero1p activation more directly, we reconstituted Ero1p activation in vitro using both Pdi1p and GSH as substrates. By itself, Pdi1p is a poor activator of Ero1p because once Pdi1p has become oxidized through Ero1p oxidase activity, there is no longer sufficient reduced Pdi1p available to keep Ero1p in an active state, and oxidized Pdi1p can accelerate inactivation of Ero1p. Therefore, fully activated Ero1p was rarely observed during reaction with reduced Pdi1p (Fig. 6 A). Glutathione alone was also a poor activator of Ero1p. 10 mM GSH did not reduce regulatory bonds in either wild-type Ero1pc (Fig. 6 B, left) or Ero1pc-C100A-C105A (not depicted). However, a mixture of 10 mM GSH and Pdi1p maintained a pool of at least partially activated Ero1p (Fig. 6 B, right). The basis of Ero1p activation in the presence of Pdi1p and GSH appeared to be that

provided by a pool of GSH in great stoichiometric excess to Pdi1p, which allowed for a significant pool of reduced Pdi1p to be maintained. This pool of reduced Pdi1p in turn could reduce the regulatory bonds of Ero1p, thus overcoming the kinetic barrier for GSH to reduce the Ero1p regulatory bonds. In this coupled reaction, oxidized glutathione (GSSG) was generated by the combined action of Pdi1p and Ero1p (Fig. 6 C), and production of GSSG depended on Ero1p enzymatic activity because even in the presence of Pdi1p, catalytically inactive Ero1pc-C100A-C105A did not produce GSSG actively, whereas hyperactive Ero1pc-C150A-C295A produced GSSG more rapidly than wild type (not depicted).

To investigate the effects of the balance of GSH to GSSG on Ero1p activity, we reconstituted an *in vitro* reaction in which recombinant Ero1p and Pdi1p were incubated in the presence of three different ratios of GSH/GSSG. When the reaction was begun in the presence of GSH only, the rate of glutathione oxidation was initially rapid but then slowed. Evidently, the concomitant generation of GSSG by the action of Ero1p and Pdi1p eventually leads to inactivation of Ero1p (Fig. 6 D, 5 mM GSH curve). Introduction of GSSG into the coupled reaction slowed the initial rate of GSH oxidation (compare the curves of 5 mM GSH with 8:1 and 3:1 GSH/GSSG; Fig. 6 D). Taking the background of slow nonenzymatic generation of GSSG into account, the Ero1p and Pdi1p catalyzed oxidation of GSH stops at when the molar ratio of GSH/GSSG is $\sim 3:1$. Thus, this ratio of GSH/GSSG appears to be the approximate steady-state set point for the coupled reaction.

Discussion

A conserved protein disulfide relay system of the ER, consisting of Ero1 and PDI, supports correct disulfide bond formation of secretory proteins (Sevier et al., 2001; Sevier and Kaiser, 2006a; Appenzeller-Herzog et al., 2008; Braakman, 2009). In *S. cerevisiae*, the Ero1-PDI relay is essential for biosynthetic disulfide bond formation and is thus essential for cell viability (Frand and Kaiser, 1998; Pollard et al., 1998; Vitu et al., 2010). We previously showed that the activity of *S. cerevisiae* Ero1p is controlled by an autoregulatory feedback loop that involves the reduction and the reoxidation of three regulatory disulfide bonds (Sevier et al., 2007). Here, we show that not only is Pdi1p the major substrate of Ero1p in the disulfide relay system but that Pdi1p is also the primary regulator of Ero1p activity by its ability carry out dithiol/disulfide exchange with the regulatory bonds. We also show that the redox state of glutathione in the ER is important for setting the regulatory state of Pdi1p, and, in this way, the disulfide bond-generating activity of Ero1p is controlled by the redox status of both Pdi1p and of glutathione.

In a reaction initiated by adding to Ero1p a stoichiometric excess of reduced Trx1, a model substrate, the regulatory bonds in Ero1p are rapidly reduced, and Trx1 is oxidized by active Ero1p. Once Trx1 has been oxidized, the regulatory bonds in Ero1p are reformed, and Ero1p returns to an inactive state. In this paper, we separated the activation and inactivation phases of this cycle to determine the biochemical requirements for each phase. As expected, the reduced form of Trx1 readily reduces regulatory bonds in Ero1p and is thus a potent activator of Ero1p.

