ANTIOXIDANTS & REDOX SIGNALING
Volume 16, Number 8, 2012

DOI: 10.1089/ars.2011.4238

FORUM REVIEW ARTICLE

Peroxides and Peroxidases in the Endoplasmic Reticulum: Integrating Redox Homeostasis and Oxidative Folding

Taichi Kakihana,[1,2] Kazuhiro Nagata,[2] and Roberto Sitia[3]

Abstract

Significance: The endoplasmic reticulum (ER), the port of entry into the secretory pathway, is a complex organelle that performs many fundamental functions, including protein synthesis and quality control, Ca^{2+} storage and signaling. Redox homeostasis is of paramount importance for allowing the efficient folding of secretory proteins, most of which contain essential disulfide bonds. ***Recent Advances:*** revealed that an intricate protein network sustains the processes of disulfide bond formation and reshuffling in the ER. Remarkably, H_2O_2, which is a known by-product of Ero1 flavoproteins in cells, is utilized by peroxiredoxin-4 and glutathione peroxidases-7 and -8, which reside in the mammalian secretory compartment and further fuel oxidative protein folding while limiting oxidative damage. ***Critical Issues:*** that remain to be addressed are the sources, diffusibility and signaling role(s) of H_2O_2 in and between organelles and cells, how the emerging redundancy in the systems is coupled to precise regulation, and how the distinct pathways operating in the early secretory compartment are integrated with one another. ***Future Directions:*** A further dissection of the pathways that integrate folding, redox homeostasis, and signaling in the early secretory pathway may allow to manipulate protein homeostasis and survival-death decisions in degenerative diseases or cancer. *Antioxid. Redox Signal.* 16, 763–771.

Introduction

Life began in a reducing environment. As a memory of those early days, the intracellular chemistry of prokaryotes requires reducing conditions. When oxygen appeared, cells faced a serious problem, that of preventing oxidative stress. Indeed, reactive oxygen species (ROS) and peroxides, obligatory by-products of many metabolic reactions, can be damaging for cells, leading to the accumulation of oxidized proteins, lipids, and nucleic acids. However, over the last decade it has become clear that if carefully handled, ROS can bring many advantages for eukaryotic cells; indeed, the redox gradients existing between the extra- and intracellular space and among organelles allow ample regulatory possibilities, and low levels of hydrogen peroxide (H_2O_2) are utilized for many signaling pathways and cell survival (13). In this connection, cysteines represent suitable molecular switches that cells utilize for various purposes. Their thiol group can undergo several modifications, most of which reversibly regulate protein function: it can be oxidized to a sulfenic (SOH), sulfinic (SO_2H) or sulfonic (SO_3H) acid, or form disulfide bonds (Fig. 1) with proteins, glutathione, or other small thiols. In eukaryotes, these issues are complicated by the existence of membrane bound compartments differing in their redox balances, and by the many kinetic and topologic constraints which influence the specificity and reactivity of oxidants with physiological targets *in vivo* (56).

This essay focuses on how H_2O_2 is produced, metabolized, and ingeniously utilized in the endoplasmic reticulum (ER) to generate and maintain an efficient and environment friendly protein factory.

Oxidative Protein Folding and H_2O_2 Production in the ER

In all eukaryotic cells, the ER is responsible for the folding of membrane and secretory proteins. Unlike those destined to the cytosol or nucleus, the majority of these proteins contain disulfide bonds, the formation of which requires net oxidative power. In the late '90s, the discovery of ER oxidoreductin-1 (Ero1) proteins provided an answer to how oxidative power can be generated in the ER (15, 43). These highly conserved flavoproteins oxidize protein disulfide isomerase (PDI) preferentially over other resident oxidoreductases (33). PDI then transfers disulfide bonds to cargo proteins. Thus, oxidative protein folding is achieved by step-by-step reactions involving several enzymes, an important feature to guarantee selectivity of oxidation. Concomitantly, electrons flow from Ero1 to molecular oxygen, yielding H_2O_2 as a by-product in

[1]Department of Molecular and Cellular Biology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan.
[2]Laboratory of Molecular and Cellular Biology, Faculty of Life Sciences, Kyoto Sangyo University, Kyoto, Japan.
[3]Division of Genetics and Cell Biology, San Raffaele Scientific Institute, Vita-Salute University, Milan, Italy.

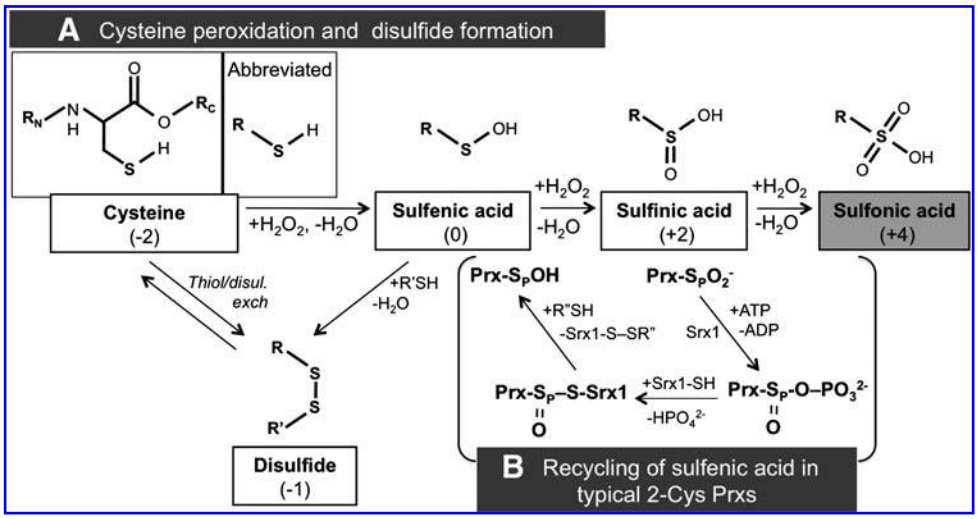

FIG. 1. Chemical modifications of cysteines. The structural formula, chemical name, and formal oxidation state of SULFUR element of the main cysteine chemotypes found in human cells are schematized. Most of them are difficult to quantify and their relative abundance may significantly differ. The picture depicts the two main reaction cycles, disulfide bond formation and oxidation. **(A)** Cysteine peroxidation and formation of disulfide bonds. A disulfide can be formed from two cysteines directly without electron flow *via* thiol/disulfide exchange reaction (thiol/disul. exch.), or *via* several intermediates, including sulfenic acid. *In vivo*, disulfide bonds are formed, isomerized and primarily reduced through thiol/disulfide exchange reactions. The sulfenic acid moieties formed in the reaction of cysteine with a H_2O_2 molecule can be further oxidized to sulfinic and sulfonic acid by additional H_2O_2. While sulfinic acid can be reduced to sulfenic acid, sulfonic acid accumulates irreversibly (*gray box*). **(B)** Recycling of sulfinic but not sulfonic acid. The reduction of sulfinic acid to sulfenic acid in human typical 2-Cys Prxs is schematized. In the presence of ATP and sulfiredoxin 1 (Srx1), sulfinylated typical 2-Cys Prx ($Prx\text{-}S_PO_2^-$) can be phosphorylated to form $Prx\text{-}S_P\text{-}O_2\text{-}PO_3^{2-}$. Then, Srx1 reacts *via* its Cys99 with phosphorylated 2-Cys Prx to form Prx-S_P-O–S-Srx1 intermediates. Reductants (R″SH), such as thioredoxin or glutathione, can subsequently reduce the Prx-S_P-O–S-Srx1 intermediates to sulfenylated 2-Cys Prx (Prx-S_P-OH) (28). Depending on the availability of Srx1, therefore, 2-Cys Prxs can be productively recycled. Prx, peroxiredoxin.