However, oxidized Trx1 has no capacity to reoxidize the regulatory cysteines of Ero1p. Instead, oxidation of the regulatory cysteines is accomplished by Ero1p itself in a reaction that probably involves the transfer of a disulfide bond from the shuttle cysteines of one Ero1p protein molecule to the regulatory cysteines of another. Thus, the inactivation phase occurs by autonomous oxidation of Ero1p once reduced Trx1 has been depleted by being converted to the oxidized form.

A similar analysis for a reaction that contains Ero1p and its natural substrate Pdi1p revealed that Ero1p is never fully activated in the presence of Pdi1p (Fig. 6 A). Whereas we initially thought that the inability of Pdi1p to activate Ero1p robustly was a consequence of some deficiency in the *in vitro* reaction, we now have evidence that the balance of the reduced and oxidized forms of Pdi1p is crucial in regulating Ero1p enzyme activity and faithfully reproduces the redox homeostasis set point of the ER.

Pdi1p has two redox-active domains containing the CxxC motifs. The a' domain is not as efficiently oxidized by Ero1p as the a domain (Vitu et al., 2010). However, the a' domain does appear to play a greater role than the a domain in oxidizing the Ero1p regulatory bonds; the partially oxidized a' domain (Pdi1p-SGCC) accelerates oxidation of Ero1p regulatory bonds more rapidly than the fully oxidized a domain (Pdi1p-CCSS). Given the relative redox potentials of the two domains, their propensity to be oxidized by Ero1p and their relative ability to oxidize the regulatory bonds of Ero1p provide a consistent explanation for why the a' domain is only partly oxidized, whereas the a domain is fully oxidized in cells under standard conditions (Vitu et al., 2010). In contrast to the yeast Pdi1p, the two active domains of the human PDI have similar reduction potentials, and the a' domain is the one preferentially oxidized by Ero1- α (Wang et al., 2009; Chambers et al., 2010). Therefore, it seems possible that the mechanism of Ero1- α regulation via the two domains of the human PDI is distinct from the one in yeast. Specificity in interaction between human and yeast Ero1 and PDI proteins has been demonstrated (Vitu et al., 2010; Masui et al., 2011). Comparison of the activity of human and yeast proteins *in vitro* shows that the highest enzyme activity is achieved using protein combinations of Ero1 and PDI from the same species (Araki and Nagata, 2011). Low catalytic activity was traced in part to a weaker binding affinity between human Ero1- α and yeast Pdi1p, relative to human PDI (Araki and Nagata, 2011). Whether the different redox potentials of the PDI domains in human and yeast PDI also contribute to the differences in Ero1 regulation between species remains to be assessed.

Several lines of evidence point to the existence of multiple disulfide relay pathways from Ero1p to Pdi1p. Best characterized is the flow of disulfides from the C352-C355 pair to Pdi1p via the C100-C105 shuttle cysteines. This pathway is necessary for full activity of Ero1pc *in vitro* and is necessary for cell viability. Nevertheless, Ero1pc-C100A-C105A retains some activity *in vitro* and shows slow autonomous reoxidation, showing that a disulfide bond can be transferred, albeit slowly, from the active site to the regulatory disulfides without the action of the C100-C105 shuttle (Fig. 2 B). Moreover, *in vivo* experiments have shown that yeast cells with an *ero1-C100A* mutation are

viable if they have the capacity to up-regulate Ero1p expression through unfolded protein response induction (Frandsen and Kaiser, 2000), and overexpression of Ero1p-C100A-C105A-C150A-C295A was able to partially suppress the temperature sensitivity of the *ero1-1* strain (unpublished data).

The pathway of dithiol/disulfide exchange reactions used by Ero1p-C100A-C105A to oxidize Pdi1p remains to be determined. Considering that autonomous oxidation of the regulatory cysteines by Ero1p-C100A-C105A is possible, we hypothesize that the active-site disulfide bond C352-355 may first be transferred to regulatory cysteine pairs, and then Pdi1p could be oxidized by reducing these regulatory bonds during the activation process of Ero1p. These pathways were revealed by experiments with Ero1p-C100A-C105A but are not limited to mutant Ero1p and are likely also used by wild-type Ero1p. By transferring one or two of the regulatory bonds to Pdi1p, Ero1p would have some capacity to oxidize a stoichiometric quantity of Pdi1p without any Ero1p becoming fully active. The C143-C166 bond may be the most labile regulatory bond, as its reduction is the first step of Ero1 activation by Pdi1p in vitro (Heldman et al., 2010) and in turn could readily contribute to Pdi1p oxidation. This pathway may allow the disulfide relay system to respond to modest increases in load with a minimum of excess hydrogen peroxide generation.

When Ero1p is incubated with reduced Pdi1p and GSH, Ero1p can be shown to be catalytically active by its ability to oxidize a stoichiometric excess of GSH, although little or no Ero1p can be detected in a state in which all regulatory cysteines are in a reduced state (Fig. 6 B). As previously noted (Heldman et al., 2010), this observation indicates that the partially reduced forms of Ero1p have at least some oxidase activity. Thus, the regulatory bonds in Ero1p may act less like an on/off switch than a proportional controller with different degrees of activity according to the redox states of the three regulatory bonds. We have not been able to devise a quantitative test of this attractive hypothesis because it is not possible to isolate in pure form Ero1p in a partially reduced state.

Toward a biochemical understanding of the mechanism of Ero1p regulation in a highly simplified reaction, we successfully reconstituted the disulfide relay system and the basic properties of redox homeostasis in vitro with just their components, Ero1p, Pdi1p, and glutathione (Fig. 6). The capacity of Pdi1p to sustain Ero1p in an activated state can be increased by addition of an excess of GSH in the in vitro reaction. This coupled reaction appears to approximate conditions in the ER (Hwang et al., 1992), in which transfer of disulfide bonds to nascent secretory proteins and to GSH keeps enough Pdi1p in a reduced state to allow for some capacity to activate Ero1p. In the coupled reaction, Ero1p enzyme activity is controlled by the ratio of GSH/GSSG, and a 3:1 ratio of GSH/GSSG appears to be close to the steady-state set point at which Ero1p can be activated. Pdi1p, which has both reductase and isomerase activities, can serve as the key link between thiol substrates, including glutathione, and Ero1p to activate the disulfide relay system only when substrates are present (Fig. 7). In the mammalian cell, it has also been suggested that oxidation of PDI and glutathione is well regulated by an equilibrium between Ero1- α and PDI

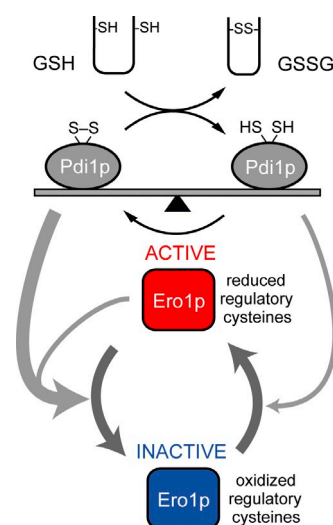


Figure 7. A balance between reduced and oxidized Pdi1p determines ER redox homeostasis. The thiol substrates (reduced protein thiols or reduced glutathione) present in the ER can be oxidized by Pdi1p, leading to formation of oxidized Pdi1p. If sufficient reduced Pdi1p has formed, Ero1p is activated by direct reduction of the Ero1p regulatory bonds by reduced Pdi1p. Once activated, Ero1p oxidizes Pdi1p until a steady-state balance of oxidized and reduced Pdi1p is reestablished. The balance of reduced and oxidized Pdi1p determines the degree of Ero1p activation; once reduced Pdi1p has declined sufficiently, autonomous oxidation of the regulatory bonds of Ero1p will lead to Ero1p inactivation, preventing overoxidation of the ER. Under hyperoxidizing conditions, oxidized Pdi1p would rapidly inactivate Ero1p.

(Appenzeller-Herzog et al., 2010). It seems likely that ER redox homeostasis is maintained by the same basic mechanism in yeast and mammalian cells.