stoichiometric amounts to the disulfides formed (54) (Fig. 2A). Two members of the family are present in mammals, *Ero1α* and *Ero1β*, with different tissue distribution and transcriptional control. The reader is referred to excellent reviews in this Forum for updated information on the evolution and regulation of the Ero1 family (3, 44). In yeast, *ERO1* is an essential gene. The unexpectedly mild phenotype of *Ero1α* and *Ero1β* compound mutant mice, therefore, raises the question of what sustains oxidative folding in the absence of these flavoproteins (60). Possibly disulfide bonds can be inserted into proteins by sulfydryl oxidases as overexpressed Erv2 can rescue *ero1*-defective yeast cells (49). Interestingly, besides a flavin-containing domain endowed with oxidase activity, quiescin-sulfhydryl oxidase contains a thioredoxin-like domain through which it can directly transfer disulfide bonds to its substrates (Fig. 2B). Unlike Ero1 proteins, these enzymes are normally secreted by cells and are hence present in the entire exocytic compartment (25). It is presently unclear whether mammalian sulfydryl oxidases act also downstream of the ER and/or in the extracellular space.

The above flavoprotein-driven reactions sustaining disulfide bond formation are all associated with the production of luminal H_2O_2. Moreover, other systems can generate or supply H_2O_2. For instance, NADPH oxidase-4 (Nox4) has been reported to reside in the ER membrane: this enzyme produces H_2O_2 without generating $O2^{\bullet -}$ (Fig. 2C) and has been implicated in activating apoptosis after prolonged ER stress (58). Another potential source of oxidants might be mitochondria, where the respiratory chain can produce large amounts of H_2O_2. Close contacts are established between ER and mitochondria, at the level of the mitochondrial-associated membranes (MAM) (Fig. 2D) (4, 16). However, *ero1*-deficient murine cells did not show clear sensitivity to attenuation of mitochondrial activity (61).

H_2O_2 is Scavenged and Metabolized by ER Peroxiredoxins

Particularly in professional secretory cells, oxidative folding can become a major source of H_2O_2. A single plasma cell, for instance, inserts up to 10^5 disulfides per second into nascent immunoglobulin (6). What is the fate of the H_2O_2 generated in the process of oxidative folding, a potentially dangerous by-product? An important hint came when it was shown that H_2O_2 can promote disulfide bond formation *in vitro* with minimal oxidative damage to the substrate protein (24). It was then found that peroxiredoxin-4 (Prx4), a protein residing primarily in the early secretory compartment, can utilize H_2O_2 to catalyze PDI oxidation *in vitro* and *in vivo* (52, 61). This property seems to be shared also with glutathione peroxidases-7 and -8 (GPx7/8), two enzymes that like Prx4 reside in the ER (37). The physiological and structural aspects of these peroxidases are addressed in more details below.

The Prx Protein Family

The first member of the Prx family proteins was identified in yeast in 1994 (11). Prxs are ubiquitous enzymes: in almost all organisms they can reduce different peroxides at very high

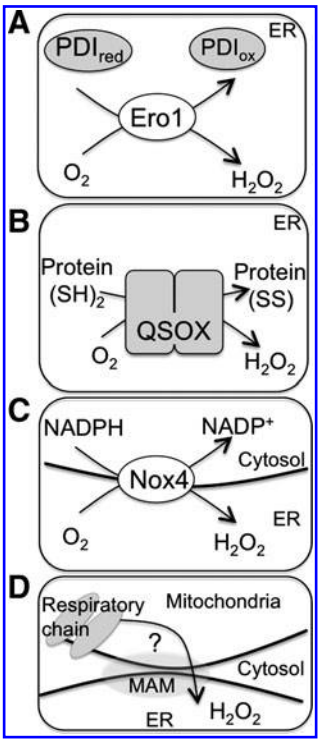

FIG. 2. Sources of H_2O_2 in the early secretory compartment. Hydrogen peroxide can derive from Ero1 or QSOX flavoproteins **(A** and **B)** or from membrane NOX4 molecules **(C)**. H_2O_2 generated by the respiratory chain could enter the ER at the level of mitochondrial-associated membrane (MAM), the sites of contact with mitochondria **(D)**. ER, endoplasmic reticulum; Ero1, ER oxidoreductin-1; QSOX, quiescin-sulfhydryl oxidase.

rates (10^6–10^7 $s^{-1}M^{-1}$) (18). They are hence one of the main lines of defense that cells deploy against excess peroxide production. There are six members of the Prx family in the human genome, residing in different compartments (46). Prxs can be divided into three classes: typical 2-cysteine (2-Cys), atypical 2-Cys, and 1-cysteine (1-Cys) Prx. Their mode of action includes similar cycles, which are schematized in Figure 3. The peroxidatic cysteine (C_P) initially reacts with H_2O_2 generating a water molecule and a sulfenic (SOH) intermediate. In 2-Cys Prxs, the cysteine sulfenic acid can then react with a resolving cysteine (C_R) to form a disulfide bond. In atypical 2-Cys enzymes an intra-chain disulfide is formed, while in typical 2-Cys Prx the resolving cysteine is located in an adjacent molecule (Fig. 3). The covalent Prx dimers are eventually reduced by thioredoxin or other reductases through thiol/disulfide exchange reactions. In the presence of excess H_2O_2, however, the SOH moiety can be further modified to the sulfinic and sulfonic derivatives (SO_2H or SO_3H). While the SO_2H form can be reversed to sulfenic acid by the catalysis of sulfiredoxin-1 in an ATP-dependent manner (8), SO_3H species are irreversibly inactivated. This mechanism, known as the floodgate model (17), may ensure that sufficient amounts of H_2O_2 locally accumulate to allow productive signaling.

Upon hyper-oxidation, Prxs may form decamers or higher order assemblies, and have been reported to lose their peroxidase activity and acquire chaperone activity (17). In this way, Prx2 can induce cell cycle arrest (42). In view of the importance of H_2O_2 in signaling, it is not surprising that many eukaryotic 2-Cys Prxs contain two motifs, the GGLG and YF sequences that decrease the flexibility of the peroxidatic cysteines, making them more susceptible to hyper-oxidation (57).