Materials and methods

Protein expression and purification

The sequence coding Ero1p (56–424) or Ero1p mutants (Ero1p-C100A-C105A, Ero1p-C355S, Ero1p-C150A-C295A, Ero1p-C90A-C349A, Ero1p-C143A-C166A, and Ero1p-C100A-C105A-C143A-C166A) was fused at the C terminus to His₆-tagged MBP linked by the Tobacco etch virus (TEV) protease cleavage sequence (ENLYFQGS) inserted into the pET17b vector (EMD) between NdeI and XhoI restriction sites. We found that C355S was the only point mutation of the active-site cysteine pair (C352-C355) that could be produced in a soluble FAD-bound state. Ero1p and all variants were overexpressed in Origami B(DE3) competent cells (EMD) by induction with 0.4 mM IPTG supplemented with 10 μ M FAD overnight at room temperature. Ero1p fusion proteins with His₆-tagged MBP were purified from cell lysates by affinity to amylose (New England Biolabs, Inc.) and then dialyzed into TEV cleavage buffer (50 mM Tris, pH 7.8, 0.5 mM EDTA, and 0.5 mM tris(2-carboxyethyl)phosphine). MBP-fused Ero1p (MBP-Ero1p) and Ero1p-C100A-C105A were also expressed and purified as described for Ero1p purification. Ero1p was cleaved from His₆-tagged MBP by incubating with recombinant His₆-tagged TEV protease (1:100 wt/wt protease to substrate; the TEV protease-encoding plasmid was provided by C. Hill, University of Utah, Salt Lake City, UT) for 15 h at 25°C. Ero1p and variants cleaved from His₆-MBP were collected by flowing through the HiTrap Ni²⁺ column (GE Healthcare) to remove uncleaved fusion protein, cleaved His₆-tagged MBP, and TEV protease. Some Ero1p variants (Ero1p-C100A-C105A-C150A-C295A and Ero1p-C100A-C105A-C90A-C349A) were subcloned in pGEX-4T vector purified by flowing through GSTrap (GE Healthcare) followed by thrombin cleavage for elution and secondary purification with the HiTrap Ni²⁺ column for removal of thrombin (Gross et al., 2004). All purified Ero1p proteins were dialyzed against PBS.

The sequence coding Pdi1p (31–522) or Pdi1p variants (C406S-C409S for CCSS, C61S-C64S for SSCC, and C61S-C64S-C406S-C409S

for SSSS) tagged with His₆ at the N terminus was inserted into pET20b between NdeI and SacI restriction sites. Recombinant His₆-tagged Pdi1p, Pdi1p variants, and His₆-tagged *E. coli* thioredoxin (Trx1; Sevier and Kaiser, 2006b) were overexpressed in BL21(DE3)pLysS competent cells (EMD) by induction with 0.4 mM IPTG for 5 h at 37°C and purified from cell lysates by affinity to the HiTrap Ni²⁺ column. Purified, naturally oxidized Pdi1p and Trx1 were dialyzed into PBS. Reduced forms of Pdi1p and Trx1 were prepared by incubating with 10 mM DTT for 30 min at room temperature and removing DTT with Micro Bio-Spin 6 (Bio-Rad Laboratories).