Prx4, a Typical 2-Cys Prx Residing in the Secretory Pathway

Prx4, also known as AOE372, AOE372-2, or TRANK (thioredoxin peroxidase-related activator of NF-κB and c-Jun N-terminal kinase), is a typical 2-Cys Prx, comprising catalytic and noncatalytic cysteines (51). Prx4 has several interesting and unique features. First, it differs from other 2-Cys Prxs in that it also utilizes reduced glutathione (GSH) as a reducing agent in addition to Trx (39). This is an important feature, because Trx is not present in the secretory pathway. However, oxidized Prx4 clearly transfers disulfide bonds much more efficiently to PDI (52, 61), ERp46 (10) and possibly other ER oxidoreductases than to GSH (Fig. 4A). This feature places Prx4 in the dual role of ER resident antioxidant and promoter of oxidative folding. Notably, an oxygen molecule can produce two disulfide bonds in the ER without H_2O_2 release, through the sequential oxidation of two oxidoreductases, that is, Ero1 and Prx4 (Fig. 4B).

Structure and Oligomerization of Prx4

Unlike other members of the Prx family, Prx4 has an additional N-terminal loop, putatively directed toward the center of the decamer rings (10, 55) (Fig. 5). A cysteine residue (Cys51) in the loop can form interchain disulfide bonds (51), which, in conjunction with those formed between the peroxidatic and resolving cysteines (Cys124 and Cys245, respectively) as part of the catalytic cycle (Fig. 4A), generate covalent decamers. Also Prx1, another typical 2-Cys Prx, forms a noncatalytic interchain disulfide bond that involves Cys83 and stabilizes decamers. In this conformation, Prx1 switches from peroxidase function to a molecular chaperone activity (26). However, Cys83 of Prx1 shows more similarity to Cys148 of Prx4, which was found irrelevant in covalent oligomer formation, than to Cys51 (51). The crystal structures of Prx4 in three different redox states confirmed that the N-terminal loop is important for stabilization of decamers, independently from interchain bonding *via* Cys51 (55). Accordingly, replacing Cys51 for alanine did not prevent decamer formation (10).

Regulating Prx4 Hyper-Oxidation

In cytosolic Prxs, sulfiredoxin 1 reverts sulfinylated subunits restoring their antioxidant properties. Is Prx4 hyper-oxidation differently regulated in the ER, where sulfiredoxin 1 is absent? The oxidized species accumulating after reaction with one H_2O_2 molecule, Prx4-SOH or Prx4-Prx4 (*via* C124-C245), undergo conformational changes that yield a locally unfolded structure (Fig. 5B, upper). Of note, and somehow counterintuitively, a Prx4 mutant lacking the resolving cysteine (C245A) was found to be resistant to hyper-oxidation even when exposed to high concentrations of H_2O_2 (10). Cao *et al*. found that this locally unfolded structure reacts less efficiently with H_2O_2, limiting hyper-oxidation (10) (Fig. 5B). It was also shown that either deleting the YF motif or mutating a

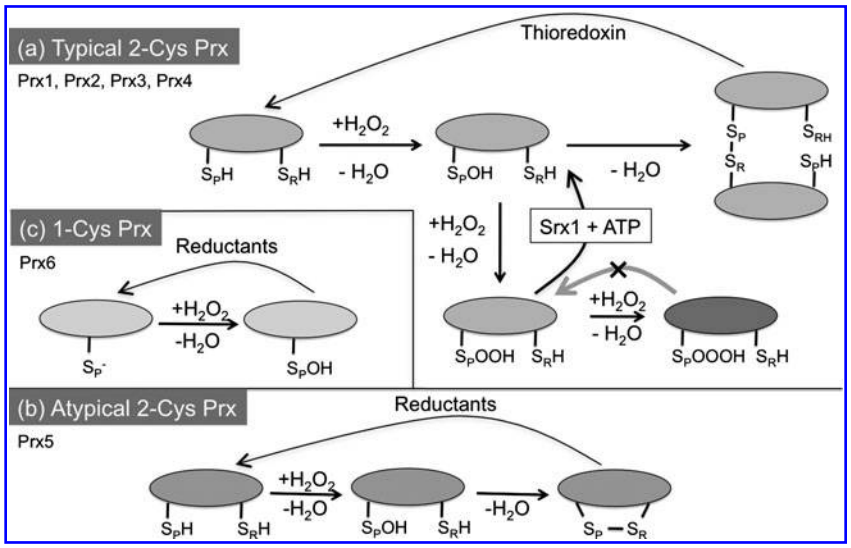

FIG. 3. The catalytic cycles of Prxs. (a) Typical 2-Cys Prxs contain a peroxidatic (P) and a resolving (R) cysteine. In the presence of hydrogen peroxide, the thiol of the peroxidatic cysteine is oxidized to a sulfenic acid. The latter can form an intermolecular mixed disulfide with the resolving cysteine within a typical 2-Cys Prx dimer. This oxidized disulfide intermediate is reduced by thioredoxin (or GSH and ER oxidoreductases in case of Prx4). Reaction with excess hydrogen peroxide further oxidizes the sulfenic acid, yielding sulfinic (SO_2H) or sulfonic (SO_3H) acid forms. The former can be reduced to a sulfenic form by sulfiredoxin (Srx). In contrast, the sulfonic acid form (depicted in *darker gray*) is irreversibly inactivated. **(b)** Atypical 2-Cys Prx also have two active cysteines (P and R). The former is oxidized to sulfenic acid by hydrogen peroxide, and then forms an intramolecular disulfide bond with the resolving cysteine. Unknown reductases may reduce this disulfide. **(c)** 1-Cys Prx have only the peroxidatic cysteine, which is oxidized to sulfenic acid. Then, unknown reductase(s) may reduce the sulfenic acid to thiol. GSH, reduced glutathione.

single amino acid (T155E) prevented Prx4 hyper-oxidation by enhancing the mobility of C124 or the dissociation of Prx4 decamer to dimers, respectively (55). These findings suggest that the susceptibility of human Prx4 to hyper-oxidation depends on multiple factors, such as (i) the stability of the enzyme active site, (ii) decamerization and (iii) the C-terminal YF motif limiting the mobility around C124. They also indicate that Prx4 retained structural features conductive to its hyper-oxidation, even though no sulfiredoxin activity has been demonstrated so far in the exocytic compartment. This raises intriguing questions as to the fate and possible significance of sulfinylated or sulfonylated forms in the ER, presumably inactive as H_2O_2 scavengers and oxidants of PDI. Do these species display increased chaperone activity? Are they degraded upon restoration of redox homeostasis in the ER? Or are they perhaps preferentially secreted, acting as stress or damage associated molecules?

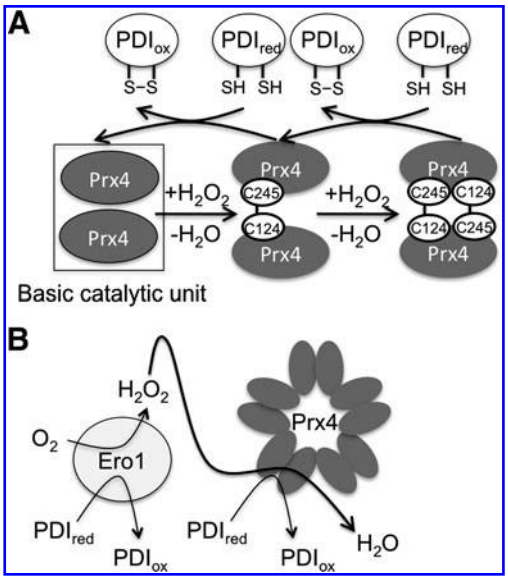

FIG. 4. Roles of Prx4 in oxidative protein folding. (A) Upon reaction with an H_2O_2 molecule, an intermolecular disulfide is formed *via* a SOH intermediate between the conserved peroxidatic and resolving cysteines (Cys124 and Cys245, respectively) of two subunits arranged in a head-to-tail dimer. These disulfide bonds are preferentially transferred to reduced PDI by interchange reactions. **(B)** In the scheme, the H_2O_2 molecule that catalyzes Prx4 activation is depicted as deriving from an Ero1 molecule, to underscore the capability of the mammalian ER to oxidize 2 PDI with a single oxygen molecule, without ROS production. PDI, protein disulfide isomerase. ROS, reactive oxygen species.

Isoforms, Topology, and Physiological Aspects of Prx4

Contrasting reports appeared in the literature concerning the subcellular localization of Prx4. Prx4 was initially identified as a cytosolic protein that regulates NF-κB (23). Soon thereafter, however, Haridas *et al*. described it as a secreted protein with a cleaved N-terminal signal sequence (19). Much remains to be understood on the precise Prx4 localization, which has been shown to depend on alternative transcription initiation and proteolytic processing. Thus, the 27-kDa species is a soluble protein accumulating in the secretory compartment of most cell types, derived from cleavage of the N-terminal signal peptide encoded in exon 1B (Fig. 6). The 31-kDa form instead is a membrane-anchored protein

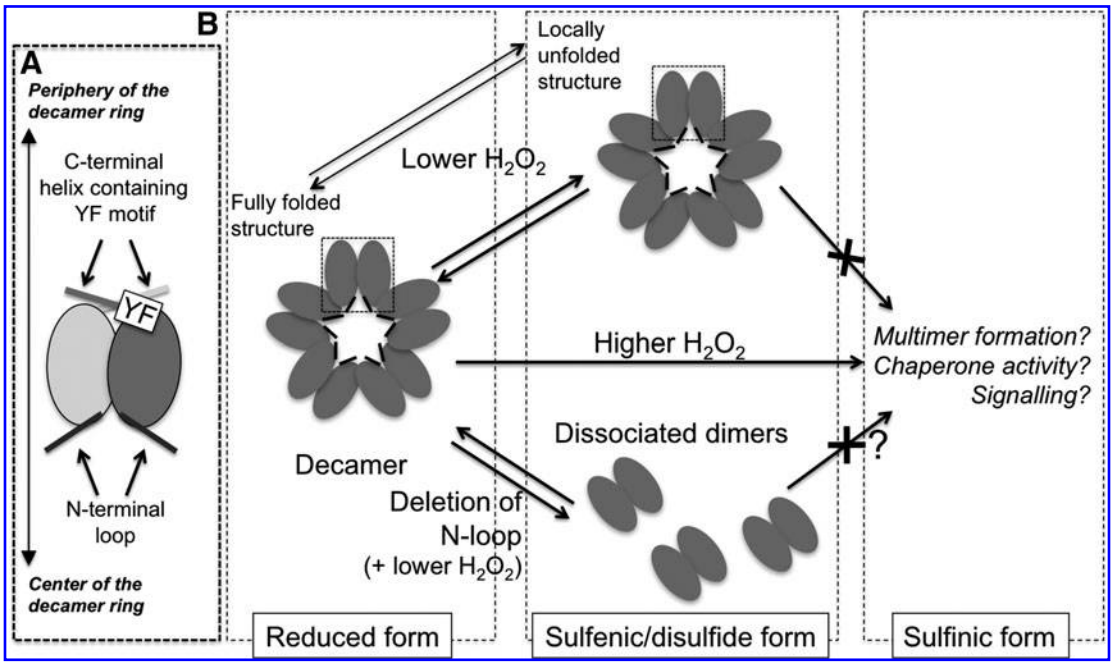

FIG. 5. Oligomeric structure of Prx4 in different redox conditions. (A) Structural studies suggest that, as schematized, the N-terminal loops and C-terminal helix of Prx4 are directed toward the center or to the outside of decamer rings, respectively. **(B)** Unlike other Prxs, Prx4 has been reported to form stable decamers irrespectively of its redox state (10, 55). At lower H_2O_2 levels, oxidized Prx4 dimers are in a locally unfolded structure, with unwound α2 and disordered α6 helices, while reduced Prx4 dimers are in a fully folded structure. Oxidized, locally unfolded Prx4 enzymes show increased resistance to hyper-oxidation. Deletion of the N-terminal loop causes the dissociation of oxidized Prx4 decamers. Dissociation increases the resistance to hyper-oxidation. In higher level of H_2O_2, Prx4 is hyper-oxidized to sulfinic or sulfonic acid. The structure of hyper-oxidized Prx4 is unknown. As other members of the 2-Cys family, Prx4 possibly forms multimers shifting its activity from peroxidase to chaperone or signal transducer.

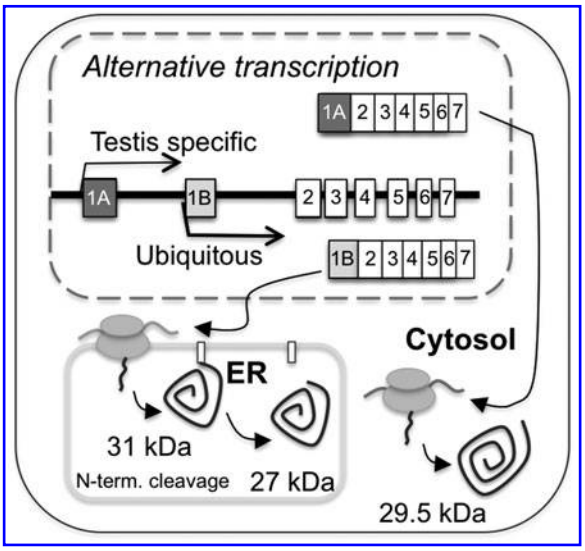

FIG. 6. Prx4 can adopt different subcellular localizations. Three isoforms can be generated from the Prx4 gene. The ubiquitous 27-kDa species originates by cleavage of the N-terminal signal sequence, encoded in exon 1B. In testis, cleavage is not efficient, yielding a 31-kDa membrane anchored isoform. In addition, alternative transcription replaces exon 1B with exon 1A, yielding a protein of 29.5 kDa that lacks a signal peptide and localizes in the cytosol.

retaining the signal sequence. This isoform is expressed in testis after puberty, being particularly abundant in the acrosomal membrane of spermatids but not in differentiated sperm. A recent study described yet an additional 29.5-kDa isoform, in which exon1-B is replaced by exon 1-A (59). The latter exon does not encode a hydrophobic N-terminal signal peptide. Accordingly, the 29.5-kDa variant was found to accumulate in the soluble fraction of mice testis homogenates, suggesting a cytosolic localization. Its functional role remains to be established.