Yeast strains and plasmids

CKY1044 and CKY1045 and MATa and MATα *pdi1Δ::KanMX* strains covered by URA3 *CEN PDI1* plasmid pCS213 have been previously described (Vitu et al., 2010). To facilitate scoring of crosses with the *pdi1Δ* strain, a NatMX-marked *pdi1Δ* strain was constructed by swapping the KanMX marker in CKY1045 with a NatMX4 cassette by homologous recombination (Goldstein and McCusker, 1999). Individual disruptions of the genes encoding the yeast PDI homolog proteins were made by one-step gene replacement of the entire ORF with KanMX by homologous recombination. The compound deletion strain CKY1086 with the genotype MATa *GAL2 ura3-52 leu2-3,112 pdi1Δ::NatMX mpd1Δ::KanMX mpd2Δ::KanMX eug1Δ::KanMX eps1Δ::KanMX* [pCS213] was constructed by sequential rounds of mating, sporulation, and scoring of the initial individual disruption mutant strains. Disruption of all five genes encoding the PDI1 homologs in CKY1086 was confirmed by PCR analysis. Some *pdi1Δ* strains also contained a genomic-tagged copy of *BGL2*. Tagging of *BGL2* with a triple HA epitope at its C terminus was performed by homologous recombination with a 3HA-KanMX6 module (Longtine et al., 1998). To construct the MATa *GAL2 ura3-52 leu2-3,112 pdi1Δ::NatMX BLG2:HA-KanMX* [pCS213] double mutant (CKY1087), individual *pdi1Δ* and *BLG2:HA* strains were mated, and an MATa NatMX⁺ KanMX⁺ segregant was selected after sporulation. Viability of CKY1086 and CKY1087 depends on a plasmid-borne *PDI1* gene provided by plasmid pCS213. CKY1088 and CKY1089 were generated by transformation of CKY1086 or CKY1087, respectively, with the pSK65 [*CEN LEU2 P_{GAL1}-PDI1*] followed by selection against pCS213 by plating on supplemented minimal medium (SMM) with 5-FOA. CKY1090 was created by transformation of CKY1044 with pSK2 [*CEN LEU2 His₆-PDI1*] followed by selection against pCS213 with 5-FOA.

To create pSK2 [*CEN LEU2 His₆-PDI1*], the sequence coding HHHH-HGG was inserted right after the signal sequence (1–22) by introducing the NheI restriction site in pCS463, which contains the *PDI1* coding region and ~875 and ~150 bp of the 5' and 3' untranslated regions. Plasmid pSK65 [*CEN LEU2 P_{GAL1}-PDI1*] contains the *PDI1* coding region and ~60 and ~150 bp of the 5' and 3' untranslated regions under the *GAL1* promoter. *ERO1*-coding URA3 2μ plasmids pSK63 [*ERO1-flag*], pSK64 [*ero1-C100A-C105A-flag*], pDPS51 [*ero1-C100A-C105A-C150A-C295A-flag*], pDPS49 [*ero1-C100A-C105A-C90A-C340A*], pDPS50 [*ero1-C100A-C105A-C143A-C166A*], and pSK66 [*ero1-C355S-flag*] were generated by insertion of sequence-encoding flag tag right after the last codon using the NotI restriction site and subcloning or site-directed mutagenesis based on the previously reported plasmid pAF84 (Fränd and Kaiser, 1998).

Analysis of protein oxidation states

For reduction of *Ero1p* regulatory bonds in vitro, 1 μM *Ero1pc* or its variants were incubated with 50 or 20 μM reduced Trx1, reduced Pdi1p, or reduced Pdi1p mutants in PBS containing 0.5 mM EDTA. After incubation at 25°C, the reactions were quenched by mixing with 1× SDS-AMS (4-acetamidido-4'-maleimidylstilbene-2,2'-disulfonic acid) buffer (1× SDS sample buffer without reducing agent and 1 mM AMS [Invitrogen]), which alkylates free cysteine thiols and adds one ~500-D moiety to each free thiol. For reactions that contained glutathione or DTT, proteins were first precipitated by 10% chilled TCA. Protein precipitants were washed with 0°C acetone and were redissolved with 1× SDS-AMS buffer. *Ero1p* and Pdi1p were analyzed by nonreducing SDS-PAGE followed by immunoblotting with anti-*Ero1p* serum or anti-HDEL (Santa Cruz Biotechnology, Inc.). The oxidation state of Trx1 was analyzed by nonreducing SDS-PAGE followed by staining with SimplyBlue SafeStain (Invitrogen).

The in vivo oxidation state of *Ero1p* was assessed in cells growing in SMM medium incubated with 0, 0.2, or 1 mM DTT for 30 min and then lysed in the presence of chilled 10% TCA by agitating with glass beads. Protein pellets from cell lysates were washed with chilled acetone and redissolved with 1× SDS-AMS buffer. Oxidation states of *Ero1p* and variants were analyzed by nonreducing SDS-PAGE and immunoblotting with anti-*Ero1p*. Immunoblots were visualized with an Odyssey imaging system (LI-COR Biosciences). The fully reduced or the oxidized band of *Ero1pc* was quantified by the Odyssey program (v2.0; LI-COR Biosciences), and reduced fraction

was calculated by dividing reduced band intensity with the sum of reduced and oxidized band intensity.

To determine the effect of hydrogen peroxide on the oxidation state of *Ero1p* regulatory bonds (Fig. S1), 1 μM *Ero1pc* was incubated with 100 μM reduced His₆-tagged Trx1 in PBS containing 0.5 mM EDTA for 2 min, and Trx1 was selectively removed by affinity for Ni²⁺ beads. To remove hydrogen peroxide generated during action of *Ero1p* oxidase, 80 μg/ml catalase was added to the reaction. Removal of hydrogen peroxide by catalase was confirmed by measuring the amount of hydrogen peroxide in the reaction with Amplex UltraRed reagent (Invitrogen). 1 μM *Ero1pc* was incubated with 100 μM reduced thioredoxin, 50 μM Amplex red, and 0.1 U/ml HRP in the presence or absence of 80 μg/ml catalase for 30 min at room temperature. Fluorescence was measured in a microplate reader with wavelength settings of 530/590 nm. Inactivation of *Ero1p* under anaerobic conditions was analyzed as described above except being performed in the anaerobic chamber flowing N₂ and CO₂ gases. To test whether exogenous hydrogen peroxide or oxidized Pdi1p can promote inactivation of *Ero1p*, 100 μM hydrogen peroxide or 20 μM oxidized Pdi1p was added to *Ero1pc*, *Ero1pc-C100A-C105A*, or *Ero1pc-C355S* after Trx1 depletion. Oxidation states of *Ero1pc* and its mutants were analyzed after cysteine modification with 1 mM AMS by nonreducing SDS-PAGE and immunoblotting with anti-*Ero1p* serum.

The oxidation states of each catalytic domain of recombinant Pdi1p were determined by introducing a thrombin cleavage site that allows separation of the two catalytic domains (Fig. S2). The sequence coding N-terminal His₆-tagged Pdi1p (31–522) with a thrombin recognition sequence in the X-linker was subcloned from pSK42 (Vitu et al., 2010) for *E. coli* expression. His₆-Pdi1p-thrombin was overexpressed in BL21(DE3)pLysS competent cells by IPTG and purified from cell lysates with the HiTrap Ni²⁺ column. Purified His₆-Pdi1p-thrombin was dialyzed into PBS. 10 μg His₆-Pdi1p-thrombin was incubated with 1 mM AMS for 30 min at room temperature followed by treatment with 0.1 μg thrombin supplemented with 1 mM CaCl₂ for 1 h at 37°C. Fully reduced Pdi1p control was prepared by treatment with 10 mM DTT for 30 min at room temperature. DTT was removed by using Bio-Spin 6. AMS-modified and thrombin-digested samples were analyzed by nonreducing SDS-PAGE.

The oxidation state of newly synthesized CPY in the Pdi1p-depleted *ER* was analyzed in *pdi1Δ* cells with galactose-inducible *PDI1* grown to the exponential phase for 15 h in glucose medium to deplete Pdi1p. Cells were suspended in SMM-met, pulse labeled for 10 min at 30°C, and lysed in the presence of 10% chilled TCA to block disulfide exchange. After AMS modification, CPY was immunoprecipitated and resolved by reducing SDS-PAGE. A fully reduced CPY control was prepared by treating cells with 10 mM DTT for 15 min before cell lysis.

Reoxidation of *Ero1pc* after thioredoxin depletion

1 μM *Ero1pc* was reduced by incubation with 100 μM reduced His₆-tagged Trx1 for 2 min and then passed through Ni²⁺ beads to remove Trx1. At times after Trx1 depletion, the reaction was quenched by mixing with SDS sample buffer containing 1 mM AMS. Depletion of Trx1 was confirmed by analysis with nonreducing SDS-PAGE. When necessary, 40 μM oxidized Trx1, 20 μM oxidized Pdi1p, or 20 μM oxidized Pdi1p mutant was added into *Ero1pc* or *Ero1pc* mutants immediately after Trx1 depletion.