The presence of membrane-bound and soluble isoforms seems to be a feature common to various ER-resident enzymes. For instance, ER degradation-exhancing alpha-mannosidase-like-1 (EDEM1), a lectin-like molecule accelerating ER-associated degradation, exists as a type II transmembrane protein in addition to the signal peptide-cleaved, soluble isoform. Interestingly these two isoforms have different substrate specificities (50). Similarly, transmembrane PDI family members show preferences for other client proteins than soluble PDIs (48). They may derive oxidative power from Ero1α and Ero1β, a fraction of which associates with membranes too (41). It will be of interest to determine whether membrane bound Prx4 serves a discrete role in ER redox homeostasis. In this connection, the ubiquitous, soluble 27-kDa Prx4 isoform lacks a C-terminal ER retrieval motif. Interestingly, mammalian Ero1 molecules also lack known ER localization signals. Being intracellularly retained by virtue of their interactions with ER oxidoreductases, primarily PDI and ERp44, Ero1α and Ero1β are secreted when overexpressed (40). Further experiments are needed to determine how Prx4

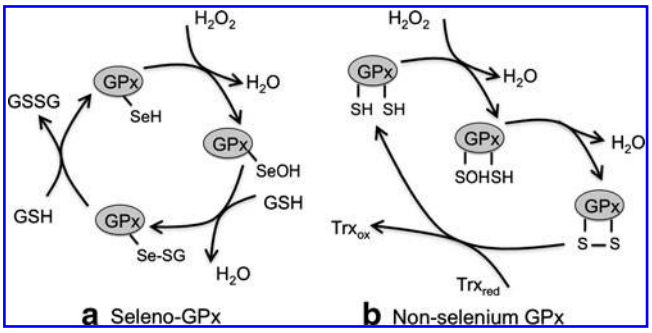

FIG. 7. The catalytic cycle of GPxs. (a) In seleno-GPx, the active selenocysteine is oxidized by H_2O_2 to form a SeOH group. A GSH molecule forms a mixed disulfide with SeOH generating water. A second GSH then interchanges, yielding GSSG and a reduced GPx, ready to reenter the cycle. Thus, two GSH are utilized for reduction of one hydrogen peroxide. **(b)** In nonselenium GPx, the active cysteine is oxidized by H_2O_2 to form a SOH group, following the formation of intramolecular disulfide with cysteine in the PCNQF motif. This disulfide is then reduced by thioredoxin, regenerating an active enzyme. GPx, glutathione peroxidase; GSSG, oxidized glutathione.

maintains its subcellular localization, and whether its retention/secretion can be regulated in living cells.

Differentiating B cells strongly upregulate *Prx4* as they embark on massive antibody production (6). Surprisingly, however, mice lacking the soluble 27-kDa Prx4 isoform had only minor defects in polymerization and secretion of IgM, a molecule harboring many disulfide bonds. These mice could produce the 29.5-kDa isoform; however, this presumably cytosolic Prx4 variant is thought to be specifically expressed in the testis (55). Therefore, the subtle phenotype of these mice implies redundancy in the ER antioxidant systems. GPx7 and GPx8 are good candidates to replace Prx4 in scavenging luminal H_2O_2 in a productive way.

GPx-7/-8, Members of Nonselenium GPxs

Discovered in 1957 by Mills (36), GPx orthologs have been found in archaea, prokaryotes, and eukaryotes (53). The activity of many GPxs depends on selenocysteine, a rare amino acid residue similar to cysteine, in which a selenium (Se) replaces sulfur in its selenothiol group. Recent reports identified also nonselenium GPxs with a cysteine at the position normally occupied by a selenocysteine residue. Biochemical studies revealed that most nonselenium GPxs depend on thioredoxin rather than glutathione (21). The working cycle of seleno-GPx and nonselenium GPx are schematized in Figure 7.

There are eight GPxs in humans. Two of them, GPx7 and GPx8 are *bona fide* ER resident proteins. The former is a soluble protein while the latter is anchored to the ER membrane (37). Both efficiently promote oxidative refolding of reduced cargo proteins in the presence of PDI and H_2O_2. GPx7 and GPx8 interact with Ero1α. GPx7 was shown to increase oxygen consumption by Ero1α. Therefore, GPx7 could promote oxidative folding, utilizing the peroxides produced by vicinal Ero1α molecules, as demonstrated for Prx4 (52, 61). Both GPx7 and GPx8 have cysteine residues in their active site. However, it remains to be established whether they share the working cycle with other nonselenium GPxs or not.

Physiology and Pathology of ER Luminal H_2O_2

The above findings suggest a scenario in which a dangerous by-product of Ero1 and other ER pathways, H_2O_2, is scavenged by Prx4, GPx7, and GPx8 which further promote oxidative folding by introducing disulfide bonds into oxidoreductases (9). However, part of the H_2O_2 generated in the ER could also enter anterograde transport vesicles to be secreted, or cross the ER membrane to reach the cytosol, potentially modulating various redox sensitive intra- and intercellular signaling pathways (12, 24, 31), including the activation and proliferation of certain cell types, such as B lymphocytes (6). An interesting question deserving more investigation is whether and to what extent biological membranes are permeant to H_2O_2 (2). Recently, evidence has accumulated for aquaporins being implicated in transmembrane H_2O_2 transport in yeast and mammalian cells (7, 35). The identification of molecules that facilitate or control H_2O_2 transport across membranes might shed a different view on redox signaling.

The unfolded protein response (UPR) is a multidimensional signaling pathway that adjusts the ER capacity to its folding demands, but becomes maladaptive and ultimately activates apoptosis if the underlying causes persist or are excessive (45). The UPR is activated by conditions that induce ER stress, such as the synthesis of aberrant proteins or of normal ones in excess, or altered Ca^{2+} homeostasis, and has been linked to many pathological conditions, including cancer, neurodegenerative diseases, or obesity. Therefore, a fundamental question is what determines the adaptive to maladaptive transition (22). In yeast, the UPR entails robust activation of Ero1p, and this oxidase has been implicated as an important determinant of the reduced growth of ER stressed cells (20). Similarly, worms expressing low Ero1 levels accumulated less ROS when exposed to ER stressors, and were more resistant to their toxic effects (30). These findings suggest that Ero1-derived H_2O_2 can sustain the maladaptive transition in ER stress responses, at least in lower eukaryotes. In mammalian cells, however, Ero1 molecules seem to have little role in sustaining oxidative stress and this may correlate with the appearance of Prx4, GPx7, and GPx8 as efficient H_2O_2 scavengers during evolution (3). Nevertheless, *Ero1α*$^{-/-}$ murine cells are less sensitive to ER stress-dependent apoptosis: the underlying mechanism seems to involve diminished Ca^{2+} release through inositol(1,4,5)triphosphate receptors around MAM (see (27) and references therein). It has been recently shown that Ero1α preferentially accumulates in MAM and regulates Ca^{2+} fluxes (1, 16). Interestingly, in hypoxic conditions Ero1α translocates from MAM to the ER (16).

Measuring H_2O_2 Production and Signaling

So far, our understanding on the role of oxidants is limited because of their rapid interconversion in the crowded cell environments and the difficulties in establishing reliable measurement methods (56). Different strategies have been followed to circumvent the difficulties in detecting H_2O_2 in cells and tissues (47). Chemical probes have been developed containing a boronate-based cage that releases a fluorochrome upon reacting with H_2O_2 (34). These probes are highly specific, but suffer two limitations: (i) they react rather slowly with H_2O_2 and (ii) they are not reversible, impeding dynamic measurements. On the other hand, protein-based sensors

were obtained by fusing a permutated fluorescent protein with the bacterial transcription factor OxyR the activation of which is regulated by H_2O_2. The H_2O_2-dependent formation of a disulfide bond in the OxyR moiety causes conformational changes in HyPer sensors that lead to a ratiometric switch between two fluorescence excitation peaks (32).

HyPer sensors rapidly and reversibly react with H_2O_2 but not with $O_2^{\bullet -}$, GSSG, NO, or $ONOO^-$ (5), and allow the measurement of H_2O_2 production and fluxes within and between living cells. For instance, transgenic zebrafish expressing HyPer allowed to demonstrate a signaling role for extracellular H_2O_2 in recruiting inflammatory cells to the vicinity of wounds (38). Organelle targeted HyPer have been successfully engineered: several labs produced ER-localized HyPer (HyPer-ER) appending a signal sequence and a KDEL motif onto the HyPer N- and C-termini, respectively (14, 29, 58). By using HyPer-ER, Wu *et al*. provided evidence implicating the ER membrane localized Nox4 and downstream Ras/ERK signaling in the production of H_2O_2 in response to tunicamycin or HIV-tat treatment of endothelial cells (58).

However, a limit in the utilization of HyPer-ER is that its wavelength shifts depend on the formation of a disulfide bond. In the secretory compartment, therefore, HyPer could report on the efficiency of oxidative folding, besides on the H_2O_2 levels, by directly reacting with oxidized PDIs. Indeed, in reducing compartments such as the cytosol, HyPer readily reacts with thioredoxins for its disulfide recycling. It will be of interest to identify the system(s) that can oxidize/reduce the ER luminal HyPer sensors. These studies will likely be relevant to determine the role and regulation of ER peroxidases during the UPR.

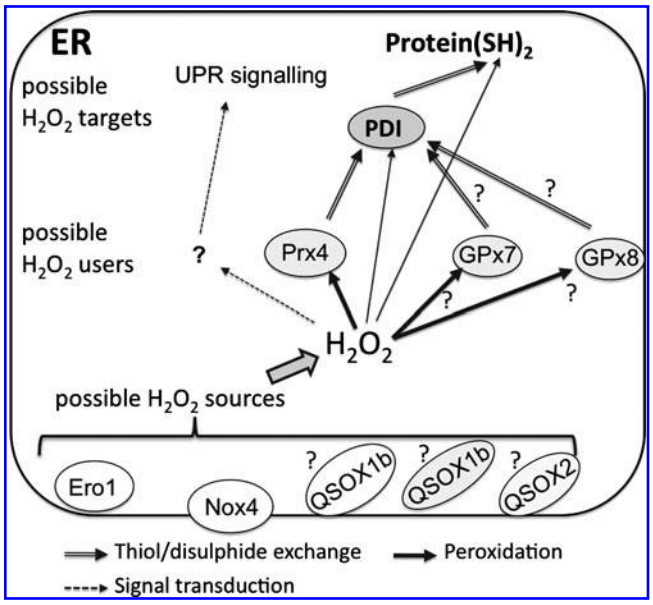

FIG. 8. Sources, users, and targets of H_2O_2 in the mammalian ER. The cartoon depicts a simplified scheme of the molecular networks operating in the ER to maximize efficiency of protein folding while preventing oxidative stress. The bottom part shows three protein families that could act as potential H_2O_2 sources: the Ero1 and QSOX flavoproteins, and the NADPH oxidases located in ER membrane. Mitochondria could provide oxidative equivalents as well. Prx4, GPx7, and GPx8 scavenge incoming H_2O_2 to catalyze the oxidation of PDI, ERp46, and possibly other resident oxidoreductases. H_2O_2 could oxidize PDI or cargo protein directly, but with low efficiency (24). Upon ER stress H_2O_2 could also act as a second messenger. Unknown molecules may sense the change in H_2O_2 concentration in the ER to activate apoptosis in UPR signaling (58). NADPH, nicotinamide adenine dinucleotide phosphate. UPR, unfolded protein response.

Concluding Remarks

As the mechanistic dissection of Ero1 molecules proceeded, the ER emerged as a potential source of abundant H_2O_2 particularly in the process of *de novo* disulfide bond formation (54). However, it became clear that a potential foe, H_2O_2 can act in a friendly and useful way, further fueling oxidative folding (Fig. 8). The presence of several ER-resident peroxidases, Prx4, GPx7, and GPx8, couples the scavenging of H_2O_2 to oxidation of PDI, and in turn of cargo proteins (37, 52, 61). Mammalian cell and animal models tolerate well the absence (or extremely low levels) of molecules that are essential in lower eukaryotes (3). On the one hand, redundancy contrasts with the notion that specific protein-based relays—rather than a general redox poise—are active in the ER to allow the coexistence of disulfide bond formation, isomerization, and reduction. On the other hand, redundancy implies the evolution in metazoan of parallel pathways devoted to form disulfides without eliciting oxidative stress (9). Indeed, a lower sensitivity to Ero1 hypo- or hyperactivity coincides with the appearance of enzymes capable of productively scavenging peroxides. Since metabolism and redox signaling are associated with key pathophysiological events including cancer and degenerative disorders, further investigation on the handling of H_2O_2 in and from the ER could lead to novel approaches for therapy.

Acknowledgments

We thank Christian Appenzeller-Herzog, Kazutaka Araki, Milena Bertolotti, and Anna Rubartelli for their most helpful suggestions and critical reading of the manuscript; all the members of our laboratories for helpful discussions and Tina Scacciante and Tamami Ishida for secretarial assistance. This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC), Regione Lombardia and Telethon (to R.S), and by a Grant-in Aid for Creative Scientific Research and for Scientific Research on Priority Area from MEXT (to K.N). We apologize to the many colleagues whose pioneering work could not be cited owing to the strict space limits imposed by the editors.