Purification of mixed disulfides from yeast

CKY1090 expressing His₆-Pdi1p was transformed with pSK64 [*ero1-C100A-C105A-flag*], pDPS51 [*ero1-C100A-C105A-C150A-C295A-flag*], pDPS49 [*ero1-C100A-C105A-C90A-C340A*], and pDPS50 [*ero1-C100A-C105A-C143A-C166A*]. Exponentially grown cells were lysed by agitation with glass beads in the presence of chilled 10% TCA. TCA-precipitated protein pellets were washed with acetone and redissolved with 2% SDS and 50 mM Tris, pH 8.0, containing 50 mM NEM to block free cysteines. For efficient affinity purification, SDS was removed by SDS-Out reagent (Thermo Fisher Scientific). After clarification by centrifugation, *Ero1p-C100A-C105A-flag* was released from anti-flag affinity beads using 100 μg/ml flag peptide (Sigma-Aldrich) followed by deglycosylation with PNGaseF for 3 h at 37°C. Samples purified by affinity to anti-flag beads were passed through an Ni²⁺ column to capture mixed disulfides with His₆-Pdi1p. Both Ni²⁺ unbound fraction and bound fraction were collected and analyzed by nonreducing SDS-PAGE and immunoblotting with anti-flag (Sigma-Aldrich) or anti-Pdi1p.

Depletion of Pdi1p and glutathione in yeast

To determine the oxidation states of *Ero1p* and mutants in Pdi1p- or glutathione-depleted cells, pSK63, pSK64, and pSK66 were transformed into CKY1088 and/or CKY1089. Cells exponentially grown in SMM containing

1.5% galactose and 1% raffinose were incubated in SMM containing 2% glucose for 15 h to shut off expression of Pdi1p. Glutathione was depleted by growing cells in the presence of 5 mM BSO, an inhibitor of glutathione biogenesis, for 15 h. Pdi1p depletion was confirmed by immunoblotting with anti-Pdi1p (Fig. 5 E), and glutathione depletion was confirmed using Ellman's reagent.

The maturation of CPY was evaluated in *pdi1Δ* cells with galactose-inducible PDI1 grown to exponential phase for 15 h in glucose medium to deplete Pdi1p. For fully reduced cell environment, cells were incubated with 1 mM DTT for 1 h after a 14-h incubation in glucose medium. Cell lysates were prepared in the presence of chilled 10% TCA, and mature and immature CPY were analyzed by immunoblotting with anti-CPY serum.

Measurement of oxidized glutathione

GSSG produced by Ero1p and Pdi1p was measured by using Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid) [DTNB]]. 1 μM Ero1p, Ero1p-C150A-C295A, or Ero1p-C355S was incubated with 10 mM GSH with or without 10 μM oxidized Pdi1p. For extended reaction, 5 mM GSH, 4 mM GSH/0.5 mM GSSG, or 3 mM GSH/1 mM GSSG was mixed with Ero1p and Pdi1p. At various time points after mixing, 10 μl of reactants was quenched by chilled 5-sulfosalicylic acid (1% final concentration), and precipitated protein pellet was removed. GSH was alkylated by 2% 2-vinylpyridine after neutralizing sulfosalicylic acid with a drop of triethanolamine (Sigma-Aldrich) for 1 h at 25°C followed by a 20-fold dilution in DTNB reaction buffer containing 100 mM sodium phosphate, pH 7.0, 1 mM EDTA, 0.2 mM DTNB, and 0.2 mM NADPH. The change in absorbance at 405 nm was recorded after adding 1 U of glutathione reductase.

Online supplemental material

Fig. S1 shows that autonomous oxidation and inactivation of Ero1p is not a side effect of hydrogen peroxide produced by aerobic catalysis by Ero1p. Fig. S2 shows that the endpoint of air oxidation yields a form of recombinant Pdi1p with the α domain almost fully oxidized, and the α' domain is partially oxidized. Fig. S3 shows that air-oxidized Pdi1p can contribute to the oxidation of the Ero1p regulatory bonds. Fig. S4 shows that when cells are depleted of Pdi1p by growing *pdi1Δ* cells with glucose-repressible PDI1 in glucose medium, newly synthesized CPY remains largely in a reduced state. Fig. S5 shows that when cells are depleted of Pdi1p, autonomous oxidation of the regulatory bonds of Ero1p can occur. This oxidation requires active-site cysteine C355 but does not require the shuttle cysteines C100 and C105. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201110090/DC1>.

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