References

1. Anelli T, Bergamelli L, Margittai E, Rimessi A, Fagioli C, Malgaroli A, Pinton P, Ripamonti M, Rizzuto R, and Sitia R. Ero1α regulates Ca2+ fluxes at the endoplasmic reticulum-mitochondria interface. *Antioxid Redox Signal* 2011 [Epub ahead of print]; DOI: 10.1089/ars.2011.4004.
2. Appenzeller-Herzog C. Glutathione- and non-glutathione-based oxidant control in the endoplasmic reticulum. *J Cell Sci* 124: 847–55, 2011.
3. Araki K and Inaba K. Structure, mechanism and evolution of Ero1 family enzymes. *Antioxid Redox Signal* 16: 790–799, 2012.
4. Araki K and Nagata K. Protein folding and quality control in the ER. *Cold Spring Harb Perspect Biol* 3: a007526, 2011.

5. Belousov VV, Fradkov AF, Lukyanov KA, Staroverov DB, Shakhbazov KS, Terskikh AV, and Lukyanov S. Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. *Nat Methods* 3: 281–6, 2006.
6. Bertolotti M, Yim SH, Garcia-Manteiga JM, Masciarelli S, Kim YJ, Kang MH, Iuchi Y, Fujii J, Vene R, Rubartelli A, Rhee SG, and Sitia R. B- to plasma-cell terminal differentiation entails oxidative stress and profound reshaping of the antioxidant responses. *Antioxid Redox Signal* 13: 1133–44, 2010.
7. Bienert GP, Moller AL, Kristiansen KA, Schulz A, Moller IM, Schjoerring JK, and Jahn TP. Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. *J Biol Chem* 282: 1183–92, 2007.
8. Biteau B, Labarre J, and Toledano MB. ATP-dependent reduction of cysteine-sulphinic acid by S. cerevisiae sulphiredoxin. *Nature* 425: 980–4, 2003.
9. Bulleid NJ and Ellgaard L. Multiple ways to make disulfides. *Trends Biochem Sci* 36: 485–92, 2011.
10. Cao Z, Tavender TJ, Roszak AW, Cogdell RJ, and Bulleid NJ. Crystal structure of reduced and of oxidized peroxiredoxin IV reveals a stable oxidized decamer and a non disulfide-bonded intermediate in the catalytic cycle. *J Biol Chem* 2011.
11. Chae HZ, Chung SJ, and Rhee SG. Thioredoxin-dependent peroxide reductase from yeast. *J Biol Chem* 269: 27670–8, 1994.
12. Csala M, Margittai E, and Banhegyi G. Redox control of endoplasmic reticulum function. *Antioxid Redox Signal* 13: 77–108, 2010.
13. D'Autreaux B and Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol* 8: 813–24, 2007.
14. Enyedi B, Varnai P, and Geiszt M. Redox state of the endoplasmic reticulum is controlled by Ero1L-alpha and intraluminal calcium. *Antioxid Redox Signal* 13: 721–9, 2010.
15. Frand AR and Kaiser CA. The ERO1 gene of yeast is required for oxidation of protein dithiols in the endoplasmic reticulum. *Mol Cell* 1: 161–70, 1998.
16. Gilady SY, Bui M, Lynes EM, Benson MD, Watts R, Vance JE, and Simmen T. Ero1alpha requires oxidizing and normoxic conditions to localize to the mitochondria-associated membrane (MAM). *Cell Stress Chaperones* 15: 619–29, 2010.
17. Hall A, Karplus PA, and Poole LB. Typical 2-Cys peroxiredoxins—structures, mechanisms and functions. *FEBS J* 276: 2469–77, 2009.
18. Hall A, Sankaran B, Poole LB, and Karplus PA. Structural changes common to catalysis in the Tpx peroxiredoxin subfamily. *J Mol Biol* 393: 867–81, 2009.
19. Haridas V, Ni J, Meager A, Su J, Yu GL, Zhai Y, Kyaw H, Akama KT, Hu J, Van Eldik LJ, and Aggarwal BB. TRANK, a novel cytokine that activates NF-kappa B and c-Jun N-terminal kinase. *J Immunol* 161: 1–6, 1998.
20. Haynes CM, Titus EA, and Cooper AA. Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death. *Mol Cell* 15: 767–76, 2004.
21. Herbette S, Roeckel-Drevet P, and Drevet JR. Seleno-independent glutathione peroxidases. More than simple antioxidant scavengers. *FEBS J* 274: 2163–80, 2007.
22. Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell* 140: 900–17, 2010.
23. Jin DY, Chae HZ, Rhee SG, and Jeang KT. Regulatory role for a novel human thioredoxin peroxidase in NF-kappaB activation. *J Biol Chem* 272: 30952–61, 1997.
24. Karala AR, Lappi AK, Saaranen MJ, and Ruddock LW. Efficient peroxide-mediated oxidative refolding of a protein at physiological pH and implications for oxidative folding in the endoplasmic reticulum. *Antioxid Redox Signal* 11: 963–70, 2009.
25. Kodali VK and Thorpe C. Oxidative protein folding and the Quiescin-sulfhydryl oxidase family of flavoproteins. *Antioxid Redox Signal* 13: 1217–30, 2010.
26. Lee W, Choi KS, Riddell J, Ip C, Ghosh D, Park JH, and Park YM. Human peroxiredoxin 1 and 2 are not duplicate proteins: the unique presence of CYS83 in Prx1 underscores the structural and functional differences between Prx1 and Prx2. *J Biol Chem* 282: 22011–22, 2007.
27. Li G, Mongillo M, Chin KT, Harding H, Ron D, Marks AR, and Tabas I. Role of ERO1-alpha-mediated stimulation of inositol 1,4,5-triphosphate receptor activity in endoplasmic reticulum stress-induced apoptosis. *J Cell Biol* 186: 783–92, 2009.
28. Lowther WT and Haynes AC. Reduction of cysteine sulfinic acid in eukaryotic, typical 2-Cys peroxiredoxins by sulfiredoxin. *Antioxid Redox Signal* 15: 99–109, 2011.
29. Malinouski M, Zhou Y, Belousov VV, Hatfield DL, and Gladyshev VN. Hydrogen peroxide probes directed to different cellular compartments. *Plos One* 6: e14564, 2011.
30. Marciniak SJ, Yun CY, Oyadomari S, Novoa I, Zhang Y, Jungreis R, Nagata K, Harding HP, and Ron D. CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes Dev* 18: 3066–77, 2004.
31. Margittai E and Sitia R. Oxidative protein folding in the secretory pathway and redox signaling across compartments and cells. *Traffic* 12: 1–8, 2011.
32. Meyer AJ and Dick TP. Fluorescent protein-based redox probes. *Antioxid Redox Signal* 13: 621–50, 2010.
33. Mezghrani A, Fassio A, Benham A, Simmen T, Braakman I, and Sitia R. Manipulation of oxidative protein folding and PDI redox state in mammalian cells. *EMBO J* 20: 6288–96, 2001.
34. Miller EW, Albers AE, Pralle A, Isacoff EY, and Chang CJ. Boronate-based fluorescent probes for imaging cellular hydrogen peroxide. *J Am Chem Soc* 127: 16652–9, 2005.
35. Miller EW, Dickinson BC, and Chang CJ. Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. *Proc Natl Acad Sci U S A* 107: 15681–6, 2010.
36. Mills GC. Hemoglobin catabolism. I. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. *J Biol Chem* 229: 189–97, 1957.
37. Nguyen VD, Saaranen MJ, Karala AR, Lappi AK, Wang L, Raykhel IB, Alanen HI, Salo KE, Wang CC, and Ruddock LW. Two endoplasmic reticulum PDI peroxidases increase the efficiency of the use of peroxide during disulfide bond formation. *J Mol Biol* 406: 503–15, 2011.
38. Niethammer P, Grabher C, Look AT, and Mitchison TJ. A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. *Nature* 459: 996–9, 2009.
39. Okado-Matsumoto A, Matsumoto A, Fujii J, and Taniguchi N. Peroxiredoxin IV is a secretable protein with heparin-binding properties under reduced conditions. *J Biochem* 127: 493–501, 2000.
40. Otsu M, Bertoli G, Fagioli C, Guerini-Rocco E, Nerini-Molteni S, Ruffato E, and Sitia R. Dynamic retention of Ero1alpha and Ero1beta in the endoplasmic reticulum by interactions with PDI and ERp44. *Antioxid Redox Signal* 8: 274–82, 2006.

41. Pagani M, Fabbri M, Benedetti C, Fassio A, Pilati S, Bulleid NJ, Cabibbo A, and Sitia R. Endoplasmic reticulum oxidoreductin 1-lbeta (ERO1-Lbeta), a human gene induced in the course of the unfolded protein response. *J Biol Chem* 275: 23685–92, 2000.
42. Phalen TJ, Weirather K, Deming PB, Anathy V, Howe AK, van der Vliet A, Jonsson TJ, Poole LB, and Heintz NH. Oxidation state governs structural transitions in peroxiredoxin II that correlate with cell cycle arrest and recovery. *J Cell Biol* 175: 779–89, 2006.
43. Pollard MG, Travers KJ, and Weissman JS. Ero1p: a novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum. *Mol Cell* 1: 171–82, 1998.
44. Ramming T and Appenzeller-Herzog C. The physiological functions of mammalian endoplasmic oxidoreductin 1: on disulfides and more. *Antioxid Redox Signal* 2012. [Epub ahead of print]; DOI: 10.1089/ars.2011.4475
45. Rao RV, Ellerby HM, and Bredesen DE. Coupling endoplasmic reticulum stress to the cell death program. *Cell Death Differ* 11: 372–80, 2004.
46. Rhee SG, Chae HZ, and Kim K. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radic Biol Med* 38: 1543–52, 2005.
47. Rhee SG, Chang TS, Jeong W, and Kang D. Methods for detection and measurement of hydrogen peroxide inside and outside of cells. *Mol Cells* 29: 539–49, 2010.
48. Schulman S, Wang B, Li W, and Rapoport TA. Vitamin K epoxide reductase prefers ER membrane-anchored thioredoxin-like redox partners. *Proc Natl Acad Sci U S A* 107: 15027–32, 2010.
49. Sevier CS, Cuozzo JW, Vala A, Aslund F, and Kaiser CA. A flavoprotein oxidase defines a new endoplasmic reticulum pathway for biosynthetic disulphide bond formation. *Nat Cell Biol* 3: 874–82, 2001.
50. Tamura T, Cormier JH, and Hebert DN. Characterization of early EDEM1 protein maturation events and their functional implications. *J Biol Chem* 286: 24906–15, 2011.
51. Tavender TJ, Sheppard AM, and Bulleid NJ. Peroxiredoxin IV is an endoplasmic reticulum-localized enzyme forming oligomeric complexes in human cells. *Biochem J* 411: 191–9, 2008.
52. Tavender TJ, Springate JJ, and Bulleid NJ. Recycling of peroxiredoxin IV provides a novel pathway for disulphide formation in the endoplasmic reticulum. *EMBO J* 29: 4185–97, 2010.
53. Toppo S, Vanin S, Bosello V, and Tosatto SC. Evolutionary and structural insights into the multifaceted glutathione peroxidase (Gpx) superfamily. *Antioxid Redox Signal* 10: 1501–14, 2008.
54. Tu BP and Weissman JS. The FAD- and O(2)-dependent reaction cycle of Ero1-mediated oxidative protein folding in the endoplasmic reticulum. *Mol Cell* 10: 983–94, 2002.
55. Wang X, Wang L, Wang X, Sun F, and Wang CC. Structural insights into the peroxidase activity and inactivation of human peroxiredoxin 4. *Biochem J* 441: 113–118, 2012.
56. Winterbourn CC. Reconciling the chemistry and biology of reactive oxygen species. *Nat Chem Biol* 4: 278–86, 2008.
57. Wood ZA, Poole LB, and Karplus PA. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* 300: 650–3, 2003.
58. Wu RF, Ma Z, Liu Z, and Terada LS. Nox4-derived H2O2 mediates endoplasmic reticulum signaling through local Ras activation. *Mol Cell Biol* 30: 3553–68, 2010.
59. Yim SH, Kim YJ, Oh SY, Fujii J, Zhang Y, Gladyshev VN, and Rhee SG. Identification and characterization of an alternatively transcribed form of peroxiredoxin IV that is specifically expressed in spermatids of the postpubertal mouse testis. *J Biol Chem* 286: 39002–39012, 2011.
60. Zito E, Chin KT, Blais J, Harding HP, and Ron D. ERO1-beta, a pancreas-specific disulfide oxidase, promotes insulin biogenesis and glucose homeostasis. *J Cell Biol* 188: 821–32, 2010.
61. Zito E, Melo EP, Yang Y, Wahlander A, Neubert TA, and Ron D. Oxidative protein folding by an endoplasmic reticulum-localized peroxiredoxin. *Mol Cell* 40: 787–97, 2010.

Address correspondence to:
Dr. Roberto Sitia
Division of Genetics and Cell Biology
San Raffaele Scientific Institute
Vita-Salute University
Via Olgettina 58
I-20132 Milan
Italy

E-mail: r.sitia@hsr.it

Date of first submission to ARS Central, December 5, 2011; date of acceptance, December 6, 2011.

Abbreviations Used

ER = endoplasmic reticulum
Ero1 = ER oxidoreductin-1
Erv2 = essential for respiration and vegetative growth 2
GPx = glutathione peroxidase
GSH/GSSG = reduced and oxidized glutathione
MAM = mitochondrial-associated membrane
NADPH = nicotinamide adenine dinucleotide phosphate
Nox = NADPH oxidase
PDI = protein disulfide isomerase
Prx = peroxiredoxin
QSOX = quiescin-sulfhydryl oxidase
ROS = reactive oxygen species
UPR = unfolded protein response

This article has been cited by:

1. Dr. Ester Zito . PRDX4, an ER-localised peroxiredoxin at the crossroads between enzymatic oxidative protein folding and non-enzymatic protein oxidation. *Antioxidants & Redox Signaling* **0**:ja. . [Abstract] [Full Text PDF] [Full Text PDF with Links]
2. Christian Appenzeller-Herzog . 2012. Updates on “Endoplasmic Reticulum Redox”. *Antioxidants & Redox Signaling* **16**:8, 760-762. [Citation] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]